# The art of making a comprehensive stability study

**Presenting the development in size-exclusion chromatography analysis of mAbs and the requirements for a comprehensive stability study.**


## Roman Lord



Master Thesis in Analytical Chemistry, 2020


Department of Chemistry
Lund University
Sweden


# The art of making a comprehensive stability study

Presenting the development in size-exclusion chromatography analysis of mAbs and the requirements for a comprehensive stability study.

Roman Lord


LUND
UNIVERSITY

Master Thesis in Analytical Chemistry

2020

**Advisor:**

Danny Mollerup Sørensen
H. Lundbeck, Copenhagen

Peter Spégel
Centre for Analysis and Synthesis, Lund

**Examiner:**

Margareta Sandahl
Centre for Analysis and Synthesis, Lund

Centre for Analysis and Synthesis
Department of Chemistry
Lund University, Box 124
SE-221 00 Lund, Sweden

# The art of making a comprehensive stability study

Antibodies do not only exist in our body, but they can also be manufactured in laboratories and cell-factories. Monoclonal antibodies (mAbs) are the type of antibodies that are used in for drug development. Their high selectivity makes them very useful in cancer treatments, but they can be used for the treatment of a large repertoire of diseases such as rheumatoid arthritis and multiple sclerosis to mention a few.

It is of great importance that the drugs that reach the market are thoroughly controlled. They should fulfil the required quality standards set by The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) or by regional authorities such as the United States Pharmacopeial Convention (USP) and the European Medicines Agency (EMA). To control an antibody's quality, stability studies are performed. These studies should be well designed and cover all stability issues that may arise. Because, if some parameters are missed, they could lead to severe consequences for the patient, or even death.

Clear standards for study designs may help to avoid unnecessary consequences by contributing to well-designed stability studies. Recently, a study found that many companies do not perform sufficiently thorough stability studies regarding mAbs. Most studies focus only on fulfilling the stated regulations by the authorities. Tests for stability in a clinical setting, such as accidental shaking of the container, short term temperature changes during transportation or accidental breaking of sterility, are often missed.

The objective of this work was therefore firstly, to define what is required today for a stability study based on guidelines and recent literature. Secondly, to propose an example of what a comprehensive, well-designed stability study for mAbs should include, based on the studied literature.

It was found that there is a need for standardised comprehensive stability study designs for mAbs. Also, it is important to understand the characteristics of the studied protein to be able to perform better studies. In order to create more comprehensive stability studies new recommendation may have to be added to the guidelines, which may contribute to a harmonisation of the stability study designs.

# Abstract

**Introduction**: There is a need for harmonisation of stability study designs of monoclonal antibodies.

**Background**: Monoclonal antibodies (mAbs) are widely used in treatments for various diseases. Like all drugs, their quality must be verified through stability studies. The ICH guidelines give general recommendations regarding stability studies of antibodies, and specifications should often be determined on a case-by-case basis. More detailed requirements may need to be defined to make better designed stability studies for mAbs.

**Aim(s):** This report aims to produce a broader understanding of the parameters that should be included in mAb stability studies and give a proposal for a comprehensive stability study design.

**Methods**: The information for this report was obtained through literature studies including amongst other the ICH guidelines and several newly published articles regarding size-exclusion chromatography and its application in stability studies.

**Results**: The existing guidelines regarding stability studies of biopharmaceutical are very general. Therefore, stability test focusing on the physicochemical stability of mAbs in clinical settings are often missed. This additional stability test can increase the understanding of the analyte and may facilitate the work of health personnel. Even though it is difficult to make a study design that fit all types of mAbs, there are opportunities for improvement in the guidelines as they are today in order to make a more comprehensive stability study design.

**Conclusion**: There is a need for development in stability study designs regarding mAbs. To create more comprehensive stability studies new recommendation may have to be added that may contribute to a harmonisation of the stability study designs



**Keywords:** Biopharmaceuticals, mAbs, SEC, stability study design

## Acknowledgement

The author gratefully thanks Danny Mollerup Sørensen and Peter Spégel for their support and guidance in the report.

# List of abbreviations

| | |
|---|---|
| **API** | active pharmaceutical ingredient |
| **CDR** | complementary determining regions |
| **CQA** | critical quality attributes |
| **DLS** | dynamic light scattering |
| **DPs** | degradation products |
| **EMA** | European Medicines Agency |
| **ESI-MS** | electrospray ionisation mass spectrometry |
| **Fab** | antigen bonding fragment |
| **Fc** | crystallisable fragment |
| **FDA** | U.S. Food & Drug Administration |
| **HPTLC** | high performance thin layer chromatography |
| **ICH** | International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use |
| **ICP-MS** | inductively coupled plasma mass spectrometry |
| **IEC** | ion exchange chromatography |
| **$K_d$** | distribution coefficient |
| **LC** | liquid chromatography |
| **mAbs** | monoclonal antibodies |
| **MALS** | multi-angle light scattering |
| **MFI** | micro-flow imaging |
| **MS** | mass spectrometry |
| **RP-HPLC** | reversed phase liquid chromatography |
| **SEC** | size-exclusion chromatography |
| **USP** | United States Pharmacopeial Convention |
| **VWD** | variable wavelength detector |

# 1 Introduction

The interest in biopharmaceuticals, or "biologics" as they often are called, has drastically increased during the last two decades. They were originally introduced to the public in the 80's much due to the development in recombinant DNA technology. As the technology developed further so did the interest for the biopharmaceuticals and new types of therapies emerged to daylight.[1,2] A big part, around 70 %, of the world-wide biopharmaceutical market revenue, counted to over US$200 million in 2018, comes from antibodies.[3,4] This is mainly to the diversity of diseases that can be mitigated or treated with antibodies.[5,6]Monoclonal antibodies (mAbs) are large proteins, so-called immunoglobulins. Of the five antibody isotopes (Figure 1), mAbs commonly have an IgG-type structure.

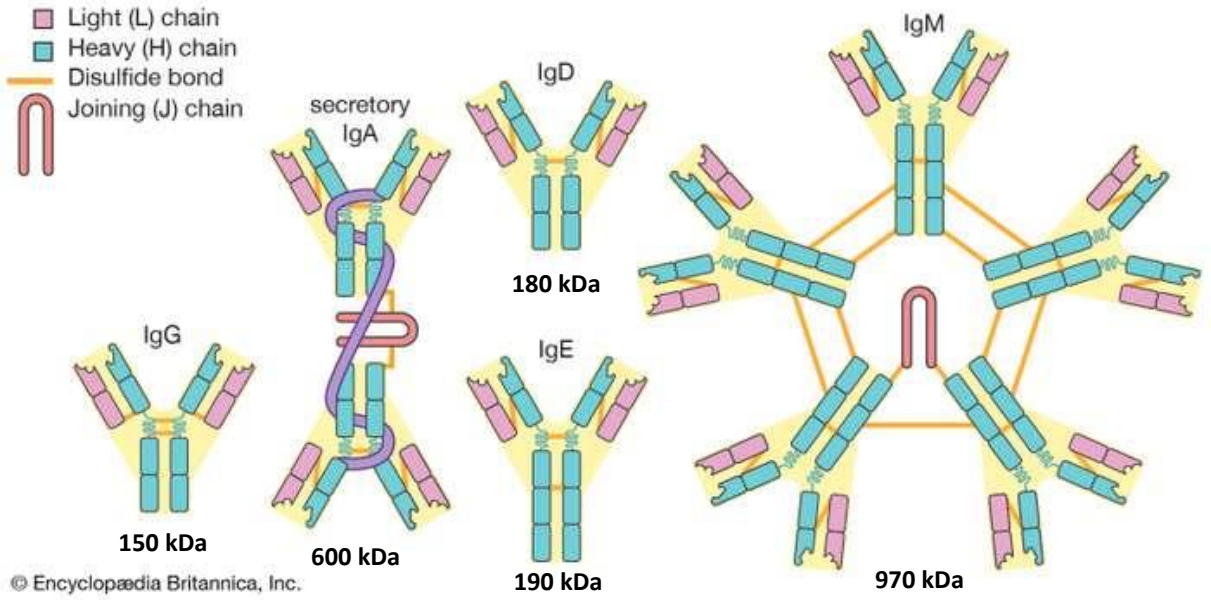


**Figure 1.** The five Isotypes of antibodies. The heavy protein chains are marked in blue while the light chains are in purple. Due to a large structural variation, the effect and mechanism of action varies between the isotypes. Adapted from: https://www.britannica.com/science/antibody

mAbs consists of four protein chains – two heavy chains and two light chains – with a total weight of around 150 kDa. It can be divided into three fragments or regions: two antigen-binding fragments (Fabs) and one crystalline fragment (Fc). The variable domains in the Fabs can attach to certain epitopes due to the complementary-determining regions (CDRs), (Figure 2). The Fc region is also known as the effector region and interacts with molecules and effector cells.[2,7,8]

