universität wien

MASTER THESIS

Titel der Master Thesis / Title of the Master‘s Thesis

„Monoclonal antibody heterogeneity – analytical characterization with main focus on multi-attribute method (MAM) “

verfasst von / submitted by

Sanja Curcic, Ph.D.

angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of

Master of Science (MSc)

Wien, 2021 / Vienna 2021

Studienkennzahl lt. Studienblatt / Postgraduate programme code as it appears on the student record sheet:	UA 992 580
Universitätslehrgang lt. Studienblatt / Postgraduate programme as it appears on the student record sheet:	Pharmazeutisches Qualitätsmanagement
Betreut von / Supervisor:	Dipl.-Ing. Dr. Manfred Schuster, MSc

Acknowledgment

First of all, I wish to express my sincere gratitude to my supervisor Dr. Manfred Schuster. I am deeply grateful for his invaluable scientific help and motivating discussions on this exciting topic, as well as for his optimism and patience.

I also wish to thank my friends and colleagues, and especially my family for their invaluable emotional support during my studies and previous schooling.

Table of contents

1 Introduction

1.1 Monoclonal antibodies

Therapeutic antibodies are a major class of biologics, with indications covering a wide range of diseases. Most of them are used to treat cancers and autoimmune diseases. Furthermore, they are also applied in the therapy of other diseases including genetic, infectious, cardiovascular and hematologic diseases, asthma as well as macular degeneration, transplant rejection, bone loss, eczema and diabetes type 2, or as antidotes (1–3). The term therapeutic antibodies comprises both monoclonal antibodies (mAb) and monoclonal antibody-related products, such as antigen-binding fragments (Fab) and Fc-fusion proteins (produced by fusing a protein/peptide with the antibody Fc fragment) (4). In recent years also innovative types of therapeutic antibodies with enhanced effector functions are emerging, such as glyco-engineered antibodies (5), or constructs which bind two targets simultaneously (bispecific antibodies) (6) and cytotoxic agents/small molecules attached to mAb (antibody-drug conjugates) (7).

1.2 Monoclonal antibodies versus small-molecule drugs

The European Medicine Agency (EMA) defines mAbs as “immunoglobulins (Ig) with a defined specificity derived from a monoclonal cell line”. mAbs exert their biological activity by “specific binding to a ligand” (antigen), and their mechanism of action may be dependent on immune effector functions such as antibody-dependent cellular cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC) (4).

The success of mAbs can be attributed to several advantages they possess in comparison to small-molecule drugs. Given that they are highly specific for target antigen, they show fewer off-target adverse events and potentially higher efficacy. In addition, mAb therapy is characterized by fewer drug-drug interactions as they do not undergo hepatic or renal metabolism. Compared to small-molecule drugs and due to a recycling mechanisms they also exhibit longer half-life (1,8).

Structure and manufacturing of small molecules and mAbs also substantially differ. Small molecules are produced by chemical synthesis which results in homogeneous products with low and measurable impurity levels. On the other hand, mAbs are produced through biological processes, involving biotechnology methods and living organisms. This leads to high complexity and inherent heterogeneity of their composition. In addition, they are susceptible to physico-

chemical modification during their entire lifespan, which makes their manufacturing and evaluation complicated and challenging. Understanding and control of the production process and product attributes are therefore highly important in order to ensure homogenous product quality, product efficacy and patient safety (1,8).

1.3 Antibody structure and function

Naturally occurring antibodies are produced by B-cells. They are found either free circulating (soluble) or bound to the B-cell surface as part of the B-cell receptor. Main role of antibodies is to neutralize pathogens (bacteria and viruses) and toxins, via specific targeting of their antigens and activating effector immune cells (8,9).

Antibodies are Y-shaped molecules consisting of two identical heavy chains and two identical light chains which are held together by disulfide bridges. Schematic antibody structure is shown in **Figure 1**. Both heavy and light chains are each composed of several domains (regions). These are variable (complementary determining) regions (CDR) and conserved (constant) regions. Heavy chain has one variable domain, three constant domains (CH1, CH2 and CH3 domain) and a hinge region between CH1 and CH2. Light chain is composed of one variable and one constant domain. Antigen-binding fragment (Fab) is formed by variable and CH1 domain of the heavy chain and light chain. The antigen-binding site is located within the variable domains (Fv) of the Fab fragment, also known as hypervariable region. On the other hand, CH2 and CH3 regions of heavy chains form the Fc domain, which determines Ab effector functions and its interaction with immune system (complement components, recycling and immune effector cells) (10,11).
Depending on the heavy chain type, human Igs are divided into 5 isotypes (classes) (IgA, IgD, IgE, IgG, IgM). Antibody isotypes can be further classified into subclasses which have very homologous constant region, but distinct characteristics such as serum half-life, ligand-binding capacity, ability to build immune complexes, avidity, activation of complement pathways and effector cells. Most of the mAbs currently used in the therapy belong to the IgG isotype, which mediates secondary immune responses, and which is also the most prevalent antibody isotype in the human organism (it accounts for about 80 % of all antibodies) (8,11,12).

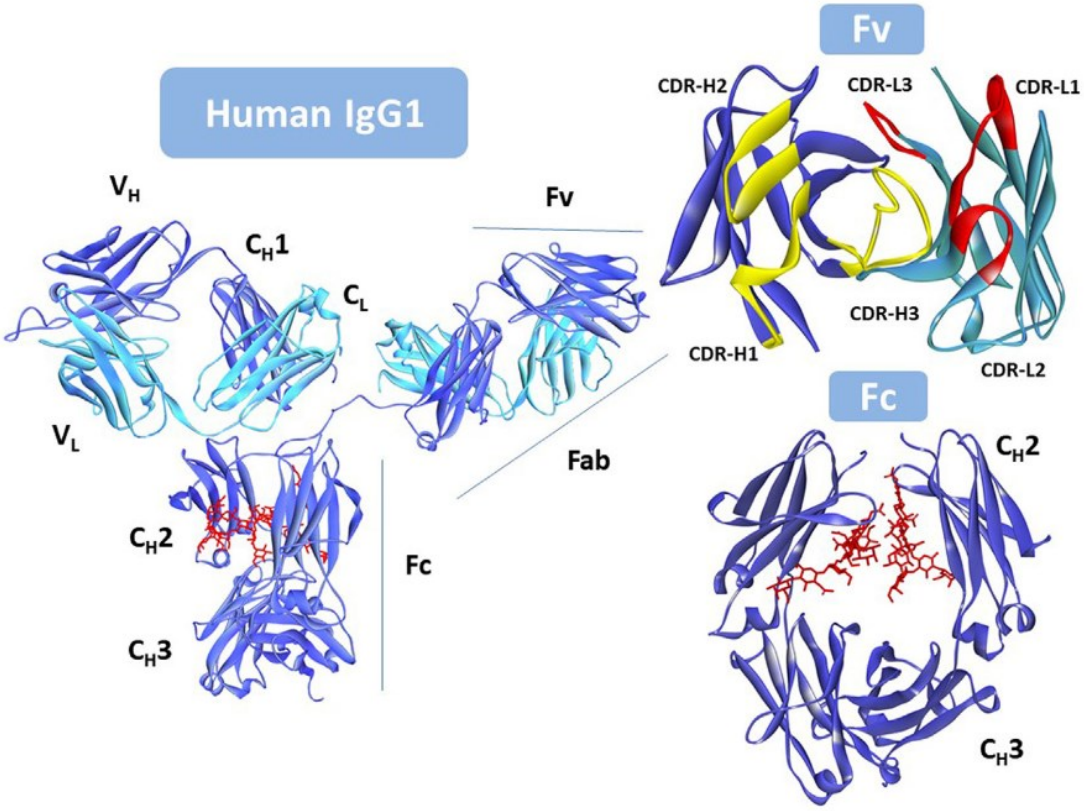

Figure 1. Human antibody structure, PDB ID: 1HZH. Left (Human IgG1): heavy chains (blue), light chains (cyan) and N-glycan (red). Upper right (variable domain (Fv)): complementary determining regions (CDR) of the light chain (V_L) in yellow, CDR of the heavy chain (V_H) in red. Bottom right (constant domain (Fc)): location of the N-glycosylation site. N-glycan is shown in red (11).

1.4 Generation of monoclonal antibodies

The first mAb produced by hybridoma technology was approved by the US FDA in 1986 and by European Medicines Agency (EMA) in 1987 as an immunosuppressant for the treatment of acute renal transplant rejection (muromanab, murine mAb against T cell receptor CD3) (13). However, antibody-based therapy was discovered already back in the late nineteenth century by Emil Adolf von Behring. Behring and his team used the serum from animals immunized with diphtheria or tetanus toxin containing polyclonal antibodies to treat these infectious diseases in other animals (8). Since the approval of the first mAb, the field of antibody therapeutics is evolving rapidly. This can be owed to several big advances in bioengineering technologies, including hybridoma

technology, production of humanized antibodies by CDR grafting technique and especially generation of full human antibodies via transgenic mice engineered with human immunoglobulin genes and phage display technology (8,12,13).

1.5 Monoclonal antibody quality

1.5.1 QbD principle

Regulatory authorities encourage implementation of quality-by-design (QbD) principle in order to ensure that the quality of biologics is met during both development and lot release (14,15). The QbD approach means that quality is built into the product already from the start. In order to achieve this, product attributes and its intended therapeutic application need to be well-understood at the molecular level. Especially the ones which highly impact efficacy, safety and quality (known as critical quality attributes – CQAs) need to be identified and monitored. CQA is defined as “a physical, chemical, biological, or microbiological property or characteristic that should be within an appropriate limit, range, or distribution to ensure the desired product quality”. As recommended and described by ICH Q8 R2 guideline, the first step in the application of QbD principle is development of quality target product profile (QTPP) and identification of CQAs. This enables identification of the critical process parameters (CPP) whose variability can impact CQA (16,17). CPP must be tightly controlled in order to keep CQA within the pre-defined limits. Therefore, QbD approach encourages CQA-driven manufacturing process development instead of final product testing (15).

1.5.2 mAb heterogeneity as quality attribute

mAbs are not composed of a single molecule type but consist in a complex mixture of slightly different variants (18). This is a result of numerous post-translational modifications (PTM) which are influenced by different enzymatic, physical and chemical factors, and can occur during protein synthesis and maturation in the ER and Golgi, up- and downstream manufacturing processes, storage as well as *in vivo* upon final product application (18–20). The host cell line and its recombinant DNA construct, components of the culture media, and process parameters are the most important factors that impact on mAb heterogeneity and quality properties (20–23).

It is well-known that even minor structural changes can highly impact on mAb properties including biological activity, safety, immunogenicity, conformation, solubility and stability (24). Therefore,

under the QbD scope many of PTM are considered as CQA and the constant PTM profile during the entire product life-cycle is demanded by regulatory authorities (10,18). To fulfil these requirements proper analytical methods need to be applied, which can with high selectivity and specificity detect and characterize changes in PTM (18).

1.5.3 Heterogeneity characterization and analysis

Traditionally, a wide range of chromatographic and electrophoretic methods are used to support development, characterization and lot release of mAb including amongst others sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS), capillary electrophoresis using sodium dodecyl sulfate (CE-SDS), size-exclusion chromatography (SEC), ion exchange chromatography (IEX), isoelectric focusing (IEF), hydrophilic-interaction liquid chromatography (HILIC), reversed-phase high performance liquid chromatography (RP-HPLC). These techniques are mostly coupled with UV and fluorescence detection or in case of peptide mapping with a mass spectrometer (25). Most of these conventional techniques usually only indirectly assess one attribute at the time. Therefore, multiple instruments or orthogonal methods are required, which makes a full characterization of mAb complex, costly and time-consuming (26,27).

2 Aim of the thesis

In the recent years, both pharmaceutical companies and regulatory bodies have gained interest in a novel approach known as multi-attribute method (MAM) (26,28,29). MAM is based on mass spectrometric peptide mapping and represents an alternative to traditional methods. MAM uses combination of high accuracy/high resolution MS with sophisticated data analysis software. This is why it is able to identify and quantify several CQA simultaneously. This significantly reduced number of assays required for mAb analytics (27). However, before MAM can be widely adopted in the GMP environment, there are several concerns that must be addressed (26).

The aim of this master thesis is to give an overview of the current knowledge about mAb heterogeneity and how specific mAb PTM impact on pharmacology, safety and product quality. Furthermore, we will give insight into recent advancements in analytical methods, with the main focus on MAM, which would enable more simple characterization and monitoring of mAb PTM. We will critically evaluate advantages and challenges of MAM as well as regulatory considerations for its implementation in GMP settings and quality control.

3 Methods

The method used to answer the above-mentioned questions is exclusively based on literature search. In order to determine relevant sources, the following key words were used: monoclonal antibody, posttranslational modification, antibody heterogeneity, multi-attribute method. All the literature was in English language.

The main source of information were publications in PubMed database. In addition, online available interviews and official websites of respective pharmaceutical companies were used for information concerning MAM. Relevant guidelines published on the websites of authorities such as EMA and FDA were also reviewed.

4 Antibody pharmacology

4.1 Administration and absorption

mAbs are administered via parenteral routes, intravenously (i.v.), subcutaneously (s.c.) and intramuscularly (i.m.) (30). s.c. administration is especially convenient for self-administration (31). Due to their size, s.c. and i.m. administered mAbs are not able to enter the circulation through blood capillaries, but rather through lymphatic system. This results in slow absorption lasting several hours or even days, with maximum levels in plasma being reached within 3-7 days (30).

4.2 Distribution

Similar to the absorption, and due to antibody size and polarity their distribution is limited to the vascular and interstitial space. The extent of mAb distribution from circulation into most tissues ranges between 5 and 15%, except for brain where it is much lower (32). Distribution is influenced by mAb biophysical characteristics (charge and hydrophobicity) (33) as well as by other factors such as antigen binding, diffusion, pinocytosis, receptor-mediated endocytosis, and elimination from the tissue (30,33).

4.3 Metabolism and elimination

Being large molecules, antibodies do not undergo glomerular filtration. Instead, they are eliminated primarily via protein catabolism, which means that they are degraded into peptides and amino acids and re-used for protein de novo synthesis or excreted into urine (34,35). There are two possible pathways for mAb elimination, which occur simultaneously:

1) **Antigen-specific target-mediated elimination** – antibody-antigen complex is taken up by endocytosis and intracellularly undergoes lysosomal degradation. This is a non-linear saturable process (8).

2) **Non-specific elimination** – both antibody-antigen complexes and free antibodies are cleared via phagocytic and endothelial cells of the reticuloendothelial system. It is a linear non-saturable process (33). Non-specific elimination is mediated by interaction of solvent-exposed residues of Ab with blood cells, vascular system and tissues, upon which the antibody is taken up via pinocytosis or receptor-mediated endocytosis (33,36,37). Accessible terminal galactose residues on antibody glycans can bind asialoglycoprotein

receptor (ASGPR), whereas mannose and N-acetylglucosamine residues interact with mannose receptor (MR). ASGPR and MR are carbohydrate-specific endocytic receptors expressed on hepatocytes and Kupfer cells and sinusoidal endothelial cells respectively (38–40).

Antibody clearance is highly influenced by the interaction with Fc-neonatal receptor (FcRn) in the acidic endosomes, mainly expressed in endothelial and phagocytic cells, but also in renal epithelial cells. FcRn mediates antibody recycling to the cell surface and its release back into the extracellular fluid. This process is known as FcRn-mediated recycling. As a consequence of FcRn protection mechanism, antibodies escape lysosomal degradation (41–43) and have a prolonged half-life in comparison to other proteins of similar size, which in humans lasts around 11-30 days (9).

In addition, mAb elimination rates can also be altered by immunogenicity and formation of endogenous anti-therapeutic antibodies (human antibodies against therapeutic mouse, chimeric or human antibodies), which also depends on the level of humanization (33,44).

4.4 Pharmacodynamics

Antibodies can exert their effects via their variable region (direct effect) or through the Fc region (indirect effect).

1) Direct effect is mediated through selective binding of the Fab variable region to an antigen, which can be a cell surface receptor or a circulating protein (8).

2) Indirect effect involves interaction of Fc binding sites with different receptors, including Fc-γ-receptors (FcγRs) and complement component 1q (C1q). FcγRs comprise both activating (FcγRI, FcγRIIA, FcγRIIC, FcγRIIIA, and FcγRIIIB) and inhibitory (FcγRIIB) receptors, which mediate cytotoxicity/proinflammatory responses and inhibitory responses, respectively. This results in the modulation of immune responses of NK cells, monocytes/macrophages, dendritic cells, mast cells, neutrophils, eosinophils as well as adaptive immune cells such as B-cells. The final outcome of Ab interaction with the FcγRs is regulated by the affinity of the Fc part for the specific receptor and the expression pattern of those receptors on effector cells (45). Thus, Ab modifications that selectively increase the affinity of antibodies for activating FcγRs will enhance the immune effector function such as antibody-dependent cell-mediated cytotoxicity (ADCC), antibody-dependent cell-mediated phagocytosis (ADCP) and complement-dependent cytotoxicity (CDC) (8,11,45).

5 PTM and their effect on mAb pharmacology and safety

Critical residues for the mentioned pharmacokinetics and antibody effector functions are located in specific antibody regions. Therefore, depending on the type of the PTM and the affected residue(s), the heterogeneity can have different effects on mAb function. mAb binding to its target antigen is mediated via variable Fab region, whereas the hinge region enables mAb flexibility during these interactions (8). Furthermore, besides having influence on the antigen binding, changes in the variable region can impact antibody pharmacokinetics (12). CH2 region is responsible for the interaction with FcγRs (46) and C1q (47), whereas residues located near the CH2-CH3 junction interact with FcRn and determine Ab serum half-life (41).

5.1 Glycosylation

Glycosylation process is a part of antibody maturation within the host cell, when glycans are formed through a series of enzymatic reactions by glycosidases and glycosyltransferases (22). In addition, upon secretion, glycosidases which are released from the lysed cells can influence glycan structure, for example by removing terminal residues (5).

Glycan can be attached to the N atome of asparagine (Asn) within the Asn-X-Serin or Asn-X-Threonine motif (N-glycosylation), which is the predominant glycosylation type, or to the oxygen atom of serine (Ser) or threonine (Thr) (O-glycosylation) (48). IgG antibodies contain conserved glycosylation at the N297 site in the Fc region of both heavy chains (49). In addition to Fc glycosylation, some of the IgG antibodies also have N-glycans located in the Fab region (22). Furthermore, several O-glycosylation sites can be found in the hinge region of IgG3, but their role is not clear (48).

The N-glycan core is composed of two N-acetylglucoseamine (GlcNAc) and three branched mannose residues (pentasaccharide), to which there are further residues attached. Depending on the residue composition, glycans can be classified into 3 types (**Figure 2**) (22):

1) **High mannose type** contains only mannose residues attached to the glycan core (22).
2) **Complex type** contains different monosaccharides attached to the core. Two GlcNAc residues are attached to the core and form biantennary branches, to which different monosaccharides can be attached such as galactose or sialic acid attached to these

galactose groups. If antibody is fucosylated, fucose is connected to the first core GlcNAc residue (22).

3) **The hybrid type** has properties of both high mannose and complex types (22).

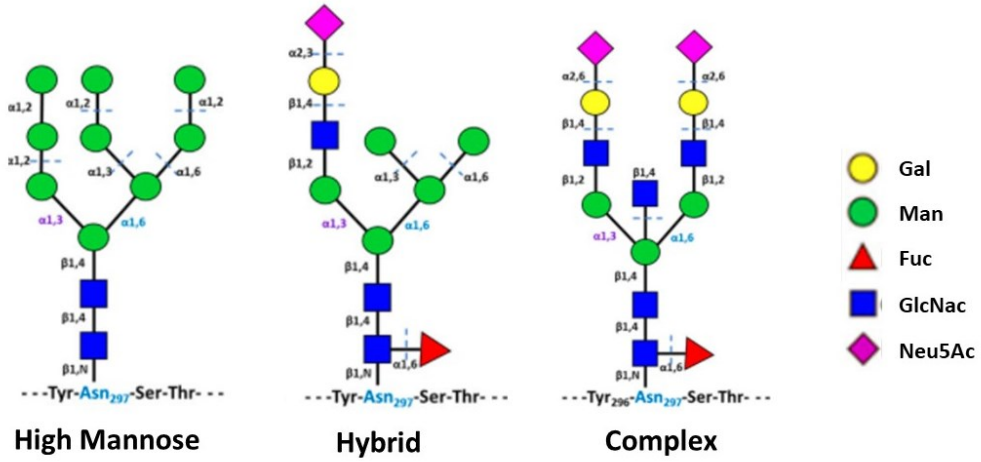

Figure 2. Heterogeneity of glycosylation at the site N297. Gal: galactose, Man: mannose, Fuc: fucose, GlcNac: N-acetylglucosamine, Neu5Ac: N-acetylneuraminic acid (5).

Given the well-known important role of glycosylation pattern in antibody function, it is not surprising that mAb glycosylation is considered as CQA (50). Variability in glycosylation of naturally occurring antibodies have been associated with several diseases including cancer, rheumatoid arthritis and inflammatory diseases (22). In line with this, glycosylation modification in mAb can affect stability, pharmacokinetics, efficacy, ADCC, CDC, and immunogenicity (50).

Modifications in N-glycan composition result in changes in binding of the Fc domain to Cq1 and FcγRs (both activating receptors FcγRI, FcγRIIA, FcγRIIC, FcγRIIIA and FcγRIIIB and inhibitory receptor FcγRIIB) as well as to FcRn. N-glycans can either have direct influence when they are directly involved in binding (for example in case of FcγRIIIa) or can act indirectly via allosteric modifications of binding sites (in case of FcγRs, C1q, FcRn, type II receptors) (5). Allosteric modifications are highly dependent on the size of the glycan. This is because the glycans regulate conformation and spacing between the two CH2 regions or CH2-CH3 regions and subsequently allosteric Fc domain-receptor interactions. By filling in the space between the two CH2 domains of the heavy chains, N297 glycans stabilize the Fc region and keep it in an open conformation (5). Smaller N-glycans result in closed antibody conformation. Moreover, complete deglycosylation results in collapsing of the CH2 region inwards which hinders the binding to most of the receptors (51). Thus, deglycosylated antibodies completely lose their effector functions.

Furthermore, N-glycans at N297 regulate opening/closing of the CH2-CH3 region which controls the opening of the binding site for FcRn and type II receptors. Glycan residues also prevent antibody aggregation by masking hydrophobic residues (5).

Effects of N-glycan presence/structure on mAb properties can be advantageous. Through so called glycoengineering process mAb lacking or enriched with certain glycans can be produced, which results in enhanced or improved effector functions. Glycoengineering can be achieved via several methods, including manipulation of the synthetic pathways of host cells or via in vitro chemo-enzymatic glycan remodeling. On the other hand, glycosylation can also pose a risk, if it is not well-monitored and controlled (5,49).

Glycosylation profile can be influenced by numerous factors such as host cell line (or example murine cell lines produce 1,3-galactose and N-glycolylneuraminic acid) (49), process parameters (pH, temperature, pCO2, production scale) (49), culture conditions (shear stress, culture reduction potential) (52) and content or availability of nutrient media components (glucose, glutamine, sodium butyrate, ammonia, threonine, dissolved oxygen) (5,21,23,49,52). For example, decreased levels of dissolved oxygen result in reduced extent of galactosylation (52). Depletion of intracellular glucose as a result of either low glucose content in the medium or high cell culture density leads to synthesis of non-glycosylated mAb or to glycosylation heterogeneity such as reduced levels of galactose and sialic acid residues (53). Changes in culture reduction potential also affect glycosylation. A possible underlying mechanism is the impairment of disulfide bond formation, and subsequent changes in accessibility of glycosylation sites (which are close to disulfide bonds) to galactotransferases (52).