Moreover, there are glycans – small carbohydrate chains – on the heavy chains of the antibody. They are added to the antibody during its maturation. Glycans control functions such as solubility, stability, and efficacy.[9] Their formation is strongly affected by external conditions such as antibody growth medium, pH and temperature. Therefore, meticulous control of the conditions during the production steps is required.[9] Between the fragments there is a hinge region, built up of polypeptide chains. It is the most flexible part of the antibody

and allows some flexibility to the Fabs. This is to contribute to better binding to the epitopes. All protein chains are connected with disulfide bonds, which also preserve the antibody's structural conformation.[2,7,8]

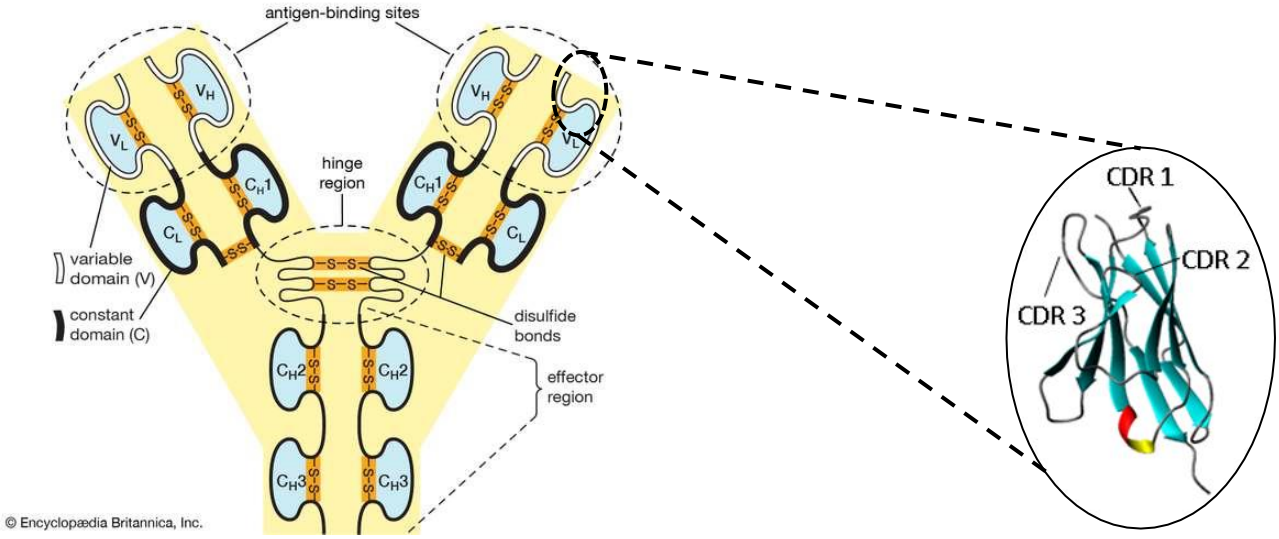


**Figure 2.** The general structure of an antibody (IgG type). Adapted from: https://www.britannica.com/science/antibody

To be able to manufacture mAbs and use them as active pharmaceutical ingredients (APIs), it is important to carefully characterise their structure and behaviour. Like with most proteins stability issues are common,[7,10,11] but there are ways of modifying the antibodies to ensure an improved quality of the product.[1,7,12,13] To ensure the quality of the product and avoid immunogenicity stability studies are performed, both during the development and for every batch produced. There are several techniques available though the most common one is size-exclusion chromatography (SEC).[14–16]

SEC has many advantages: it is a robust technique, it has often fast analysis times, the columns are commercially available, and the technique is easily coupled with different detectors such as fluorescence, diode-array and variable wavelength detector.[17–20] The main disadvantages is that SEC is mainly suitable for small size analytes, below 25 nm, and low analyte concentration. Aggregates of mAbs, which can be much larger, will be filtered out in the column frit and therefore cannot be analysed. Secondary interactions with the column, and analyte denaturation or aggregation during analysis may also occur.[15,21] These disadvantages can be minimised with thorough method optimisation and therefore the advantages of the well-known technique's availability and simplicity still makes it the most commonly used.[14]

To ensure the quality of the drug and be able to characterise all stability issues, the adopted methods need to be optimised not just on a "trial-and-error" approach but also verified by previous literature or orthogonal methods. Furthermore, the study must include the procedures and follow the requirements set by the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) and relevant agencies such as the United States Pharmacopeial Convention (USP) and the European Medicines Agency (EMA).

These organisations and agencies do not only control the quality of drugs but also the design of quality control studies.[22–25] Apart from the global organisations there are also consensuses regarding various areas in drug formulation. A relevant example of that is the European consensus regarding guidelines for the practical stability studies of anticancer drugs.[26] It was agreed upon during the European consensus conference regarding guidelines for the practical stability studies of anticancer drugs in 2010. The consensus was made to harmonise the way that stability studies should be made for anticancer drugs, including mAbs.[26]

Despite the existence of guidelines for stability studies, a recently published study that reviewed 25 mAb stability studies, found that almost half of them failed to follow the recommendations presented by European consensus[1,26]. This indicates a possible failure to studying all aspects of mAbs stability.[1] Evidently, if the published methods would be applied in clinical practice, it may lead to severe consequences for the patient.[1,26]

The objective of this review is to understand what parameters are often ignored in stability studies and to give a suggestion of comprehensive study design that covers all essential stability parameters. This will be done by studying the guidelines set by the ICH, and recommendations from the European consensus conference regarding guidelines for the practical stability studies of anticancer drugs (2010). To be able to present more detailed suggestions, recent studies regarding protein and mAb stability will be reviewed and the applications of their most important discoveries will be highlighted.

# 2 Methods

This review is based on published literature as of 2020-05-01, with a particular focus on 9 key studies and reviews. These studies cover different aspects of development in SEC and complimentary methods, with a common focus on analysis of mAbs. The studies were found on PubMed and PubMed Central by using following search strings: “SEC stability study”, “mAb stability”, “stability studies of monoclonal antibodies” and “antibody stability”.

The findings are reviewed and summarised in this report to get a better understanding of the recent development in SEC analysis. Furthermore, to provide a backbone for the presented results, the theory regarding antibody stability problems, so-called critical quality attributes (CQA), and how they can be analysed by SEC is given.

Relevant ICH guidelines[27–30] were studied and compared to the European consensus regarding guidelines for the practical stability studies of anticancer drugs [26]. This was done to provide an objective result of the existing guideline.

# 3 Antibody stability

Antibodies, like all proteins, tend to aggregate or degrade and lose their function if handled improperly. Stability problems during both development, handling, and storage are common. There is consequently a lot of research on how to maintain stability and ensure a good antibody quality.[9,14,17,31,32] Usually two types of instabilities are discussed, chemical and physical instabilities. Chemical instabilities include amongst other oxidation, deamidation, and alteration of the glycan structure which are described below in more detail. Physical instabilities are often concentrated on aggregation and denaturation of the protein. During the production, pH, structure modifications, the concentration of the mAbs in the solution and additives have a critical role on the stability of the antibody. Later, during the storage and use of the antibody therapeutics factors such as light, freeze-thaw cycles, packaging, and administration pathways have a major role. Temperature is a physical factor that affects the stability throughout the whole process from production to drug administration.[1,32] The development and production process for mAbs is complex and the substance requires a lot of caution in handling to avoid stability issues which could lead do a diminished therapeutic effect or even trigger an immunogenic response. It should also be noted that the impact of instabilities can have various consequences depending on where in the mAb structure they occur.[32] It is important to understand how antibody stability can be affected and altered to be able to fulfil the guidelines and the regulations on quality set by the legislating authorities. Furthermore, not required by the ICH guidelines, but also of great importance is the study of the effect of practical handling on stability. Stability issues that may occur during handling of the final storage container such as storage temperature changes and transportation, or preparation of the product such as dilutions and administration techniques should be included in a comprehensive study design[26]

Below is a brief description of the most common chemical and physical instabilities that can occur.

## 3.1 Chemical instabilities

### *3.1.1 Deamidation*

Deamidation is one of the most common chemical reaction causing destabilisation of the antibody. It is an reaction that can occur on asparagine and glutamine residues where the residue is removed or transformed into another functional group, with that altering the functionality of the antibody. The reaction does not need to be triggered by external factors, as also nearby amino acid residues may act as proton donors. This mechanism is pH-dependent and more neutral pH can slow down the reaction.[1,32]

### *3.1.2 Oxidation*

Oxidation is another very common cause of chemical instability. Oxidation can occur spontaneously, so-called auto-oxidation or in presence of an oxidising agent. According to Shire[32], oxidation on mAbs can occur on a variety of amino-acid residues: cysteine, histidine,

methionine, tryptophan, and tyrosine. Depending on where the oxidation occurs it may have different effects. Cysteine residue oxidation can lead to the formation of unwanted disulfide bridges that modify the protein structure.[32] Histidine residue oxidation is not well-researched but it may contribute to the formation of immunogenic aggregates according to a recent study.[32,33] Methionine residue oxidation is very common in all kinds of proteins. Methionine residue oxidation in the Fc region can alternate the binding properties of certain regions. This could affect the potency and blood circulation time of the mAb.[32] Tryptophan residue oxidation can lead to reduction and impairment of the protein function, such as decreased affinity and selectivity. This mostly affects antibodies with tryptophan residues on their CDRs. Tyrosine residue oxidation is very uncommon in mAbs. This is due to it is the ionised form of tyrosine that is susceptible to oxidation. With a pKa of around 10 for tyrosine residues, the ionised forms are rarely found in mAbs which are often formulated with a pH far below 10.[32]

### *3.1.3 Glycosylation*

Glycosylation is not causing chemical instability per se, but it impacts the chemical heterogenicity of the mAb and affect stability, solubility, efficacy, and other functional properties. Glycosylation is a post-translational modification that occurs in the eukaryotic cell during the production of mAbs.[9,13] It is an enzyme catalysed reaction[13] which ads glycans – carbohydrate chains, to specific amino acids.[9] The reaction is strongly influenced by the external conditions of the host cell, e.g. pH and temperature of the growth medium and the conditions are therefore needed to be monitored carefully. According to guidelines from EMA and USP, the glycosylation structure and patterns in mAbs should be characterised to ensure their stability.[9] Mass spectrometry (MS) combined with a chromatographic technique such as reversed-phase liquid chromatography (RP-HPLC) or SEC has previously been used for characterisation of glycans and determination of their patterns.[9,10,13]

## 3.2 Physical instabilities

### *3.2.1 Aggregation*

The most common physical cause of antibody instability is aggregation. It is the process of self-association of proteins. Aggregation process can be catalysed by chemical alteration but it may also arise due to conformational changes, such as protein folding.[32,34] The aggregates, or high molecular weights as they often are called, can form through various reactions and with different intermolecular bonding. There can both be reversible aggregates, where the proteins go back to their native form when the external conditions are changed, and non-reversible aggregates that will remain despite changes in external conditions such as pH or temperature.[13] Aggregated proteins tend too loose or change their functionality. Non-reversible aggregation may therefore lead to severe consequences for the patient.[1,9,34] Aggregates may form during any stage in an antibody's life and therefore need to be studied carefully during all steps from development to administration.[1,31,32,34] Due to that aggregation is highly dependent on both external and internal factors[1,31,32,34] the analysis methods need to be optimised not only to detect all kinds of aggregates but also to not contribute to aggregation during the analysis.

### *3.2.2 Denaturation*

Denaturation is conformational changes of the protein structure. Denaturation may have severe impacts on the stability and functionality of the protein. Proteins are folded into different structures, from the simplest alfa-helixes, which are amino acids chains linked by hydrogen bonds to the most complex quaternary structures, consisting of several amino acid chains tangled in each other due to intermolecular interactions. When a protein is exposed to stress such as mechanical forces or temperature changes it can unfold from its structure into ones of a lower order. This can also happen if the ionic strength of the solution is changed or due to spontaneous protein-solvent interactions.[32] mAbs, which are built up of quaternary structures,[1,32] can lose their functionality even with small conformational changes. Severe denaturation may contribute to fragmentation or aggregation due to exposure of unfolded protein chains. The effect of conformational changes is highly protein-dependent and need to be analysed for every protein to fully understand its instabilities.[32]

### *3.2.3 Fragmentation*

Fragmentation can both be grouped to chemical instabilities and physical instabilities. Chemical fragmentation may occur by hydrolysis or with the presence of enzymes, while physical fragmentation may occur due to mechanical stress. Fragmentation of mAbs occurs due to disruption of peptide or disulfide bonds. It results in full protein chain fragments or low molecular weight fragments. Those may later associate with other fragments and contribute to aggregation. Fragmentation in the hinge region is most common due to its flexibility and lower stability as compared to the protein chains. Fragmentation is uncommon in most formulations during recommended storage conditions and occurs mainly at extreme external conditions such as in high acidic solutions or high temperatures.[1,32]

# 4 Size-exclusion chromatography

### *4.1.1 Introduction to techniques for mAb stability studies*

There are a lot of techniques available for analysis of antibody stability. Techniques that are commonly used are ultracentrifugation, differential scanning calorimetry, dynamic light scattering, SEC and turbidity analysis.[17]

While the methods are many, SEC is the most common one both during development and for routine analysis.[14,35] This is mainly due to that it is a well-researched technique, with great robustness and fast result delivery. Apart from that, the components of a SEC system are the same as for an HPLC system, hence being available in most labs, with only the column differing. [14–18]

### *4.1.2 A brief background to size-exclusion chromatography*

SEC has been around for a long time and the increased interest in biopharmaceuticals during the 2000s gave it a further push in development and usage.[15,18] SEC is in theory based on only mechanical interactions between the sample and the packing material in the column. The SEC column is packed with porous particles. In short, the sample passes through the column and separation occurs since smaller analytes have a higher permeability through the pores compared to the larger ones. Smaller analytes spend a longer time in the columns and will therefore elute after a longer time as compared with larger analytes.[18,35] It is though hard to get the ideal SEC condition as described above and interactions between the analytes and stationary phases, so-called secondary interactions, are very common. This is one of the biggest disadvantages with SEC. The right choice of column, stationary phase, and mobile phase composition is therefore crucial to have a good separation. By making the right choice of method parameters, secondary interaction can often be minimised. Method parameters are different for every analyte, but through literature study, method optimisation and common "trial-and-error" approach they can be found for most substances of interest.[35]

### *4.1.3 SEC columns*

One of the most important parameters in the SEC method is the choice of column. Physical properties such as length, inner diameter, particle size, stationary phase chemistry, pore size and structure will affect the outcome of the analysis and should be chosen wisely for best performance. The length of the column affects both analysis times and resolution. A longer column gives a larger void volume, compared to a shorter one operating at the same flowrate, it increases retention times of the eluting analytes but also contributes to better resolution due to increased number of theoretical plates.[16,18,35] There is a frit in every column that filter out large impurities in the sample to prolong the columns life-time. In the case of aggregate studies, this is a disadvantage. Large aggregates will be filtered out by the frit which makes it impossible to detect all aggregates in a sample by only using SEC.[16,21,36]

SEC can be combined with different detectors depending on the analysis. The most common one for mAb studies is a variable wavelength detector (VWD) which can scan from 190 up to

900 nm.[37] 214 nm or 280 nm is often a good choice when studying large proteins and those wavelengths are often used in stability studies of mAbs.[10,12,14,16,19,20,32,38,39] A fluorescence detector can be used as well to be able to detect proteins at low concentrations.[16,19,32] The detector excites the analytes at a specific wavelength, often around 280 nm, and measures their emission in the excited state at a set wavelength, which is between 300 and 370 nm.[16] A study by Jirjees et al.[19] showed that it is particularly useful for tryptophan-rich antibodies at low concentration.[19] Other detectors that can be coupled with SEC columns are refractive index detector, multi-angle light scattering (MALS) detector and viscometer.[16]