5.1.1 Galactose

How galactosylation affects antibody functions is still not well-understood. Increased galactosylation results in bigger distance between the two CH2 domains and conformational changes in this region, exposing more protein residues for receptor binding (22). Full galactosylation has been shown to enhance the C1q interaction and CDC (54). It also increases binding to most of the FcγRs (55). When it comes to ADCC, there are reports of either no effect or increased ADCC, which also might be dependent on the bi-antennary glycan arm onto which galactose is bound (56) and on the fucosylation levels (57). In addition, galactose-α-1,3-galactose linkages or terminal-α-1,3-galactose are highly immunogenic (Galili-epitopes) and have been thought to be responsible for anaphylaxis in some mAbs (58).

5.1.2 Mannose

Number of mannose residues in glycans, which can vary between fine and nine, is considered to have impact on both mAb pharmacokinetics and effector functions. mAbs with the high mannose glycans type are usually reported to be faster cleared due to their interaction with MR (30). In addition, mAb with this glycan type exhibit enhanced ADCC activity due to binding to FcγRIIIA (59).

5.1.3 Sialic acid

Sialic acid mediates anti-inflammatory activity which among other factors also depends on the 2,3 linkage type, as it enables Fc domain conformational plasticity. Natural antibodies and antibodies produced by human host cells contain 2,3 linkage, whereas murine cell lines express 2,6 sialylated glycans, which results in mAbs without anti-inflammatory properties (5). Sialylated glycans exhibit anti-inflammatory activity through induced expression of inhibitory FcγRIIB receptor on immune cells (60) and diminished CDC effect due to hinderance of antibody interaction to C1q and reduced C3b disposition on the cell surface (61). In addition, it has also been shown that terminal sialic acid might reduce ADCC (62). However, sialic acid effects on ADCC also seem to differ depending on the core fucosylation status. Sialylation in the context of core fucosylation significantly decreased ADCC in a cell-based assay and suppressed antibody-mediated cell killing in vivo. In contrast, in the absence of fucosylation, sialylation did not adversely impact ADCC (63). Sialylation effects on FcγRs affinity depended on the glycan structure, whether it is partially or fully galactosylated (49). The mentioned effects of sialylation also depend on the sialic acid type. For example, sialic acid N-glycolylneuraminic acid (Neu5Gc) is linked to immunogenicity and its absence is desirable (64).

5.1.4 Core Fucose

mAbs with removed fucose linked to GlcNAc directly connected to Asn (afucosylation) exhibit several orders of magnitude higher efficacy due to increased binding affinity for FcγRIIIA and ADCC activity than fucosylated mAbs with the same structure both *in vitro* and *in vivo* (63,65,66). This is because fucose presence results in steric hinderance which interrupts glycan-glycan interactions of the Fc domain with the activating FcγRs which ultimately leads to inhibition of ADCC and ADCP (51). On the other hand the reports shows no influence of afucosylation on the C1q binding and CDC activity, as well as target antigen affinity (67).

5.1.5 N-acetylglucoseamine

GlcNAc residue interacts with MR and ASGPR, which results in increased mAb clearance. This residue is therefore one of the main factors regulating mAb serum half-life (38,68). In addition, presence of bisecting GlcNAc (GlcNAc attached to the core mannose residue) is known to improve ADCC (54), most probably by hindering core fucosylation (69).

5.2 Glycation

Glycation is a non-enzymatic reaction of lysine side chain residues with reducing sugars (such as glucose in the production culture medium, the final formulation, or *in vivo* in circulation) or breakdown products of reducing sugars (for example glyoxylic acid), which results in formation of advanced glycation end products (AGEs). For this reason, instead of glucose and maltose, sucrose is often used as an excipient in mAb formulations. However, also sucrose can hydrolyze into glucose and fructose, especially at low pH and elevated temperatures. Effects of glycation on antibody antigen binding, effector functions and pharmacokinetics do not seem to be significant (70). However, this depends on the location and extent of modification, and some reports showed possible loss of antigen binding affinity (70–72). The effects of AGEs haven't been investigated so far. However, given that AGEs can act as photo-sensitizers in the presence of UV-A light, their presence could lead to formation of reactive oxygen species and possible further antibody modifications and degradation during storage, such as formation of aggregates, cross-linking and fragmentation (73).

5.3 Oxidation

Oxidation affects several amino acids including methionine (Met), cysteine (Cys), histidine (His), tyrosine (Tyr) and tryptophan (Trp). It occurs during both mAb manufacturing and storage. Oxidation can be promoted by different factors such as buffer composition, excipients (such as polysorbates), sugars, salts, peroxides, metals, dissolved oxygen, oxygen in vial headspace, pH, light and additives. Effects of a specific factor seem also to be residue-specific (74). Depending on the extent and location of oxidized residue, oxidation can affect mAb serum half-life, stability, antigen binding and certain effector functions (74–77).

Met oxidation mostly happens in the Fc region, especially at residues Met252 and Met428 located at CH2 and CH3 respectively. Given that these residues are located close to the FcRn receptor binding sites, their oxidation affects clearance (decreases serum half-life) as well as Fc-mediated

effector functions (74). However, it seems that these two residues have different individual contribution to the changes in pharmacokinetics. Number of oxidized Met252 showed positive correlation with the decrease in FcRn binding affinity, whereas Met428 oxidation did not show significant effects, or even resulted in slightly increased binding (78). Furthermore, as these Met residues are close to N-glycans, one study showed that their oxidation can result in accelerated degradation of deglycosylated antibodies. Interestingly, it seems that deglycosylation does not have any effect on oxidation of oxidated Met residues (79). When it comes to the effects on Fc-mediated effector functions, it has been shown that Met oxidation can affect ADCC, however it seems that this results from the oxidation in the Fab region, rather than impaired FcγR binding (74).

Trp oxidation is more common in the CDR region and its oxidation leads to decreased potency and specificity for antigen binding (80). In addition to pharmacological effects, Met oxidation leads to significant reductions in antibody conformational stability, whereas Trp oxidation promotes formation of aggregates (81).

5.4 Deamidation and aspartate isomerization

Hydrolysis of asparagine (Asn) and glutamine (Gln) at acidic pH and elevated temperatures results in formation of aspartic (Asp) and glutamic (Glu) acid. On the other hand, at increased pH levels, this is a slow reaction and it is possible for Asn to undergo modification to an imide intermediate (succinimide) with Asp or isoaspartate (iso-Asp) being the end products. Deamidation can occur at any stage of mAb manufacturing, but it is also very common modification *in vivo*. Main factors influencing deamidation are temperature, pH, buffer composition (especially content of glycerol, sodium chloride and iron) (10,82,83).

Both deamidation and aspartate isomerization lead to changes in mAb charge and formation of acidic variants. This process also happens *in vivo* and if modifications are located in the CDR they result in decreased antigen binding (84,85). Furthermore, iso-Asp has been considered to have immunogenic potential (86).

The impact on pharmacokinetics is not clear. For long time it was considered that deamidation of asparagine in the Fc region does not have any impact on pharmacokinetics. However, recent findings suggest that changes in the charge distribution can impact local structural conformation, structural flexibility/dynamics, and solvent accessibility and subsequently clearance (87).

In addition to pharmacological effects, changes in mAb stability as well as effect on antibody conformation and aggregation have been reported (88).

5.5 C-terminal lysine heterogeneity

mAbs are synthesized with two C-terminal lysine (Lys) residues, each of them on one of the heavy chains. C-terminal lysine heterogeneity comes from partial removal of these residues by carboxypeptidases, and this also occurs *in vivo* (89,90). In the cell culture, prolonged exposure to pH 6.8 can lead to reduced host cell viability and higher release of carboxypeptidases into the culture medium and thus increased cleavage of lysine residues (20). Given that C-terminal Lys is neither involved in Fc nor in Fab interactions with their receptors/targets, it is considered that this modification does not affect mAb function and pharmacokinetics. In line with this, in their most recent publication, Faid et al. used highly pure charge variants and were able to confirm the common belief that C-terminal Lys clipping has no impact on FcRn and FcγIIIA receptor binding (91).

5.6 N-terminal pyroglutamate

Pyroglutamate (Pyro-Glu) is formed through conversion of N-terminal glutamic acid or N-terminal glutamine. This modification happens both *in vivo* and *in vitro* in the cell culture, during purification, formulation, storage, or even under analytical procedures (19,92). The final effect on the charge variant nature depends on the origin of Pyro-Glu, whether it comes from Glu or Gln. Cyclization of Gln clearly results in formation of acidic variants, due to the loss of the N-terminal amine. On the other hand, although conversion of Glu is considered not to produce a change of the net charge, recently it has been demonstrated that that cyclization of Glu produces a basic variant (93). However, Pyro-Glu formation is considered not to have a significant effect on mAb structure, binding and pharmacokinetics (94).

5.7 Disulfide bond heterogeneity

Normally all cysteine residues in mAbs are involved in disulfide bond formation. There are two types of disulfide bonds – intra-chain bonds and inter-chain bonds between the two heavy chains and between the light and the heavy chain. They can have different location depending on the antibody isotype. As inter-chain bonds are more exposed to the solvent, they are also more susceptible to modifications. There are several possible variants of disulfide bonds including free

Cys, alternative disulfide bond linkage (scrambling), trisulfide bonds, cysteinylation and formation of thioether, which can all be found in both natural as well as in recombinant Ab (95,96).

Free sulfhydryl groups can commonly occur in both endogenous as well as in recombinant antibodies (95). They result from incomplete processing within the host cell or reduction during the manufacturing process (97). Disulfide bonds are mostly reduced by released intracellular components (mostly thioredoxin and glutathione systems) upon cell damage due to mechanical stress during mAb harvest (98). Free sulfhydryl groups might lead to decreased stability, dimer and aggregate formation as well as loss of potency, which depends on the antibody structure and specific location of free SH groups (18,97).

Alternative disulfide bond linkage has been so far reported in IgG4 and IgG2 molecules, and results in formation of variants which are in equilibrium. One example for IgG4 is variant with intrachain disulfide bonds in the hinge region, which is in equilibrium with the variant with inter-heavy chain disulfide bonds. Depending on each case, some variants may have impact on potency or stability (18,99,100).

Trisulfide bonds can be present in all classes of mAbs, and its formation is highly dependent on cell culture parameters, especially on the hydrogen sulfide in the culture medium. When present, trisulfide bonds are mostly located between light and heavy chain. It is considered that these bonds do not to have any effect on stability and potency (18,101).

Thioether formation is promoted by high pH levels and occurs through beta elimination reaction. The effects of this bond on mAb function are not well examined. However, although this modification is distant from the antigen binding site, due to shorter bond length it could result in changes in Fab orientation and subsequently antigen binding (101).

Cysteinylation involves formation of a disulfide bond between an antibody cysteine residue and free cysteine molecules in the culture medium (96). It can be located in the hinge region (96,102) as well as in the CDR (103). Cysteinylation in the variable region resulted in tertiary and quaternary structural perturbations, decreased stability and increased tendency to aggregation as well as decreased activity (102,104). On the other hand, not much is known about the effects of the cysteinylation in the hinge region. Recently published study identified this modification in endogenous IgG2 and concluded that hinge cysteinylation is not expected to impact antibody safety and immunogenicity (96).