Combing SEC with MS is another possibility to collect detailed information on the separated mAbs, including aggregate types, glycosylation patterns and other chemical modifications.[10,13,16] Although being an exceedingly powerful detector in mAb analysis, it requires a lot of optimisation, in particular, to find a mobile phase that works well in both SEC and MS. While stability analyses with SEC benefits from aqueous non-volatile mobile phases with high ionic strength, MS require volatile mobile phases with low ionic strength to avoid ionisation suppression and salt precipitation in the ionisation source.[16,20,40]

#### *4.1.3.1 The stationary phase*

The SEC columns are packed with small porous beads with well-defined pore size. The analytes can diffuse in and out of these pores and the analytes with a smaller hydrodynamic radius can penetrate deeper into the pores, thus increasing its elution volume.[16,18,35] The right choice of particle size and pore size is essential for good analysis. If the pores are too small a lot of particles will be eluted with the interstitial volume leading too poor resolution.[16] It is also important to have a large pore volume per unit column volume. This increases the volume in the particles where the analytes can diffuse into and thus contribute to better resolution between peaks.[35] The typical particle sizes to use when analysing proteins around 150kDa, such as mAbs, are 3–10 µm with a pore size of 200–300 Å, but even smaller particles exist on the market.[16] The smaller the particles, the better resolution and peak shape can be acquired, but the cost of that is a higher back pressure which has a negative effect on separation due to band broadening. High backpressure can also create leakage.[16,35]

The two most common stationary phase types are inorganic silica-based particles and cross-linked polymeric particles. The silica-based stationary phases can be naked or coated with modification such as dextrans.[16,40,41] The polymeric particles are available in many different variations and can be both hydrophobic, hydrophilic, or ionic in nature.[16] The stationary phase should therefore be chosen with consideration to the analytes' hydrodynamic volume, and their chemical properties.[16,35,40]

#### *4.1.3.2 The mobile phase*

The mobile phase transports the analyte through the column. Its composition strongly impacts the outcome of the analysis. While some mobile phases preserve the stability of the analytes others can contribute to secondary interactions and impair the peak symmetry.[19,20,42] Buffers consisting of an acid and a salt containing the conjugate base are common to use in SEC

analysis of mAbs to preserve the proteins' stability.[14,15,19,20,33,42] Mobile phase effects are analyte dependent and should therefore be evaluated for each analyte.[20,42] The ionic strength and pH of the mobile phase will directly affect the analytes distributions between the stationary and mobile phase, leading to variations in the distribution constant ($K_d$).[42] While low ionic strength may be favourable when using SEC/MS it may contribute to secondary interactions and therefore shift the $K_d$ between analyses.[20,33,40,42]

# 5 Regulatory guidelines on stability testing of new drugs

## 5.1 ICH, USP and EMA

ICH is a global organisation with a mission to harmonise the production and registration of drugs to be able to ensure safe and efficient drug development with high-quality drugs as an outcome.[25] ICH has set up guidelines regarding quality, safety, and efficacy of drugs and other multidisciplinary guidelines that do not fit in only one category.[43] At regional levels there are agencies around the world such as EMA and USP. These agencies adapt the ICH guidelines to regional standards. They set standards and supervise drug development and productions, to ensure the safety and quality of the products. EMA and USP have their own pharmacopoeias that contain the required standards that every product in the pharmaceutical industry must fulfil, together with descriptions of the ICH guidelines, adapted for the specific region.[44,45] Even though guidelines are not legally binding, the companies in charge of a medicine can be held responsible for if the guidelines are not followed. Local agencies such as the U.S. Food & Drug Administration (FDA) control the compliance with the ICH guidelines and may stop medicine from reaching the market. To give an overview of what is expected from ICH to perform a well-designed stability study, the relevant guidelines are summarised in section 6.2.1.

## 5.2 European consensus regarding guidelines for the practical stability studies of anticancer drugs

In 2010, a European conference consensus was held with the goal was to define specific requirements that need to be met when designing a stability study for anticancer drugs. This was due to that they found that most stability studies only focus on fulfilling the legislation requirements, thereby often failing to evaluate the stability issues in the clinical setting. As these parameters are not included in the general guidelines the consensus proposed more specific requirements for anticancer drugs, including mAbs.[26]

The recommended requirements from the consensus are presented in section 6.2.2.

# 6 Results

## 6.1 Results and findings in recent studies

### *6.1.1 The importance of a comprehensive stability study*

In an extensive review by Le Basle et al.[1], the authors gathered information from 25 studies on mAb stability to get an overview of how companies perform their stability studies to address the recommendations from regulatory agencies. The authors suggest that a lot of the companies, which were responsible for the reviewed studies, failed in making a suitable and sufficient stability analysis on their drugs. Of the 25 studies, just about half were found to, at least to a reasonable level, follow the guidelines in the European consensus for stability[1,26], with the rest only partially covering relevant stability issues. There most likely are more studies made regarding a specific drug that are not in the public domain. They may complement some studies and contribute to a more extensive analysis. Either way, it is of great importance that protein stability studies, for commercially used drugs, cover as much stability characteristics as possible.[1]

### *6.1.2 The power of complementary methods to cover all types of aggregates*

Soluble aggregates can cover a particle size range from nanometres to visible particles (>10μm).[21] SEC alone cannot cover all types of impurities no matter what detector is used. This due to that large particles can get stuck in the frit in the inlet of the column and will therefore be filtered out from the analysis. A combination of analytical techniques can be used to cover the whole spectrum of aggregates. In a recent study, Bansal et al.,[21] suggested combining SEC, dynamic light scattering (DLS) and micro-flow imaging (MFI). The setup is shown in Figure 3. A prior analysis using DLS was first performed on the test sample, to determine the aggregates sizes. They were later analysed by one of the three techniques depending on their size. An advantage of using this combination of techniques is the overlapping analysis ranges: 10–25 nm for SEC-DLS and 2–5 μm for DLS-MFI, (Figure 3). The methods credibility can therefore be tested by a linear correlation analysis in those ranges, which will enhance the credibility of the analysis.

In summary, using complementary methods that cover the whole spectrum of aggregates can give a great advantage in understanding the effects of different buffers, formulations, and methods. Even though a combination of other analytical techniques are possible, the SEC-DLS-MFI platform is the newest and perhaps the most powerful one for mAb stability analyses.[21]

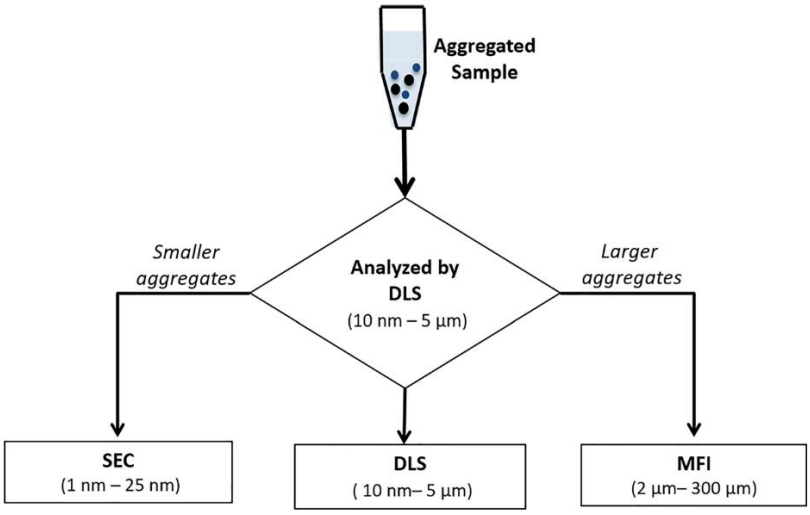


**Figure 3.** Flowchart depicting the procedure of analysis of aggregated samples via different characterization tools according to their sizes (Bansal et al. 2019)