5.8 Charge heterogeneity

Isoelectric point (pI) is the pH at which the protein carries no net electric charge. Charge variants are not a single component, they contain several different PTM which together contribute to introduction of certain charge (105). Negative charge variants have decreased isoelectric point (pI) and are formed when the following PTM are introduced into the mAb structure: deamidation, sialylation, trisulfide bonds, cysteinylation, and glycation (105,106). On the other hand, basic variants are formed from molecules containing C-terminal lysine, non-cyclized N-terminal glutamine, C-terminal amidation and succinimide formation from aspartic acid residues (105).
mAb charge heterogeneity mostly influences antibody pharmacokinetics (30). Increase in positive charge increases clearance and distribution because basic variants have higher tendency to adhere to the negatively charged cell surfaces and are being faster taken up by the tissues and eliminated from the blood (33).

Effects on pharmacokinetics are mostly measurable when bigger changes in charge such as 1-2 pI units occur (30). For changes in charge less than 1 unit, results are contradictory, with earlier reports showing no effect of smaller changes. However, more recent studies show that not only overall charge, but also local charge changes within the Fab and CDR can impact clearance and pharmacokinetics (30,87).

5.9 Aggregates

Aggregates can occur in all protein therapeutics during manufacture, storage and administration. They are very diverse and vary in solubility, reversibility and structure (covalent/non-covalent, native/denatured) (18,23). Aggregates lead to changes in bioavailability, loss of biological activity and especially concerning immunogenicity and changes in safety (8,18,19,23,107). Thus, many drug product formulations contain detergents to avoid aggregation (108).

6 Multi-attribute methods (MAM)

6.1 Conventional methods for mAb characterization/routine analysis

Oligosaccharide profiling is used to determine the type and level of N-linked glycan residues. The traditional strategy for analyzing glycans uses hydrophilic interaction liquid chromatography (HILIC), where sample is denatured and oligosaccharides are enzymatically released. The released oligosaccharides are derivatized with fluorophore, separated by liquid chromatography (LC) (normal or reverse phase) and detected with a fluorescence detector. The percentage of each species is determined relative to the total area of all detected species. Site-specific profiling of oligosaccharides is challenging using HILIC (109).

Charge isoform distribution is routinely determined by capillary isoelectric focusing (cIEF) and ion exchange chromatography (IEC). IEC separates proteins based on their affinity to an ion exchanger. IEF is based on separation by the pI. Species with different net charges are separated by their isoelectric point (pI) on a pH gradient through an applied electric field. Results are reported as % acidic, neutral and basic isoforms. In case that further analysis of the peaks is needed (for example in case of increased % of certain charge isoform) this is done by peptide mapping with MS and glycan analysis (18,110,111).

Product-related impurities (fragments) are assessed by gel electrophoresis (reducing and non-reducing), capillary electrophoresis using sodium dodecyl sulfate (CE-SDS) and by the below-mentioned methods for aggregation analysis such as SEC (18,112).

Process-related impurities (host cell DNA, host cell protein and protein A) are assessed by qPCR and ELISA respectively (113).

C-terminal lysine and other **terminal modifications** are assessed by methods used to assess **charge heterogeneity** such as ion exchange chromatography (IEC) and capillary isoelectric focusing (cIEF) (18,111).

Disulfide bond variants are assessed by the peptide mapping. The sample is denatured and then enzymatically digested under non-reducing conditions. Following digestion, the peptides are separated by LC, and are monitored using MS in positive ion mode. The method can assess

integrity of the disulfide bonds and provides information on any mispaired disulfides or free cysteine residues (18,114).

Aggregates are assessed by combination of several methods. Standard method is SEC analysis with UV, refractive index (RI) or light scattering detection. Peaks eluting earlier are considered as aggregates. In addition, analytical ultracentrifugation, nanoparticle tracking analysis, light obscuration and micro-flow imaging are used (18,115,116).

6.2 MAM principle

MS-based methods are used through the whole mAb life cycle, from initial clinical trial to marketing authorization applications. One study showed that MS analysis is a part of about 80% of FDA biologics license applications. In line with this, number of quality attributes which are assessed by MS has increased from 2 to 11 in recent years (117). MS enables investigation of the nature of the modification and on which residue the modification is located (by analyzing peptide fragments and their mass changes) and mechanisms associated with modifications (by comparing different types of samples, storage conditions and formulations) (118).

MA-based MAM was introduced for the first time by Amgen in 2015. Rogers at al. had an aim to develop an analytical workflow that is in accordance with QbD requirements and can provide a better quantitative information about PQAs in comparison to currently used methods. They used a combination of high mass accuracy/high resolution MS and automated identification and relative quantification of PTM with dedicated software. The method is based on peptide mapping using bottom-up approach. This means that mAbs are digested into smaller peptides which are then analyzed (27). This is the main difference between the MAM and conventional techniques such as CEX and CE-SDS, which assess the intact mAb or intact subunits (26). In comparison to traditional LC-MS/MS peptide mapping, where algorithms are applied to search for peaks with known identities, MAM is able to both quantitatively monitor defined CQA as well as to differentially analyze test sample versus reference and identify any new, missing or changed peak (119).

MAM has a potential to be used through different product phases – for clone screening, characterization, process development, comparability studies, stability assessment as well as in lot release (27). It is able to analyze a wide range of PQAs including:

1) Primary structure including sequence modifications and mutations (26,27).

2) PTM: glycosylation, glycation, deamidation, isomerization, succinimide formation, oxidation, signal peptide identification, C-terminal lysine truncation, tryptophan degradation, N-terminal pyroglutamate, fragmentation, hydroxylysine, thioether, cysteine adducts, C-terminal amidation (27,120,121).

3) Impurities such as product related impurities (sequence variants) and process-related impurities (host cell proteins and residual protein A) (120,121).

An overview mAb quality attributes and methods used for their analysis is given in **Table 1**.

A typical MAM workflow consists of the sample preparation and the MS-analysis. Therapeutic protein samples are denatured by heating or by using a surfactant (SDS or RapiGest) and reduced by dithiothreitol or tris(2- carboxyethyl)phosphine to break disulfide bonds between the cysteine residues. Subsequently sample is alkylated by iodoacetamide and digested by a protease, such as trypsin, to generate smaller peptides carrying PQAs. The resulting peptides are separated and analyzed by LC-MS (122).

In the MAM application in recombinant protein analytics there are two stages – characterization and monitoring stage.

Table 1. Overview of analytical tools used for mAb characterization and lot release

Quality attribute	Standard method	Lot Release	Covered by MAM	Comment	Ref
Color, clarity, particles	Visual inspection (appearance)	Yes	No		(123,124)
Identity	Immunoassay, MS (Peptide mapping) cIEF, CGE	Yes	Yes	Choice of identity tests is based on specific product properties	(123,124)
Residual host cell proteins	ELISA	Yes	Yes	Can be omitted if proper clearance studies were performed	(123,124)
Residual protein A	ELISA	Yes	Yes	Can be omitted if proper clearance studies were performed	(123,124)
Residual DNA	PCR	Yes	No	Can be omitted if proper clearance studies were performed	(123,124)s
Glycosylation, Oligosaccharide Profiling	HILIC-Flour, MS	Yes	Yes	Included in lot release only if it is defined as CQA	(18,123,124)

Charge heterogeneity	IEC, IEF	Yes	No	MAM does not measure directly pI, but specific PTM modifications, N- and C- terminal, variants, sialylated species	(117,123)
Deamidation	Charge heterogeneity methods (IEC, IEF), MS (Peptide mapping)	No, assessed within charge heterogeneity (IEC, IEF)	Yes	Peptide mapping is used for characterization; MAM can assess site-specific deamidation	(117,123)
Glycation	Charge heterogeneity methods (IEC, IEF), Intact and peptide level LC-MS, boronate affinity chromatography	No, assessed within charge heterogeneity (IEC, IEF)	Yes	Intact LC-MS measures overall glycation; Peptide level LC-MS and MAM are site specific.	(18,26)
Oxidation	Peptide mapping	No	Yes	Peptide mapping is used for characterization; MAM can assess	(117,123)

				site-specific deamidation	
Fragments	SEC, IEC, IEF, CGE	Yes	Yes	For lot release SEC and CGE recommended	(26,123)
Aggregation	SDS, CGE, SEC, analytical ultracentrifugation, MALDI-TOF MS, ESI-MS (intact molecule)	Yes	No	For lot release usually SEC under native conditions; analytical ultracentrifugation, ESI-MS and MALDI-TOF used for characterization	(26,123)
C-terminal lysine	Peptide mapping	No	Yes	Assessed in characterization and development	(26,123)
PyroGlu	Peptide mapping	No	Yes	Assessed in characterization and development	(26,123)
Disulfide bond variants	LC-MS (reducing and non-reducing)	No	No	Assessed during development	(18,26,123)

Characterization stage (method development and identification of target peptides). The main aim of this step is to develop a full list of PQAs by identifying all peptides and their PTM. This is achieved by performing peptide mapping by tandem MS (LC-MS/MS). A software (for example Pinpoint) is used to target MS1 precursor ions by retention time, accurate mass and isotopic distribution. In depth characterization of the molecules using MS2 is essential to create a workbook for each of the modifications. Upon risk-assessment studies, a subset of PQAs are identified as CQAs and used for development of target peptide workbook (125). In this way a panel with all the relevant peptides and all CQA that will be monitored in the future are established (126).

Monitoring stage (quantitative monitoring of CQA at peptide level). At the monitoring stage reduced amount of MS data is collected. The analysis is performed in MS1 mode or with an MS1 only MS (27) and experimental samples are compared to a characterized reference standard (125). The specific mass-to-charge ratios (m/z) of modified and unmodified peptides that contain the selected CQAs, are monitored (26,126,127). Here there are 2 aspects to be considered - targeted attribute quantification and new peak detection (NPD).

For **targeted attribute quantification** the processing method developed in the characterization stage is applied for relative quantification of modifications for PQAs which were determined during the method development. Relative abundance values for each CQA are calculated based on the ratio of modified to total peptide abundance. MAM employs site-specific quantitation, where for a particular amino acid modification, the readout is percent modified relative to the unmodified peptide (26). The equation for calculation of the modification level is shown below:

$$\text{Modification level (\%)} = \frac{\text{Area modified peak}}{\text{Area unmodified peak + Area modified peak (Total peak area)}} \times 100$$

NPD feature is on the other hand applied to identify impurities and potential changes in PTMs (26,126,127). Here a differential algorithm is applied to identify any changes of LC-MS peak or any new peak (“impurity”). In order to minimize false-positive peak detection, pre-set peak selection criteria (pre-set detection limit) must be implemented. Therefore, NPD function detects any new peaks (impurities) which are above the pre-set detection limit, missing peaks (peaks not present in test sample compared to a reference standard) as well as changes in existing peaks.

New peaks are defined as peaks with unique mass-to-charge ratio and retention times. On the other hand, changed peaks can be identified in both test and reference samples, but differ in abundance. In case new peak/changes in the peak are identified, the experimental sample will be rerun in the MS/MS mode for detailed characterization (125).

Schematic overview of the MAM principle and stages is shown in **Figure 3**.

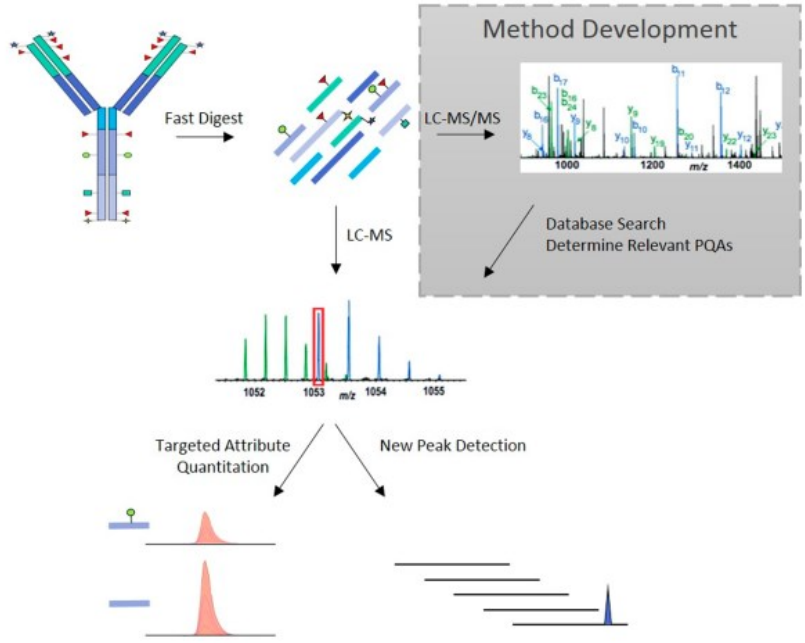

Figure 3. MAM workflow. mAb sample is digested into peptides and during method development analyzed in the LC-MS/MS mode, CQA are determined. In the monitoring stage only LC-MS mode is applied for the NPD and target attribute quantification (26).