### *6.1.3 Effects of forced degradation of mAbs*

Forced degradation studies are performed to measure the extreme point of different parameters and how they affect the mAb. Two recently published studies found that the effects are antibody-dependent. This highlights the importance of antibody characterisation to be able to understand the effects. The results of the forced-degradation study showed that all types of mAbs were affected by both aggregation and fragmentation. Dilution has a strong negative effect on stability and the most diluted mAb solutions showed severe stability issues.[14] Freeze-thaw cycles affected some mAbs more than others with fragmentation as the most common cause of instability. Freeze storage, without freeze-thaw cycles, was found to cause degradation to the mAbs in itself. Thus, making it an essential parameter to include in stability studies.[14] Long term storage was found to impact the stability differently depending on the storage temperatures. mAbs at low-temperature storage (~5°C) forms mostly non-reversible aggregates while at 37°C only fragmentation has been observed, (Figure 4).[19]

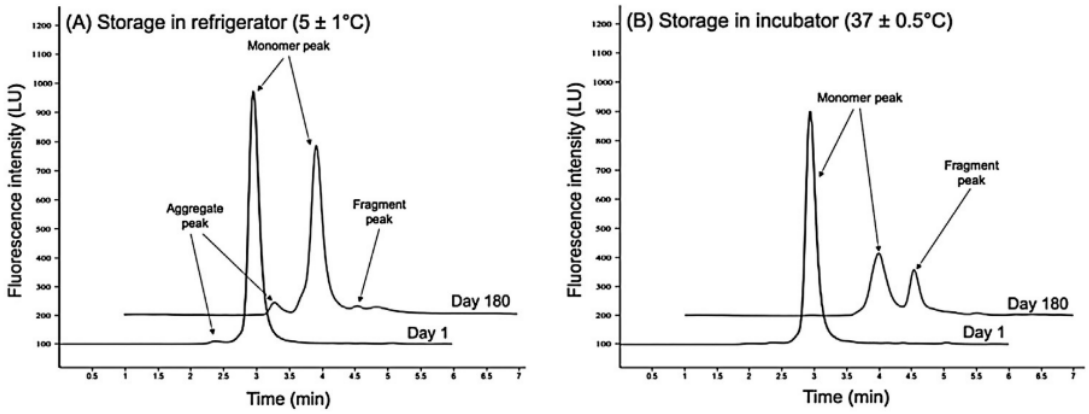


**Figure 4.** Chromatograms of bevacizumab solutions (10 µg/mL) stored in (A) refrigerator at 5 ± 1 ◦C and (B) incubator at 37 ± 0.5 ◦C (Jirjees et al. (2019))

Mechanical stress can often occur during the handling of antibodies. mAbs, like many other proteins, are especially susceptible to mechanical stress. Therefore, it is important to handle mAbs with care and avoid shaking and stirring to preserve their stability

### *6.1.4 Excipients effect on stability*

Excipients are often added to the formulation to maintain mAb stability. In one study, the impact of several different dextrans and sucrose on mAb stability was investigated using SEC coupled to electrospray ionisation mass spectrometry (SEC/ESI-MS). Interestingly, they found sucrose to be without impact on mAb stability, whereas all investigated dextrans caused a time- and temperature-dependent aggregation mainly due to increased covalent glycation (Figure 5). Hence, the impact of excipients on mAb stability need to be assessed, not only during the developmental phase but also continuously during storage and handling.[12]

### *6.1.5 Metal-protein interactions and their effects on the protein stability*

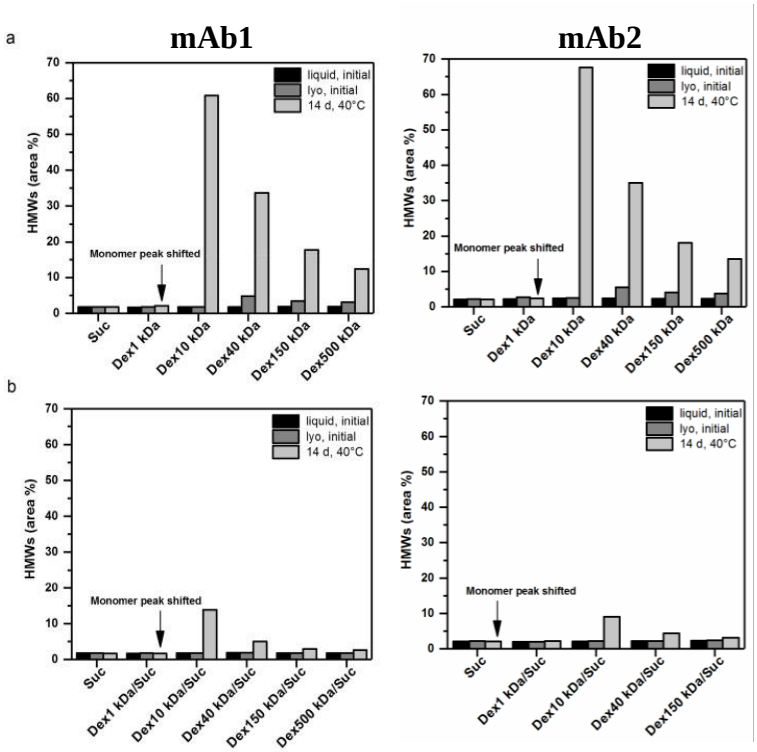


**Figure 5.** Amount of high molecular weights of mAb1(left) and mAb2 (right) by SE-HPLC. 10 mg/mL mAb2 formulated with 80 mg/mL of either (a) pure dextran or sucrose or (b) 1:1 dextran/sucrose mixtures. (Haeuser et al., (2020))

While some proteins require metals for stability and functionality, others like mAbs can get severe stability problems if metals are accidentally introduced during production or storage.[33] mAb-metal interactions can be assessed by SEC/inductively coupled plasma mass spectrometry (SEC/ICP-MS). [33,40] Two recently published studies in this subject found that the right mobile phase concentration, pH, and stationary phase composition is beneficial for a successful analysis. [33,40] While a high ionic strength is favourable for SEC analysis to avoid secondary interactions, it may contribute to ionisation suppression and contamination of the

ion source. A mobile phase buffer concentration of 100 mM yields the best results during the analysis of mAb-metal complexes by SEC/ICP-MS. [33,40] The effect of the pH of the mobile phases were found to be antibody-dependent. Some studies recommend a pH which is close to the isoelectric point of the protein for mAb stability analysis by SEC,[19,20,42] but a lower pH can be beneficial for some mAbs when studying mAb-metal interactions. It should be noted though that a low pH may suppress metal-protein interactions and cause aggregation which will lead to inaccurate results. [33,40] mAb-metal analysis should be performed on dextran-based columns. Silica-based columns can contribute to secondary interactions and unwanted metal-mAb interactions and are therefore not recommended.[40]

### *6.1.6 Impact of buffers and solvents on mAb stability*

As shortly mentioned above, the choice of mobile phase composition strongly affects both the analysis and the mAb stability.[19,20,33,40,42] Ventouri et al.[42] compared the effect of various mobile phases on myoglobin (Figure 6), and found that lower ionic strength (< 100 mM) gives a variation in distribution coefficients ($K_d$) when the pH is changed. This indicates that interactions take place between the protein and the stationary phase.[42] Even though a higher ionic strength seems favourable for the SEC analysis (Figure 6) it is unsuitable in an analysis by MS.[16,20,40] This should be considered when development of the analytical method

Looking at the pH of the buffers, a pH close to the isoelectric point of the mAb is beneficial for peak symmetry and mAb stability.[20,42] Same results have been observed in a study where SEC-fluorescence was used.[19] Though, this observation may not be applicable for all mAbs.

The composition of the buffer salts plays a major role for the analysis, especially when using MS. Ventouri et al.[42] who studied the effects of ammonium acetate, ammonium formate, and ammonium bicarbonate based buffers, found that ammonium acetate is the most effective one when it comes to protein preservation and system compatibility. This is regardless of the ionic strength and the pH conditions. Ammonium formate showed a much higher denaturation effect of the proteins during the analysis. Ammonium bicarbonate was the worst buffer of the three and gave a high contribution to irreversible denaturation. It should be noted that Ventouri et al.[42] used pH 7.5 when studying effects of ionic strength on the chromatographic profiles (Figure 6 (A)). pH 7,5 is not covered by any of the ammonium salt-based buffers[46,47] which means that small changes in the pH may alter the charge of the protein. This can influence the protein's interaction with the stationary phase and affect the chromatographic profile. This aspect and an explanation to the specific effects of the ammonium salts based buffers is not further described in the article.[42] Phosphate-based buffers have a buffer range around 7[46,47] and can be used in analyses by SEC. They have been found to give sharp peaks which allow detection at very low concentrations but[20] they are though not volatile and may easily may soil the ion source.[20,42]

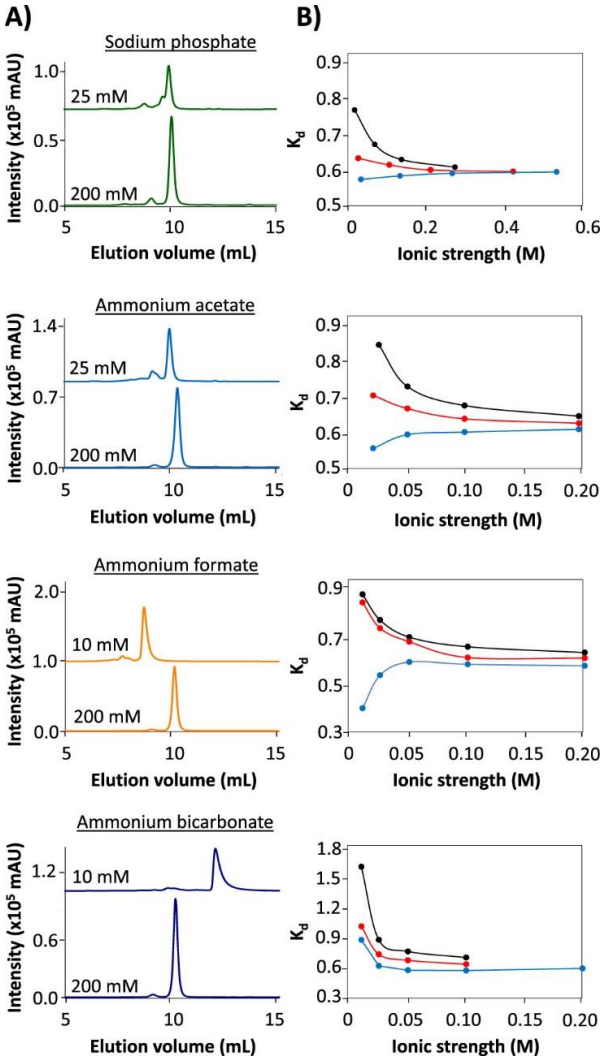


**Figure 6.** SEC-UV of myoglobin using different eluents varying in nature of salt, ionic strength, and pH. (A) Typical chromatograms obtained with eluents of low and high ionic strength, all at pH 7.5. (B) Plots representing the Kd observed for myoglobin versus the eluent ionic strength using the indicated salt. Eluent pH: 5.9 (black), 6.9 (red), and 7.5 (blue). The connecting lines between the points highlight trends and are not

The effects of the mobile phase will strongly depend on the physical and chemical properties of the protein.[20,42] It is therefore important to get a broader understanding of these interactions on an individual level of each protein. The choice of mobile phase should therefore be carefully thought out.

## 6.2 Relevant requirements and guidelines to perform a comprehensive stability study of mAbs

### *6.2.1 Summary of ICH guidelines regarding design of stability studies*

There are general requirements regarding new drugs stated in guideline Q1A(R2).[27] Examples of that are as follows: both the API and the final product should undergo stability studies; tests should be done on three separate batches; stress testing should be performed for at least one batch and should test pH, temperature and humidity effects, along with oxidation and

photolysis.[27] Furthermore, accelerated stability tests and long-term storage test should be included and performed in containers similar to the final container. The recommendations for the storage test conditions regarding new drugs are though difficult to apply on biopharmaceuticals due to a high variety in their shelf-life, from days to years.[27,29]

The guidelines Q5C and Q6B addresses quality specifications and stability tests of biopharmaceuticals.[29,30] They state that biopharmaceuticals require a variety of assays in order to characterise their physicochemical properties and thus it is difficult to recommend specific analysis methods. The suggested stability test specifications stated in guideline Q1A(R2)[27] may not be appropriate for all types of biopharmaceuticals.[29] Q5C recommends that characteristics such as potency, purity, structural characterisation (including impurities), conformational changes, and sterility should be included. Effect of additives, stress conditions, impurities and interactions with storage container should also be investigated.[29]

Regarding the testing conditions for storage testing, accelerated studies and testing frequency the guideline states that they should be "carefully selected on a case-by-case basis" by the manufacturer. This of course, after agreement with relevant legislation agencies.[29] Q5C and Q6B guidelines highlight the importance of a credible analysis and therefore recommends that more than one technique is used for physicochemical characterisation of the drug. [29,30] Note that all methods used in the study design need to be validated in compliance with the ICH guideline Q2(R1).[28]

### *6.2.2 Additional requirement regarding anticancer drugs recommended by the European conference consensus (2010)*

The European conference consensus found a great need for additional stability data regarding these drugs.[26] The consensus proposed that the stability study design regarding anticancer drugs should include tests to evaluate the effect of practical handling on stability. This refers to condition changes during transportation, preparation of drugs and storage in non-sterile environments. The consensus suggests that light, temperature, and humidity in the study facility should be defined. Ambient light is recommended to use where applicable. In addition to existing storage specifications, the drug should be analysed in syringes, plastic polyethene bags and other relevant containers used for administration of the drug. Concentrations variations and interactions with the storage container such as adsorptions should be evaluated. Accidental condition changes in temperature, humidity or light exposure which can occur in practical situations should also be included. Moreover, the stress test should assess all parameters stated in the guidelines thoroughly, along with an evaluation of API-formulation solution interactions and various relevant mechanical stress tests.[26]

Regarding the study of chemical stability, there are several recommendations presented in the consensus. In conformity with the ICH guidelines, the consensus recommends using several techniques to increase credibility, due to the possibility of studying the whole spectrum of aggregates. The degradation products (DPs) should be identified and quantified to the highest degree possible due to that some DPs can be toxic even in smaller amounts. A successively increasing forced degradation is strongly recommended to be able to observe the progression

of degradation. Furthermore, sterility studies should be enhanced to get a better understanding of the drugs' resistance to microbiological contaminants.

Due to that mAbs are a big part of the cancer therapeutics sector many of the presented recommendations can directly be applied to them. These recommendations may thus also fit for general mAbs stability studies. The additional requirements presented in the consensus may allow hospital staff may to prepare the drugs in advance and use the same preparation during a longer time, for example for long-time therapies. This will not only facilitate their work but also be economically favourable for the hospitals.[26]

### 6.3 Proposed stability study design

With the information presented above, it has been found that a comprehensive stability study, include the presented parameters in Table 1. Note that this stability study design is directed primarily to the studies done during the development process, which are more extensive than routine controls. The proposed study design also gives examples of what respective analytical techniques can be used. The presented techniques are the ones used in or recommended by the reviewed literature, but other suitable techniques can be used as well. As highlighted by the ICH guidelines[27,29], all test parameters should be checked both for the API in the bulk and for the final product in its storage container. For specifications such as the length of the study, testing frequency, sample and batch selection, it is advised to follow the set recommendations from ICH[27,29,30] and proposed suggestions by the European conference consensus regarding stability studies for anticancer drugs[26].

**Table 1**
Proposal of essential testing parameters for a comprehensive stability study and examples of analysis methods available.

| General Physicochemical test parameters | Analytical techniques |
|---|---|
| **Characterisation of the API** | ELISA, SEC-MS, LC-MS, capillary electrophoresis (CE), SDS-PAGE[26,29,48] |
| **Conformational changes (aggregation, fragmentation, denaturation etc.)** | SEC,[12,21] DLS, MFI,[21], turbidimetry[26]<br><br>Visual examination for opalescence and colour change[26,29,49] |
| **Characterisation of impurities** | Metal – SEC-ICP MS[33,40]<br><br>DPs – SEC-MS, Ion exchange chromatography (IEC), CE[26]<br><br>Others - SEC-MS, high performance thin layer chromatography (HPTLC), CE[26] |

| | |
|---|---|
| **Potency** | SEC,[12] ELISA[48] |
| **Stability of solutions and excipients** | SEC-ESI-MS for both quantification and characterisation[12] |
| **Light** | Photostability assays[29,50] |
| **Forced degradation studies (temperature, photostability, freeze-thaw cycles, mechanical stress, oxidation, low/high pH, glycation)** | SEC,[14,26,51] LC-MS[51] RP-HPLC,[7,51]IEC,[26,51] CE,[26,51] |
| **Mechanical stress (stirring, shaking, and shearing) in practical stability studies** | SEC,[19,20,42] MALS, LC-MS[16] |
| **Temperature effects (long term)** | See Conformational changes and Characterisation of impurities |
| **Humidity effects (long term)** | See Conformational changes and Characterisation of impurities |
| **Sterility (long term)** | For sterility studies: Sterility test assays provided by USP or EMA.<br><br>To study effects of broken sterility: See Conformational changes and Characterisation of impurities. |
| **Concentrations variations of the API (long term)** | Weighing of containers and analysis by SEC-MS[26] |
| **Frozen storage (long term, if applicable)** | See Conformational changes and Characterisation of impurities |