Since MAM protocol introduction by Amgen, which is based on orbitrap technology and bottom-up approach, several different workflows were proposed by other groups (which use combination of different methods/instruments or MS with middle-down and top-down approach). Some of them are described below.

3D multi-attribute analyzer – This workflow combines protein A affinity chromatography, SEC, and Q-TOF LC/MS. It allows the fully automated determination of mAb titer and structural aspects such as aggregation, fragmentation, molecular weight, amino acid sequence, and PTM directly from cell culture supernatants. The titer is measured by mAb separation via affinity chromatography (protein A column) and subsequent UV or DAD detection. In the next step the

sample is loaded onto the SEC column for mAb size variants separation and their detection by a second DAD. The final step in the described MAM workflow is generation of mass spectra by high resolution MS (Q-TOF LC/MS equipped with ESI source) (128).

Middle-down approach (subunit mass analysis) – Most of the MAM are performed on digested proteins. However, there are also MAM workflows based on the protein analysis at subunit level. It is known as middle-down LC/MS approach, where antibody subunits are generated by reduction or enzymatic cleavage. It uses intact mass analysis by quadruple time of flight mass spectrometry (Q-TOF). It was shown as suitable for use as a method for identity testing, glycosylation profiling, cysteinylation, glycation, oxidation and protein ratio determination for co-formulated drugs such as ADC (129,130).

6.3 Advantages and challenges of MAM application

6.3.1 Advantages

More targeted assessment of CQAs and product quality profile – Chromatography and electrophoresis methods can monitor only one type of CQA and they often provide only a general information about mAb variants. On the other hand, MAM identifies the exact site of modification. For example, CEX gives information about mAb charge via assessment of the amounts of main, acidic and basic peaks. However, it is common to have more mAb variants within one peak, and CEX cannot distinguish between these variants (28). Another example is N-glycan profiling via HILIC assay. This method gives a general snapshot of combined all N-glycans in the molecule. Therefore, if there are more than one glycosylation site in a mAb molecule, this can pose a problem, as it is not capable to give the information about on which residue is the modification located (109). In contrast to standard methods, by analyzing site-specific modifications, MAM enables more precise differentiation between different species/variants and provides detailed and quantitative information at the molecular level. Importantly, MAM can provide a better understanding and control of the product and the process, which is in accordance with QbD requirements (26,106,127).

MAM enables assessment and monitoring of unknown impurities and modifications – This is known as “new peak detection” (NPD) feature. Importantly, MAM’s NPD is more sensitive and robust than the conventional purity assays and it is capable of identifying unexpected new modifications (26).

LC-MS data allows retrospective data processing – Traditionally, when a new CQA is identified later at any stage of the product life-cycle, the analysis of the attribute from archived samples might be needed. Therefore, there might be a need to develop a new analytical method to quantify the attribute. Moreover, historical batches may or may not be available. If done in the full-scan mode, MAM, on the other hand, collects the large amount of product quality information. This is why no additional data need to be collected. Instead, MAM generates a holistic dataset from each run, batch or stability sample. These data can be re-analyzed to obtain necessary information about the specific quality attributes. This gives MAM the potential to essentially change approaches for establishing product control strategies (26).

Improved cycle times – Due to high structural complexity, fast mAb characterization poses a constant challenge. MAM is capable of collecting required attribute information five times faster than array of traditional assays. Interestingly, when time estimation is performed, including sample preparation, incubation time and instrument set up, data collection, analysis and interpretation, one samples can be analyzed by MAM in only 2 days in comparison to traditional approach which takes 10 days (29,120,131,132).

Reduced costs – Given that MAM monitors multiple quality attributes simultaneously, it can replace several assays and instruments (28,127).

6.3.2 Challenges

MAM clearly has potential to improve understanding and control of mAb manufacturing process and product attributes. However, there are still some challenges that need to be overcome before the MS-based MAM can be fully accepted by regulatory authorities and implemented in the GMP environment.

Artificial modifications – There are several steps during sample preparation and analysis which can generate artificial modifications. Artifacts can be misinterpreted as modification or could lead to overestimation or underestimation, or even complete loss of detection of actual PTM. Given that MAM's purpose is to evaluate mAb variants, formation of artifacts needs to be reduced to be able to reliably quantify PTM. This can be achieved by optimization and improvement of sample preparation and analysis protocols in order to generate low artifact peptides (126,133).
Artifact formation can be divided into two groups – sample preparation-induced artifacts and MS-induced artifacts (133). Below we will give an overview of the artifacts and possible ways to control them.

1) Sample preparation-induced artifacts

Carbamylation – can occur as consequence of urea application as the denaturation reagent. Urea's degradation product, isocyanic acid, can react with protein N-terminus, lysine side chain or free cysteines (133,134). Cysteine residues can also be modified by some surfactants used to increase digestion efficiency such as ProteaseMAX (135).

Reduction of Met oxidation – can lead to underestimation of actual oxidation levels. This artifact can occur during reduction of disulfide bonds, which is sample preparation step required to increase digestion efficacy (133).

Increase of Met oxidation – can be catalyzed in the presence of trace metals in digestion buffers or sample contact with metal surfaces of the analysis instruments. This can be avoided by introduction of EDTA or Met in the digestion buffer (136).

Formation of peptide fragments - tris(2-carboxyethyl) phosphine (TCEP) used for reduction of disulfide bonds can cleave cysteine peptide bonds (137). Furthermore, heating sample in the presence of TCEP can also cause peptide bond cleavage (138).

Formation of alanine from cysteine – can occur as a result of heating sample in the presence of TCEP (138).

Alkylation – Normally cysteine is alkylated after sample reduction in order to prevent reformation of disulfide bonds. Undesired alkylation of Met and Lys can happen as artifact (139).

Elimination of reduction-labile modifications including cysteinylation, glutathionylation (140), and trisulfide bonds can occur during reduction sample preparation step (141).

Asn deamidation during enzymatic digestion. This artifact is highly dependent on digestion conditions such as pH, temperature and incubation time, and can be controlled by digestion at lower temperature, lower pH and for a shorter incubation time (142). Another possibility is to distinguish pre-existing and artificial deamidation by using ^{18}O-water during sample preparation (143).

Amino acid cyclization - Finally, digestion procedures can also cause the cyclization of amino acids, such as formation of succinimide from Asp isomerization and formation of pyroglutamate from N-terminal glutamine (133).

Mis-cleavage – is related to protease (trypsin) digestion and can be improved by using trypsin/lysyl endopeptidase (Lys-C) mixture (126,133).

2) Mass-spectrometry-induced artifacts

Ion generation via ESI can generate a wide range of artifacts such as oxidation of methionine, tryptophan, and histidine, formation of peptide-metal adducts (144), peptide cleavage (144), loss of water from Ser, Asp, Glu, and N-terminal Gln (145). The same as with sample preparation, oxidation occurs due to presence of trace metals or by contact with metal surfaces (136). Oxidation can also occur as a result of water electrolysis during analysis by capillary zone electrophoresis coupled to ESI as well as due to higher potential applied to the electrospray needle causes higher levels of oxidation when above the onset of coronal discharge (146). Oxidation of Met can also occur in the gas phase, caused by discharge of eroded emitter during the process of electro-spray ionization (147).

MAM is not suitable for assessment of certain PQA – MAM cannot assess **disulfide bonds, disulfide isoforms and trisulfide levels**. Furthermore, direct assessment of **succinimide** is not possible when trypsin digestion is performed at pH above 7.5, as under these conditions succinimide rapidly hydrolyses to iso Asp and Asp. MS-based MAM is also not able to measure **DNA levels, self-association and aggregation**. MAM may not be able to provide information if there was an overall shift in the **pI** of the product, which could affect pharmacokinetics, especially of s.c. administered products (29). MAM can identify and quantify specific **PTMs and sequence variants, but not if they are evenly distributed** across molecules or only on one part of the molecule population. In addition, certain variants such as clipped species that include a cleavage at lysine and arginine may not be captured by MAM due to masking by the digested peptides (26).
Complicated hardware and software and complex data analysis – MS is very sensitive method and gives complex and detailed information which generates high amount of data. This can pose an additional burden during analysis, especially as not all data are relevant (132).

MAM implementation in QC – Although MS is used during the development phase of almost all mAb, options for fully GMP-compliant LC/MS are limited. LC/MS application in the quality control (QC) environment is complex and so far limited to more simple products, mainly for the mass assessment (26). This is due to several specific challenges for implementation of MS and MS-based MAM in commercial QC laboratories. These challenges are mostly associated with robustness, GPM-compliance of MS and required extensive analyst expertise (148).

Validation of MAM – MAM uses high-maintenance mass spectrometers and equipment which are difficult to implement for product-release testing in the quality control. One of the reasons is that substantial MS expertise is needed for method execution. In addition, due to complex

instrumentation, there is a high instrument-to-instrument variability and it can be challenging to align instrument performance across multiple laboratories. However, recently MAM platforms which instead of high-end mass spectrometers use more simple MS instruments have become available. These instruments could be easier implemented in quality control laboratories to replace several conventional assays (106,127,132,148). One example is MAM based on a single quadrupole MS instrument, which applies peptide mapping under non-reducing conditions, electrospray ionization source and Quadrupole Dalton (QDa) mass detector. The authors showed that the method was successfully validated for the assessment of several PTM such as Asn and Asp isomerization, deamidation, Met oxidation and glycosylation as well as disulfide bond-related modifications. It was cost effective, robust and simple for maintenance and handling. However, this MAM approach does not detect PTM on all amino acid residues, but the pre-selected CQA list defined during the development phase. Therefore, it is not suitable for the NPD and changes in process would require additional methods in order to detect unexpected modifications and impurities (106). In addition, structure-based MS techniques are advancing. Several groups were recently successful in development and validation of QC-friendly MAM platforms based on subunit assessment (129,130,148). Examples are a GMP-compliant LC/MS method for monitoring antibody oxidation (148) and TOF spectrometer-based method for identity, glycan quantification and protein ratio determination (129).

6.4 Regulatory aspects

Amgen has presented the MAM concept for the first time at the CASSS CMC Strategy and Mass Spec Forums in 2014 (149) and its development was first published by Rogers at al. in 2015 (27). Since then, MAM has been a hot topic in the pharmaceutical industry. It has been discussed at several conferences including 8th Annual Biotherapeutic Analytical Summit in 2016 (150), 13th Symposium on the Practical Applications of Mass Spectrometry in the Biotechnology Industry in 2016 (151) and USP Biologics Stakeholder Forum in 2020 (120). Importantly, also regulatory agencies show high interest in novel analytical approaches for biologics such as MAM. In line with this, MAM is being constantly discussed with FDA's Emerging Technology Team (ETT), which also works on adaptation and implementation of MS in biologics development and routine analysis (26). Furthermore, USP is already working on MAM-based performance standards that may help ensure the sensitivity of MAM-based methods and support transfer of methods across laboratories (152). In order to bring leaders from biopharmaceutical companies, regulatory bodies, MS and software vendors together, a MAM Consortium was formed. It currently has more than

320 members. The Consortium is involved in different activities including organization of monthly meetings, development of an open-source MAM software and evaluation of data similarity between companies and vendors (153).