Furthermore, it is important to have a well-developed and validated method in order to conduct credible analysis and to understand how the protein behaves during analysis.[20,42] The quality of analysis is directly dependent on the method parameters. SEC is recommended to use for stability studies during both development and routine analysis.[14,16,18,19] Correctly adjusted parameters such as the mobile phase composition, temperature, and analysis time is not only essential for the analysis but also for the preservation of the mAb stability. For example, a mobile phase pH far from a mAbs physiological pH or too high column temperatures may degrade the antibody during the analysis and give inaccurate results of its stability.[14,19,20,33,40,42] Dilution solutions and sample buffers used to store the mAb may have a major effect on the mAbs' stability and should therefore always be evaluated. This can be done by comparing several buffers or alternating the buffer's pH to find the one that affects the mAb the least.[19] Mobile phase buffers may contribute to secondary interactions and decrease the stability of an antibody during analysis. The choice of mobile phases is both method and analyte dependent, but several studies recommend mobile phases with a pH close to but not above the physical pH.[19,20,42]SEC coupled with MS, ICP or fluorescence detectors can be favourable for improved analyses but important to remember to use mobile phase

buffers with volatile salts when using MS or ICP. [20,33,40,42] By altering pH and composition, mobile phase buffers can be evaluated to find the most suitable one for the analysis.

# 7 Discussion

The problems of insufficient stability studies that Le Basle et al.[1] mention, which is also brought up in the European conference consensus,[26] seem to originate from the lack of descriptive study designs by the ICH. The guidelines regarding biopharmaceuticals often state that the methodologies used or the specification set should be determined on a "case-by-case basis".[29,30] Due to a large characteristic, functional and structural variation among biopharmaceuticals it is understandable why the ICH chose to generalise the descriptions. The regional legislation agencies may have stricter, more detailed regulations that the guidelines but according to the consensus they are not enough to fulfil the evaluation of stability issues in practical situations.[26] According to the consensus it is of great interest to see how a certain protein reacts to stresses that can be found in a clinical environment where the drugs are prepared and administered.[26] As mentioned earlier, the recommendations from the consensus regards mainly the drugs used for anticancer treatment, but due to that many of them are biopharmaceuticals, or more precisely mAbs, it is possible to assume that these test can be adapted for mAbs in general. The proposed stability study design focuses on including all important parameter in order to make a comprehensive stability study of mAbs. Due to a lot of similarities between mAbs regarding storage and handling, the proposed study design should be applicable to the vast majority of mAbs. It should be noted that well-designed stability studies may signify more work for the manufacturer meaning higher production costs. On the other hand, it may also contribute to a better understanding of the drugs CQA and perhaps a more descriptive labelling of the product, which may facilitate the work of hospital personnel. To achieve better stability studies the guidelines and regulations from legislation agencies need to be improved and that may take a long time. Though are consensuses such as the European consensus regarding guidelines for the practical stability studies of anticancer drugs a good step on the way for better stability studies.

Hopefully, this report may contribute to the development of better stability studies which include all essential parameters and may shine a light of the methods that can be used to study them.

# 8 Conclusion

There is a need for development in stability study designs regarding mAbs with more focus on the stability during routine clinical use and storage. It is difficult to make precise guidelines that will suit all biopharmaceuticals, or all mAbs for that matter, due to their complexity and difference in structural and functional characteristics. However, the ICH guidelines are too general about the stability study design of pharmaceuticals, which leave a lot of freedom to the manufacturer. In order to create more comprehensive stability studies new recommendation may have to be added, which may contribute to a harmonisation of the stability study designs.

# 9 Future aspects

For future studies, it should be further researched if there are any more important parameters, that could have been missed in this report, to include in stability studies of mAbs. It could be further investigated if it is possible to provide a general method for all mAbs or if it better to separate the biopharmaceuticals by structure or by disease, as done in the article by Bardin et al.[26] Furthermore, it would be interesting to find out if the European conference consensus regarding stability studies for anticancer drugs has been approved by the EMA and how it has affected the stability studies of anticancer drugs since it was published. Finally, a further examination whether there are consensuses like this for other diseases or for certain types of mAbs would be relevant to do, to get a broader understanding of the problem and how authorities work to harmonise the stability study designs.

# 10 References


1. Le Basle Y, Chennell P, Tokhadze N, Astier A, Sautou V. Physicochemical Stability of Monoclonal Antibodies: A Review. *J Pharm Sci*. 2020;109(1):169-190. doi:10.1016/j.xphs.2019.08.009

2. Chiu ML, Goulet DR, Teplyakov A, Gilliland GL. Antibody Structure and Function: The Basis for Engineering Therapeutics. *Antibodies*. 2019;8(4):55. doi:10.3390/antib8040055

3. La Merie Publishing. Biologics sales reach another all time high in 2018 driven by innovative antibodies. Published April 4, 2019. Accessed October 25, 2020. https://lamerie.com/biologics-sales-reach-another-all-time-high-in-2018-driven-by-innovative-antibodies/

4. IndustryARC. Biopharmaceutical Market Research Report: Market size, Industry outlook, Market Forecast, Demand Analysis,Market Share, Market Report 2019-2025. Accessed October 25, 2020. https://www.industryarc.com/Report/9586/biopharmaceutical-market.html

5. Tsumoto K, Isozaki Y, Yagami H, Tomita M. Future perspectives of therapeutic monoclonal antibodies. *Immunotherapy*. 2019;11(2):119-127. doi:10.2217/imt-2018-0130

6. ACTIP. Monoclonal Antibodies Approved by the EMA and FDA for Therapeutic Use. Accessed October 25, 2020. https://www.actip.org/products/monoclonal-antibodies-approved-by-the-ema-and-fda-for-therapeutic-use/

7. Wang W, Singh S, Zeng DL, King K, Nema S. Antibody Structure, Instability, and Formulation. 2006;101(7):2271-2280. doi:10.1002/jps

8. Janeway CAJ, Travers P, Walport M, Shlomchik MJ. The structure of a typical antibody molecule. *Immunobiol Immune Syst Heal Dis*. 2001;5. Accessed October 25, 2020. https://www.ncbi.nlm.nih.gov/books/NBK27144/

9. Oshinbolu S, Wilson LJ, Lewis W, Shah R, Bracewell DG. Measurement of impurities to support process development and manufacture of biopharmaceuticals. *TrAC - Trends Anal Chem*. 2018;101:120-128. doi:10.1016/j.trac.2017.10.026

10. Wang Y, Li X, Liu YH, et al. Simultaneous monitoring of oxidation, deamidation, isomerization, and glycosylation of monoclonal antibodies by liquid chromatography-mass spectrometry method with ultrafast tryptic digestion. *MAbs*. 2016;8(8):1477-1486. doi:10.1080/19420862.2016.1226715

11. Razinkov VI, Treuheit MJ, Becker GW. Accelerated formulation development of monoclonal antibodies (MABS) and mab-based modalities: Review of methods and tools. *J Biomol Screen*. 2015;20(4):468-483. doi:10.1177/1087057114565593

12. Haeuser C, Goldbach P, Huwyler J, Friess W, Allmendinger A. Impact of dextran on thermal properties, product quality attributes, and monoclonal antibody stability in freeze-dried formulations. *Eur J Pharm Biopharm*. 2020;147(February 2019):45-56. doi:10.1016/j.ejpb.2019.12.010

13. Liu H, Caza-Bulseco G, Faldu D, Chumsae C, Sun J. Heterogeneity of monoclonal antibodies. *J Pharm Sci*. 2008;97(7):2426-2447. doi:10.1002/jps.21180