In order for MAM to be accepted by regulatory agencies there are several points which need to be considered. They are classified into risk assessment, method validation, capabilities and specificities of new peak detection feature and comparisons to conventional methods.

1) **Risk assessment** – If MAM is intended to replace conventional methods, risk/benefit ratio when using MAM needs to be evaluated for a specific product and quality attribute. The risk assessment is based on the following points: the severity potential of a CQA, occurrence (the process capability of controlling CQA) and the detection. MAM in comparison to most of conventional methods shows benefits by acting on the detection (28). CQA detection by MAM is amino acid-specific, whereas chromatographic methods assess a whole molecule, which can possibly result in overlooking of specific PTM. However, MAM also shows some limitations here. For example, it is not capable of distinguishing PTM distribution, meaning if a modification is present on both chains or on one chain on approximately half molecules. Therefore, in this case through risk assessment it needs to be evaluated if distribution of a certain modification has effect on product quality and safety. Furthermore, due to digestion, some information about the intact protein might be lost, such as clipped species that include a cleavage at lysine and arginine (which are trypsin cleavage sites) or information about pI. pI is especially important for s.c. or i.m. administration, as charge variants in these products may affect bioavailability. Therefore, if pI or clipped species are classified as CQA for a product, risk assessment and demonstration that the attributes assessed by MAM can replace CEX and reducing CE-SDS are needed before MAM can be implemented (26).

2) **Method validation** – As it is the case for other analytical methods, validation of MAM is necessary for its licensing and application in QC (28) This might be challenging, as there is not much experience with validating MS-based methods (126). The guidelines which can be used for validation are ICH guidelines and FDA guidelines. These are ICHQ2 (R1) – Validation of Analytical Procedures, ICHQ6B – Specifications: Test procedures and Acceptance Criteria for Biotechnological/Biological Products, FDA Guidance on Validation of Chromatographic Methods, FDA Guidance on Analytical Procedures and Methods Validation of Drugs and Biologics. Based on the mentioned guidelines, for a quantitative

method validation specificity, linearity, accuracy, precision, range, quantitation limit, detection limit, robustness, and system suitability testing need to be assessed. Precision, robustness, LOD/LOQ and system suitability are considered as the most challenging aspects in validating MAM (28,127).

Linearity – To test if MAM shows a linear response to a PTM, approach already used for LC-MS systems is applied. Calibration curve is generated by analyzing control samples spiked with a range (for example 0%, 25%, 50%, 75% and 100%) of modified sample. MAM linearity with R^2 above 0.99 has been shown by several studies (106,154).

Specificity – MAM specificity is highly influenced by the chromatographic and mass spectral resolution (155). It is usually assessed by comparing different samples including buffers, stressed samples and samples from different purification steps (106).

Accuracy – Accuracy is assessed by applying MAM protocol to samples spiked with known quantities stressed material and subsequent recovery calculation. In addition, MAM accuracy can be evaluated by comparison of results with the ones obtained by traditional assays (106,154).

Precision – Given that MS is not routinely used in the QC of therapeutic proteins, assessment of precision is essential for showing system suitability (26). Different precision levels should be considered. Repeatability evaluation or intra-assay precision for the monitored PTM is conducted by measuring sample replicates during a single analytical run under identical conditions (27,109,154). Furthermore, intermediate precision (inter-assay, day-to-day, operator-to-operator, column-to-column, instrument-to-instrument) as well as reproducibility are also part of the method validation (106,154). In addition, for MAM it is recommended to ensure its discrimination power, which means that it is able to measure inherent product variability such as low-level differences between batches. This is assessed by comparing the precision acquired from a single batch to the intrinsic batch variability. Furthermore, precision should be compared to conventional methods which are planned to be replaced by MAM (26).

Robustness – MS-based MAM is a highly sensitivity method which is able to detect a thousand of species in a single run. However, it needs to be assured that small variations in sample preparations and LC/MS conditions do not affect results. Robustness in detecting changes in different runs and lots and on different days needs to be ensured (28).

Detection and quantitation limit – DL and QL should be determined for both targeted attribute quantitation and NPD. This is done by using peptides which are representative of the one being measure. Of high importance is to cover expected variations in peptide size, retention time and abundance. Especially challenging is the determination of DL and QL for NPD feature. This is done by spiking of the sample with potential impurities and modifications that are not actively monitor (26).

System suitability testing is used to demonstrate acceptable assay performance and that an analytical method is suitable for its intended purpose. Setting appropriate system suitability criteria can ensure acceptable sample preparation and LC/MS analysis. The proposed metrics for system suitability testing for mAb characterization by LC/MS are variation of peak areas, accuracy and variation of relative quantitation results, mass accuracy/resolution change, signal-to-noise ratio (26,156).

Software validation – Software used for MAM data acquisition and processing also need to be validated and GMP compliant according to relevant guidelines for the use of computerized systems in a GMP regulated activities including EU GMP Guidelines Annex 11 Computerized Systems and FDA Guidance for Industry Part 11 (157,158). The relevant GMP compliance items for MAM software are audit trail, security and limited system access to authorized individuals, electronic signatures, data backup and recovery as well as full validation of the LC/MS system including hardware and software (installation qualification (IQ), operational qualification (OQ) and performance qualification (PQ)) (148). In line with this, several vendors offer semi-automated GMP compliant software, which are delivered with detailed user documentation, certificates of software validation, and support documentation for on-site system validation by end-users (159).

3) **Comparison to conventional methods** – From a regulatory perspective MAM needs to be compared to the conventional methods in order to justify that MAM is capable to control the CQAs. However, MAM and conventional methods are not always directly comparable as they sometimes do not measure the same isoform species. Therefore, by introducing MAM some information might be lost and some gained. The differences should be part of the risk assessment and their impact on product quality and safety should be thoroughly evaluated. Before the existing methods can be completely replaced by MAM, it is expected that there will be a period where both methods will be side-by-side included in the submissions (26). One of the possible regulatory strategies used by some pharmaceutical companies is to first present MAM to authorities as a characterization method. The next

step would be, based on successful acceptance, to replace standard methods by MAM as a single method for the late-stage clinical products (28).

4) **New peak detection capability and specificity** – The most critical and challenging parameter for NPD feature is peak intensity threshold. Setting a too low threshold may lead to false positive detection. On the other hand, caution needs to be taken also not set the threshold too high as some peaks could be missed (121). The threshold is set experimentally and needs to be optimized for the specific product and instrument/software. There are several approaches that can be applied for the threshold determination. For example, samples with known expected modifications (spike samples) might be used to prevent false negative detection. In this way the limit is set at the level which enables identification of all known modifications in the spike samples. In addition, a sample with no expected modifications is used to set the threshold to the level which prevents false positive detection. Another approach is to run traditional assays and MAM side by side and to use “failed samples” (samples which would not meet specification requirements when assessed by traditional methods) to set the MAM threshold. The threshold is being decreased to the level which enables detection of new peaks in the failed samples and lowered even further until false positive peaks appear. Recently published MAM Consortium interlaboratory study showed that when fold-change is used as criterion to determine new peaks, most laboratories use 5-fold or 10-fold limit (119).

7 Discussion and conclusion

Regulatory authorities recommend the QbD approach for the manufacturing of therapeutic antibodies. This approach requires that CQAs are identified based on the severity of harm to a patient or lack of efficacy resulting from failure to meet that quality attribute. To fulfill these requirements in-depth understanding of CQA at molecular level is required.

Final structure of recombinant proteins together with PTM is influenced by many factors as well as their mutual interactions. This makes it nearly impossible to achieve an identical mAb structure, even across the batches of the same manufacturer produced in the same facility using the same processes (22). Because they highly impact mAb pharmacology and safety, many of these PTM are considered as CQA. Therefore, under the QbD scope the authorities demand PTM analysis during product development, quality control as well as a part of biosimilarity assessment (160–162). Therefore, choice and application of proper analytical methods to identify and quantify PTM are essential for identification of CQA and implementation of control strategies. However, none of the standard methods are able to characterize all aspects of heterogeneity. This is why PTM are commonly assessed by orthogonal methods, multiple assays and instruments, which makes mAb analytics complex and time- and resource-consuming (163).

In order to reduce number of methods required for drug characterization, innovative approaches which could possible assess several CQA in one run are being proposed. One of them is bottom-up MS-based MAM. Owing to rapid advances in the MS field, this approach has been adopted by several pharmaceutical companies and its development is highly supported by regulatory authorities. Compared to conventional methodologies, the LC-MS-based MAM provides direct and amino acid-specific identification and quantification of the biologically relevant PQA. In addition, it enables establishment of correlation between CQA and critical process parameters (CPPs) which fulfils high regulatory expectations. The application of MAM assays provides quantitative information to support mAb development from early phases such as clone selection and process development to pre-clinical and clinical studies. Moreover, the LC-MS MAM approach has been demonstrated by some groups to be useful to monitor potential changes to the PTM profile of a given therapeutic mAb *in vivo*, which has also recently been recommended by some regulatory bodies. The potential susceptibility of a therapeutic protein to modifications *in vivo* may result in loss of efficacy or induction of immune responses. Obtaining this information

early in design stage, candidate selection, and drug development may facilitate therapeutic protein engineering to increase the stability of the product in the *in vivo* milieu (164).

In the short run, MAM has the biggest potential in the areas of biopharmaceutical development, in the beginning mostly as a complementary assay. Looking toward the future, MAM has potential to replace many of the standard assays. However, adoption of MAM in the QC environment requires further optimization and numerous challenges to be addressed (120). Some of the challenges connected to MAM application could be overcome by development of suitable sample preparation protocols (reduction of artifacts), optimization of the LC-MS conditions (such as S-Lens RF, Capillary temperature and auxiliary gas temperature) (126), implementation of system suitability controls and GMP-compliant software, automatization of the sample preparation and data analysis (92,132).

Compared to traditional methods, development of a LC-MS-based MAM approaches is more challenging, due to complex sample preparation, LC separation, MS instrumentation, and data acquisition and analysis. Each step requires careful adjustment and optimization of multiple critical conditions or parameters. Depending on the antibody type, development of different sample preparation protocols might be required. Denaturation and enzyme digestion conditions, such as pH, digestion time, and denaturants, need to be optimized in order to ensure specific cleavages and minimize possible artifacts. When it comes to separation and MS acquisition, the strategy should be carefully chosen and adjusted. For each workflow, column parameters (type, temperature), LC gradient, buffers need to be determined in order to achieve optimal separation. Mass spectrometry parameters, such as tuning method and acquisition method, must also be optimized. Setting of data analysis parameters including search parameters, ion peak extraction parameters and peak intensity threshold levels is critical for obtaining reliable and solid data.

In accordance with the above-mentioned challenges, since the first MAM publication, several research groups have been working on the method development, its improvement and new applications. For example, application of mico-LC compared to UHPLC, assessment of glycosylation sites, application for process monitoring and *in vivo* testing (26,165).

Most MAM developed so far are based on high-resolution/high-mass accuracy MS instruments, due to their high mass resolving power to distinguish analyte signals from interferences (132). However, several recently published works suggest application of lower-resolution instruments characterized which are easy to handle, reliable, generate small footprint and are low cost. If these spectrometers indeed demonstrate to be suitable for MAM purposes, this may further

facilitate the adoption of the MAM technology for the routine application in analytical laboratories (155).

In conclusion, with the advancement of mass spectrometry field and instrument and analysis software automation, fully automated LC-MS MAM platform could be developed in the near future. This approach would provide more in-depth qualitative and quantitative information of CQAs than current standard analytical methods. It could support the QbD-compliant therapeutic protein analysis during through the whole product life-cycle.

8 Abstract

Therapeutic monoclonal antibodies (mAb) play an important role in the treatment of several diseases such as cancer, autoimmune disorders, and infectious diseases. In comparison to small-molecule drugs, they are much larger and by far more complex, which makes their development and regulatory evaluation challenging.

One of the biggest challenges is a high variability of mAbs, known as (micro-)heterogeneity. It originates from post-translational modifications (PTM) which are happening during mAb synthesis in the cell culture or from chemical and physical modifications during purification, formulation and storage. Hundreds of different modifications may co-exist such as glycosylation (which includes galactosylation, fucosylation, high mannose derivatives and sialylation), oxidation, phosphorylation, sulfation, disulfide bond formation and deamidation.

Heterogeneity pattern can significantly impact pharmacokinetics, pharmacodynamics and safety of mAbs. Therefore, some of PTM belong to critical quality-defining attributes and their characterization, quantification and monitoring during development and quality control release is essential.

The conventional approach uses a panel of different analytical methods to evaluate the heterogeneity and the other quality attributes of mAbs. These are high-performance liquid chromatography (HPLC), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), capillary gel electrophoresis and liquid chromatography/mass spectrometry (LC/MS). Taken together, all these methods give a kind of method specific fingerprint of respective physic-chemical properties of the mAb. However, they do not give insight into composition and structures at molecular level. In the recent years, both industry and regulatory bodies have gained interest in a novel approach known as multi-attribute method (MAM). MAM combines advanced state-of-the-art mass spectrometry methods with liquid chromatography (LC-MS) in order to simultaneously assess several critical quality attributes related to PTM in one assay. Main MAM advantages are high specificity and resolution, lower costs and saving time as it has potential to replace several conventional methods.

The aim of this master thesis is to give an overview of the current knowledge about mAb heterogeneity and how specific mAb PTM impact mAb pharmacology and product quality. Furthermore, we will give insight into recent advancements in analytical methods, with the main focus on MAM, which would enable more simple characterization and monitoring of mAb PTM.

Finally, advantages and challenges of MAM as well regulatory considerations for its implementation in GMP settings and quality control will be thoroughly evaluated.

9 Zusammenfassung

Therapeutische monoklonale Antikörper (mAbs) spielen eine wichtige Rolle bei der Behandlung verschiedener Krankheiten wie Krebs, Autoimmunerkrankungen und Infektionskrankheiten. Im Vergleich zu niedermolekularen Arzneimitteln sind sie viel größer und weitaus komplexer, was ihre Entwicklung und behördliche Evaluierung erschwert.

Eine der größten Herausforderungen ist die hohe Variabilität von mAbs, bekannt als (Mikro-) Heterogenität. Sie entsteht durch posttranslationale chemische und physikalische Modifikationen (PTM), die bei der Herstellung in der Zellkultur oder bei der Reinigung, Formulierung und Lagerung auftreten. Hunderte von verschiedenen Modifikationen können nebeneinander existieren, wie Glykosylierung (Galaktosylierung, Fucosylierung, Sialylierung, mannosereiche Strukturen), Oxidation, Phosphorylierung, Sulfatierung, Bildung von Disulfidbindungen und Desamidierung.

Heterogenitätsmuster können Pharmakokinetik, Pharmakodynamik und Sicherheit von mAbs signifikant beeinflussen. Daher gehören einige der PTM zu kritischen Qualitätsattributen und ihre Charakterisierung, Quantifizierung und Überwachung während der Entwicklung und der Freigabe nach der Qualitätskontrolle ist wesentlich.

Der konventionelle Ansatz verwendet ein Panel verschiedener analytischer Methoden, um die Heterogenität und die anderen Qualitätsmerkmale von mAbs zu bewerten. Dies sind Hochleistungsflüssigkeitschromatographie (HPLC), Natriumdodecylsulfat-Polyacrylamidgelelektrophorese (SDS-PAGE), Kapillarelektrophorese und Flüssigchromatographie mit Massenspektrometrie-Kopplung (LC/MS). Zusammengenommen ergeben alle diese Methoden eine Art methodenspezifischer Fingerabdruck der jeweiligen physikalisch-chemischen Eigenschaften des mAb. Sie geben jedoch keinen Einblick in Zusammensetzung und Strukturen auf molekularer Ebene. In den letzten Jahren haben sowohl die Industrie als auch die Behörden zunehmend Interesse an einem neuartigen Ansatz, der als Multi-Attribute-Methode (MAM) bekannt ist. MAM kombiniert modernste Massenspektrometrie-Methoden mit Flüssigchromatographie (LC-MS), um gleichzeitig mehrere kritische Qualitätsmerkmale im Zusammenhang mit PTM in einem Assay zu bewerten. Die Hauptvorteile von MAM sind hohe Spezifität und Auflösung, geringere Kosten und Zeitersparnis, da es das Potenzial hat, mehrere konventionelle Methoden zu ersetzen.

Das Ziel dieser Masterarbeit ist es, einen Überblick über das aktuelle Wissen über die Heterogenität von mAbs und den Einfluss spezifischer PTM auf die Pharmakologie und Produktqualität von mAbs zu geben. Darüber hinaus werden wir Einblicke in die neusten Fortschritte bei analytischen Methoden mit dem Schwerpunkt auf MAM geben, die eine einfachere Charakterisierung und Überwachung von PTM ermöglichen würden. Schließlich werden die Vorteile und Herausforderungen von MAM sowie regulatorische Überlegungen zu ihrer Implementierung in GMP-Umgebung und Qualitätskontrolle gründlich diskutiert.

10 Abbreviations

ADCC	Antibody-dependent cell-mediated cytotoxicity
AGE	advanced glycation end product
ASGPR	Asialoglycoprotein receptor
Asn	Asparagine
C1q	Complement component 1q
CDC	Complement-mediated cytotoxicity
CDR	Complementarity-determining region
CE	Capillary electrophoresis
CPP	Critical process parameters
CQA	Critical quality attribute
Cys	Cysteine
EMA	European Medicines Agency
FcRn	Fc-neonatal receptor
FcγRs	Fc-γ-receptors
FDA	Food and Drug Administration
GlcNAc	N-acetylglucosamine
HILIC	Hydrophilic-interaction liquid chromatography
IEF	Isoelectric focusing
IEX	Ion exchange chromatography
Ig	Immunoglobulin
mAb	Monoclonal antibody
MAM	Multi-attribute method
Met	Methionine
MR	Mannose receptor
Neu5Gc	N-glycolylneuraminic acid
pI	Isoelectric point
PQA	Product quality attribute
PTM	Posttranslational modifications
PyroGlu	Pyroglutamate
QbD	Quality by design
QTPP	Quality target product profile
RP-HPLC	Reversed-phase high performance liquid chromatography
SDS	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC	Size-exclusion chromatography
Ser	Serine
Thr	Threonine
Trp	Tryptophan
Tyr	Tyrosine

11 References

1. Castelli MS, McGonigle P, Hornby PJ. The pharmacology and therapeutic applications of monoclonal antibodies. Pharmacol Res Perspect. 2019 Dec;7(6):e00535–e00535. Available from: https://pubmed.ncbi.nlm.nih.gov/31859459
2. Kaplon H, Muralidharan M, Schneider Z, Reichert JM. Antibodies to watch in 2020. MAbs. 2020;12(1):1703531. Available from: https://pubmed.ncbi.nlm.nih.gov/31847708
3. Kaplon H, Reichert JM. Antibodies to watch in 2021. MAbs. 2021;13(1):1860476. Available from: https://pubmed.ncbi.nlm.nih.gov/33459118
4. Medicines Agency E. Committee for medicinal products for human use (CHMP) Guideline on development, production, characterisation and specification for monoclonal antibodies and related products Guideline on development, production, characterisation and specification for monoclonal antibodies and related products. 2008 [cited 2021 Mar 17]. Available from: www.ema.europa.eu/contact
5. Mastrangeli R, Palinsky W, Bierau H. Glycoengineered antibodies: Towards the next-generation of immunotherapeutics. Glycobiology. 2018;29(3):199–210.
6. Labrijn AF, Janmaat ML, Reichert JM, Parren PWHI. Bispecific antibodies: a mechanistic review of the pipeline. Nat Rev Drug Discov. 2019 Aug;18(8):585–608.
7. Birrer MJ, Moore KN, Betella I, Bates RC. Antibody-Drug Conjugate-Based Therapeutics : State of the Science. 2019;
8. Foltz IN, Karow M, Wasserman SM. Evolution and emergence of therapeutic monoclonal antibodies: what cardiologists need to know. Circulation. 2013 Jun;127(22):2222–30.
9. Ovacik M, Lin K. Tutorial on Monoclonal Antibody Pharmacokinetics and Its Considerations in Early Development. Clin Transl Sci. 2018 Nov;11(6):540–52.
10. Xu X, Qiu H, Li N. LC-MS multi-attribute method for characterization of biologics. J Appl Bioanal. 2017;3(2):21–5.
11. Almagro JC, Daniels-Wells TR, Perez-Tapia SM, Penichet ML. Progress and Challenges in the Design and Clinical Development of Antibodies for Cancer Therapy. Front Immunol. 2017;8:1751.
12. Chiu ML, Gilliland GL. Engineering antibody therapeutics. Curr Opin Struct Biol. 2016 Jun;38:163–73.
13. Liu JKH. The history of monoclonal antibody development - Progress, remaining challenges and future innovations. Ann Med Surg. 2014;3(4):113–6. Available from: http://dx.doi.org/10.1016/j.amsu.2014.09.001

14. Finkler C, Krummen L. Introduction to the application of QbD principles for the development of monoclonal antibodies. Biologicals. 2016 Sep;44(5):282–90.
15. Yu LX, Amidon G, Khan MA, Hoag SW, Polli J, Raju GK, et al. Understanding pharmaceutical quality by design. AAPS J. 2014 Jul;16(4):771–83.
16. European Medicines Agency [EMA]. ICH guideline Q8 (R2) on pharmaceutical development. 2009. Available from: https://www.ema.europa.eu/en/documents/scientific-guideline/international-conference-harmonisation-technical-requirements-registration-pharmaceuticals-human-use_en-11.pdf
17. U.S. Food and Drug Administration (FDA). Q8(R2) Pharmaceutical Development. [cited 2021 Mar 18]. Available from: https://www.fda.gov/regulatory-information/search-fda-guidance-documents/q8r2-pharmaceutical-development
18. Beyer B, Schuster M, Jungbauer A, Lingg N. Microheterogeneity of Recombinant Antibodies: Analytics and Functional Impact. Biotechnol J. 2018 Jan;13(1).
19. Kunert R, Reinhart D. Advances in recombinant antibody manufacturing. Appl Microbiol Biotechnol. 2016 Apr;100(8):3451–61.
20. Brunner M, Fricke J, Kroll P, Herwig C. Investigation of the interactions of critical scale-up parameters (pH, pO(2) and pCO(2)) on CHO batch performance and critical quality attributes. Bioprocess Biosyst Eng. 2017 Feb;40(2):251–63.
21. Rathore AS, Kumar Singh S, Pathak M, Read EK, Brorson KA, Agarabi CD, et al. Fermentanomics: Relating quality attributes of a monoclonal antibody to cell culture process variables and raw materials using multivariate data analysis. Biotechnol Prog. 2015;31(6):1586–99.
22. Batra J, Rathore AS. Glycosylation of monoclonal antibody products: Current status and future prospects. Biotechnol Prog. 2016;32(5):1091–102.
23. Torkashvand F, Vaziri B. Main Quality Attributes of Monoclonal Antibodies and Effect of Cell Culture Components. Vol. 21, Iranian biomedical journal. 2017. p. 131–41.
24. Alter G, Ottenhoff THM, Joosten SA. Antibody glycosylation in inflammation, disease and vaccination. Semin Immunol. 2018;39(May):102–10.
25. Ahrer K, Jungbauer A. Chromatographic and electrophoretic characterization of protein variants. J Chromatogr B, Anal Technol Biomed life Sci. 2006 Sep;841(1–2):110–22.
26. Rogstad S, Yan H, Wang X, Powers D, Brorson K, Damdinsuren B, et al. Multi-Attribute Method for Quality Control of Therapeutic Proteins. Anal Chem. 2019;
27. Rogers RS, Nightlinger NS, Livingston B, Campbell P, Bailey R, Balland A. Development of a quantitative mass spectrometry multi-attribute method for characterization, quality