14. Hernández-Jiménez J, Martínez-Ortega A, Salmerón-García A, et al. Study of aggregation in therapeutic monoclonal antibodies subjected to stress and long-term stability tests by analyzing size exclusion liquid chromatographic profiles. *Int J Biol Macromol*. 2018;118:511-524. doi:10.1016/j.ijbiomac.2018.06.105

15. Arakawa T, Ejima D, Li T, Philo JS. The critical role of mobile phase composition in size exclusion chromatography of protein pharmaceuticals. *J Pharm Sci*. 2010;99(4):1674-1692. doi:10.1002/jps.21974

16. Fekete S, Beck A, Veuthey JL, 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

17. Lowe D, Dudgeon K, Rouet R, Schofield P, Jermutus L, Christ D. *Aggregation, Stability, and Formulation of Human Antibody Therapeutics*. Vol 84. 1st ed. Elsevier Inc.; 2011. doi:10.1016/B978-0-12-386483-3.00004-5

18. Hong P, Koza S, Bouvier ESP. A review size-exclusion chromatography for the analysis of protein biotherapeutics and their aggregates. *J Liq Chromatogr Relat Technol*. 2012;35(20):2923-2950. doi:10.1080/10826076.2012.743724

19. Jirjees F, Soliman K, Wang Y, et al. A validated size exclusion chromatography method coupled with fluorescence detection for rapid quantification of bevacizumab in ophthalmic formulations. *J Pharm Biomed Anal*. 2019;174:145-150. doi:10.1016/j.jpba.2019.05.038

20. Vandenheede I, Sandra P, Sandra K. Denaturing and Native Size- Exclusion Chromatography Coupled to High-Resolution Mass Spectrometry for Detailed Characterization of Monoclonal Antibodies and Antibody–Drug Conjugates. *LCGC Eur*. 2019;32(June):304-312. https://web.a.ebscohost.com/ehost/detail/detail?vid=0&sid=1902c702-cf36-4594-9cae-dc3b3df85b10%40sessionmgr4006&bdata=JnNpdGU9ZWhvc3QtbGl2ZQ%3D%3D#db=bth&AN=136972471

21. Bansal R, Gupta S, Rathore AS. Analytical Platform for Monitoring Aggregation of Monoclonal Antibody Therapeutics. *Pharm Res*. 2019;36(11):1-11. doi:10.1007/s11095-019-2690-8

22. European Medicines Agency. EMA official website: What we do. Accessed October 25, 2020. https://www.ema.europa.eu/en/about-us/what-we-do

23. European Medicines Agency. EMA official web site: Who we are. Accessed October 25, 2020. https://www.ema.europa.eu/en/about-us/who-we-are

24. United States Pharmacopeial Convention. USP offisial website: USP and FDA Working Together to Protect Public Health. Accessed October 25, 2020. https://www.usp.org/about/public-policy/usp-fda-roles

25. The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use. ICH official web site: Mission. Accessed October 25, 2020. https://www.ich.org/page/mission

26. 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

27. International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use. ICH Q1A (R2) Stability testing of new drug substances and drug products. Published online 2003. Accessed October 25, 2020. https://www.ich.org/page/quality-guidelines

28. International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use. ICH Q2 (R1) Validation of analytical procedures: text and methodology. Published online 2005. Accessed October 25, 2020 https://www.ich.org/page/quality-guidelines

29. International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use. ICH Q5C Stability testing of biotechnological/biological products. Published online 1995. Accessed October 25, 2020 https://www.ich.org/page/quality-guidelines

30. International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use. ICH Q6B Specifications: Test procedures and acceptance criteria for biotechnological/biological products. Published online 1999. Accessed October 25, 2020 https://database.ich.org/sites/default/files/Q6B Guideline.pdf

31. Johnson M. Antibody storage and antibody shelf life. *Mater methods*. 2012;2(120):1-9. doi://dx.doi.org/10.13070/mm.en.2.120

32. Shire SJ. Stability of Monoclonal Antibodies (MAbs). *Monoclonal Antibodies.* 2015. doi:10.1016/b978-0-08-100296-4.00003-8

33. Luo Y, Ronk M, Joubert MK, Semin D, Nashed-Samuel Y. Determination of interactions between antibody biotherapeutics and copper by size exclusion chromatography (SEC) coupled with inductively coupled plasma mass spectrometry (ICP/MS). *Anal Chim Acta*. 2019;1079:252-259. doi:10.1016/j.aca.2019.06.047

34. Wang W, Roberts CJ. Protein aggregation – Mechanisms, detection, and control. *Int J Pharm*. 2018;550(1-2):251-268. doi:10.1016/j.ijpharm.2018.08.043

35. Chakrabarti A. Separation of monoclonal antibodies by analytical size exclusion chromatography. *Intech*. 2016;i:13. doi:http://dx.doi.org/10.5772/57353

36. Carpenter JF, Randolph TW, Jiskoot W, Crommelin DJ, Middaugh C, Winter G. Potential inaccurate quantitation and sizing of protein aggregates by size exclusion chromatography: essential need to use orthogonal methods to assure the quality of therapeutic protein products. *J Pharm Sci*. 2009;99:2200-2208. doi:10.1002/jps

37. Thermo Fisher Scientific. The collective power of chromatography LC. *Thermo Sci Prod Specif*. Published online 2019. doi:10.1353/dss.2018.0031

38. Bond MD, Panek ME, Zhang Z, Wang D, Mehndiratta P, Zhao H, Gunton K, Ni A, Nedved ML, Burman S, Volkin D. Evaluation of a Dual-Wavelength Size Exclusion HPLC Method With Improved Sensitivity to Detect Protein Aggregates and Its Use to Better Characterize Degradation Pathways of an IgG1 Monoclonal Antibody. *J Pharm Sci*. 2009;101(7):2271-2280. doi:10.1002/jps

39. 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

40. Lago L, Thomas ORB, Roberts BR. Choice of mobile phase: Implications for size exclusion chromatography-inductively coupled plasma-mass spectrometry analyses of copper, zinc and iron metalloproteins. *J Chromatogr A*. 2020;1616:460806. doi:10.1016/j.chroma.2019.460806

41. Santarelli X, Muller D, Jozefonvicz J. Dextran-coated silica packings for high-performance size-exclusion chromatography of proteins. *J Chromatograpy*. 1988;443:55-62.

42. Ventouri IK, Malheiro DBA, Voeten RLC, et al. Probing Protein Denaturation during Size-Exclusion Chromatography Using Native Mass Spectrometry. *Anal Chem*. 2020;92(6):4292-4300. doi:10.1021/acs.analchem.9b04961

43. The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use. ICH official web site: ICH Guidelines. Accessed October 25, 2020. https://www.ich.org/page/ich-guidelines

44. EDQM - European Directorate for the Quality of Medicines. European Pharmacopoeia (Ph. Eur.) 10th Edition. Accessed October 25, 2020. https://www.edqm.eu/en/european-pharmacopoeia-ph-eur-10th-edition

45. United States Pharmacopeial Convention. USP offisial website: About U.S. Pharmacopeia. Accessed October 25, 2020. https://www.usp.org/about

46. Sigma-Aldrich. Non-volatile and volatile buffer systems. Accessed November 4, 2020. https://www.sigmaaldrich.com/technical-documents/protocols/biology/ion-exchange-chromatography/non-volatile-and-volatile-buffer-systems.html

47. Kromasil. Frequently asked questions - Which are common buffers used in HPLC? Accessed November 4, 2020. https://www.kromasil.com/support/faq.php?FAQelkzz

48. Wang X, An Z, Luo W, Xia N, Zhao Q. Molecular and functional analysis of monoclonal antibodies in support of biologics development. *Protein Cell*. 2018;9(1):74-85. doi:10.1007/s13238-017-0447-x

49. Counsil of Europe. Physical and physiochemical methods. In: *European Pharmacopoeia*. 8th ed. Druckerei C. H. Beck, Nördlingen, Germany; 2013:21-25.

50. International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use. ICH Q1B Photostability testing of new drug substances and products Q1B. Published online 1996. Accessed October 25, 2020. https://www.ich.org/page/quality-guidelines

51. Nowak C, K. Cheung J, M. Dellatore S, et al. Forced degradation of recombinant monoclonal antibodies: A practical guide. *MAbs*. 2017;9(8):1217-1230. doi:10.1080/19420862.2017.1368602