control testing and disposition of biologics. MAbs. 2015;7(5):881–90.
28. Amgen. Amgen “Analytics of the Future” Initiative Develops MS-based “Multi-Attribute Methodology” to Streamline Biotech Development and QC Testing. IPQ Publ. 2015; Available from: https://ipq.org/amgen-analytics-of-the-future-initiative-develops-ms-based-multi-attribute-methodology-to-streamline-biotech-development-and-qc-testing/
29. Swann P. Regulatory Considerations When Developing Relevant and Sustainable Analytical Technologies. Amgen. 2018; Available from: https://cdn.ymaws.com/www.casss.org/resource/resmgr/at_europe_speaker_slides/2018_ATE_Swann_Slides.pdf
30. Liu L. Pharmacokinetics of monoclonal antibodies and Fc-fusion proteins. Protein Cell. 2018 Jan;9(1):15–32.
31. Richter WF, Jacobsen B. Subcutaneous absorption of biotherapeutics: knowns and unknowns. Drug Metab Dispos. 2014 Nov;42(11):1881–9.
32. Shah DK, Betts AM. Antibody biodistribution coefficients: inferring tissue concentrations of monoclonal antibodies based on the plasma concentrations in several preclinical species and human. MAbs. 2013;5(2):297–305.
33. Kamath A V. Translational pharmacokinetics and pharmacodynamics of monoclonal antibodies. Drug Discov Today Technol. 2016;21–22:75–83.
34. Tabrizi MA, Tseng C-ML, Roskos LK. Elimination mechanisms of therapeutic monoclonal antibodies. Drug Discov Today. 2006 Jan;11(1–2):81–8.
35. Keizer RJ, Huitema ADR, Schellens JHM, Beijnen JH. Clinical pharmacokinetics of therapeutic monoclonal antibodies. Clin Pharmacokinet. 2010;49(8):493–507.
36. Khawli LA, Goswami S, Hutchinson R, Kwong ZW, Yang J, Wang X, et al. Charge variants in IgG1: Isolation, characterization, in vitro binding properties and pharmacokinetics in rats. MAbs. 2010;2(6):613–24.
37. Li B, Tesar D, Boswell CA, Cahaya HS, Wong A, Zhang J, et al. Framework selection can influence pharmacokinetics of a humanized therapeutic antibody through differences in molecule charge. MAbs. 2014;6(5):1255–64.
38. Stockert RJ. The asialoglycoprotein receptor: relationships between structure, function, and expression. Physiol Rev. 1995 Jul;75(3):591–609.
39. Ashwell G, Harford J. Carbohydrate-specific receptors of the liver. Annu Rev Biochem. 1982;51:531–54.
40. Lee SJ, Evers S, Roeder D, Parlow AF, Risteli J, Risteli L, et al. Mannose receptor-mediated regulation of serum glycoprotein homeostasis. Science. 2002

Mar;295(5561):1898–901.
41. Roopenian DC, Akilesh S. FcRn: the neonatal Fc receptor comes of age. Nat Rev Immunol. 2007 Sep;7(9):715–25.
42. Borvak J, Richardson J, Medesan C, Antohe F, Radu C, Simionescu M, et al. Functional expression of the MHC class I-related receptor, FcRn, in endothelial cells of mice. Int Immunol. 1998 Sep;10(9):1289–98.
43. Kobayashi N, Suzuki Y, Tsuge T, Okumura K, Ra C, Tomino Y. FcRn-mediated transcytosis of immunoglobulin G in human renal proximal tubular epithelial cells. Am J Physiol Renal Physiol. 2002 Feb;282(2):F358-65.
44. Schellekens H. Immunogenicity of therapeutic proteins: clinical implications and future prospects. Clin Ther. 2002 Nov;24(11):1720–40; discussion 1719.
45. Nimmerjahn F, Ravetch J V. Fcgamma receptors as regulators of immune responses. Nat Rev Immunol. 2008 Jan;8(1):34–47.
46. Jefferis R, Lund J. Interaction sites on human IgG-Fc for FcgammaR: current models. Immunol Lett. 2002 Jun;82(1–2):57–65.
47. Duncan AR, Winter G. The binding site for C1q on IgG. Nature. 1988 Apr;332(6166):738–40.
48. Zhang Y, Fan C, Zhang L, Ma X. Glycosylation-dependent antitumor therapeutic monoclonal antibodies. Prog Mol Biol Transl Sci. 2019;163:471–85.
49. Cymer F, Beck H, Rohde A, Reusch D. Therapeutic monoclonal antibody N-glycosylation – Structure, function and therapeutic potential. Biologicals. 2018;52(July):1–11. Available from: http://dx.doi.org/10.1016/j.biologicals.2017.11.001
50. Reusch D, Tejada ML. Fc glycans of therapeutic antibodies as critical quality attributes. Glycobiology. 2015 Dec;25(12):1325–34.
51. Arnold JN, Wormald MR, Sim RB, Rudd PM, Dwek RA. The impact of glycosylation on the biological function and structure of human immunoglobulins. Annu Rev Immunol. 2007;25:21–50.
52. Dionne B, Mishra N, Butler M. A low redox potential affects monoclonal antibody assembly and glycosylation in cell culture. J Biotechnol. 2017;246:71–80. Available from: http://dx.doi.org/10.1016/j.jbiotec.2017.01.016
53. Villacrés C, Tayi VS, Lattová E, Perreault H, Butler M. Low glucose depletes glycan precursors, reduces site occupancy and galactosylation of a monoclonal antibody in CHO cell culture. Biotechnol J. 2015 Jul;10(7):1051–66.
54. Hodoniczky J, Zheng YZ, James DC. Control of recombinant monoclonal antibody effector

functions by Fc N-glycan remodeling in vitro. Biotechnol Prog. 2005;21(6):1644–52.
55. Subedi GP, Barb AW. The immunoglobulin G1 N-glycan composition affects binding to each low affinity Fc γ receptor. MAbs. 2016;8(8):1512–24.
56. Chung AW, Crispin M, Pritchard L, Robinson H, Gorny MK, Yu X, et al. Identification of antibody glycosylation structures that predict monoclonal antibody Fc-effector function. AIDS. 2014 Nov;28(17):2523–30.
57. Thomann M, Reckermann K, Reusch D, Prasser J, Tejada ML. Fc-galactosylation modulates antibody-dependent cellular cytotoxicity of therapeutic antibodies. Mol Immunol. 2016 May;73:69–75.
58. Chung CH, Mirakhur B, Chan E, Le Q-T, Berlin J, Morse M, et al. Cetuximab-induced anaphylaxis and IgE specific for galactose-alpha-1,3-galactose. N Engl J Med. 2008 Mar;358(11):1109–17.
59. Zhou Q, Shankara S, Roy A, Qiu H, Estes S, McVie-Wylie A, et al. Development of a simple and rapid method for producing non-fucosylated oligomannose containing antibodies with increased effector function. Biotechnol Bioeng. 2008 Feb;99(3):652–65.
60. Bruhns P, Samuelsson A, Pollard JW, Ravetch J V. Colony-stimulating factor-1-dependent macrophages are responsible for IVIG protection in antibody-induced autoimmune disease. Immunity. 2003 Apr;18(4):573–81.
61. Quast I, Keller CW, Maurer MA, Giddens JP, Tackenberg B, Wang L-X, et al. Sialylation of IgG Fc domain impairs complement-dependent cytotoxicity. J Clin Invest. 2015 Nov;125(11):4160–70.
62. Scallon BJ, Tam SH, McCarthy SG, Cai AN, Raju TS. Higher levels of sialylated Fc glycans in immunoglobulin G molecules can adversely impact functionality. Mol Immunol. 2007 Mar;44(7):1524–34.
63. Li T, DiLillo DJ, Bournazos S, Giddens JP, Ravetch J V, Wang L-X. Modulating IgG effector function by Fc glycan engineering. Proc Natl Acad Sci U S A. 2017 Mar;114(13):3485–90.
64. Borys MC, Dalal NG, Abu-Absi NR, Khattak SF, Jing Y, Xing Z, et al. Effects of culture conditions on N-glycolylneuraminic acid (Neu5Gc) content of a recombinant fusion protein produced in CHO cells. Biotechnol Bioeng. 2010 Apr;105(6):1048–57.
65. Shields RL, Lai J, Keck R, O'Connell LY, Hong K, Meng YG, et al. Lack of fucose on human IgG1 N-linked oligosaccharide improves binding to human Fcgamma RIII and antibody-dependent cellular toxicity. J Biol Chem. 2002 Jul;277(30):26733–40.
66. Shibata-Koyama M, Iida S, Misaka H, Mori K, Yano K, Shitara K, et al. Nonfucosylated rituximab potentiates human neutrophil phagocytosis through its high binding for

FcgammaRIIIb and MHC class II expression on the phagocytotic neutrophils. Exp Hematol. 2009 Mar;37(3):309–21.

67. Chung S, Quarmby V, Gao X, Ying Y, Lin L, Reed C, 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):326–40.
68. Jones AJS, Papac DI, Chin EH, Keck R, Baughman SA, Lin YS, et al. Selective clearance of glycoforms of a complex glycoprotein pharmaceutical caused by terminal N-acetylglucosamine is similar in humans and cynomolgus monkeys. Glycobiology. 2007 May;17(5):529–40.
69. Abès R, Teillaud J-L. Impact of Glycosylation on Effector Functions of Therapeutic IgG. Pharmaceuticals (Basel). 2010 Jan;3(1):146–57.
70. Wei B, Berning K, Quan C, Zhang YT. Glycation of antibodies: Modification, methods and potential effects on biological functions. MAbs. 2017;9(4):586–94.
71. Kennedy DM, Skillen AW, Self CH. Glycation of monoclonal antibodies impairs their ability to bind antigen. Clin Exp Immunol. 1994 Nov;98(2):245–51.
72. Dolhofer R, Siess EA, Wieland OH. Nonenzymatic glycation of immunoglobulins leads to an impairment of immunoreactivity. Biol Chem Hoppe Seyler. 1985 Apr;366(4):361–6.
73. Schöneich C. Photo-Degradation of Therapeutic Proteins: Mechanistic Aspects. Pharm Res. 2020 Feb;37(3):45.
74. Shah DD, Zhang J, Hsieh M ching, Sundaram S, Maity H, Mallela KMG. Effect of Peroxide- Versus Alkoxyl-Induced Chemical Oxidation on the Structure, Stability, Aggregation, and Function of a Therapeutic Monoclonal Antibody. J Pharm Sci. 2018;107(11):2789–803. Available from: https://doi.org/10.1016/j.xphs.2018.07.024
75. Bertolotti-Ciarlet A, Wang W, Lownes R, Pristatsky P, Fang Y, McKelvey T, et al. Impact of methionine oxidation on the binding of human IgG1 to Fc Rn and Fc gamma receptors. Mol Immunol. 2009 May;46(8–9):1878–82.
76. Dashivets T, Stracke J, Dengl S, Knaupp A, Pollmann J, Buchner J, et al. Oxidation in the complementarity-determining regions differentially influences the properties of therapeutic antibodies. MAbs. 2016;8(8):1525–35.
77. Cymer F, Thomann M, Wegele H, Avenal C, Schlothauer T, Gygax D, et al. Oxidation of M252 but not M428 in hu-IgG1 is responsible for decreased binding to and activation of hu-FcγRIIa (His131). Biologicals. 2017 Nov;50:125–8.
78. Stracke J, Emrich T, Rueger P, Schlothauer T, Kling L, Knaupp A, et al. A novel approach to investigate the effect of methionine oxidation on pharmacokinetic properties of

therapeutic antibodies. MAbs. 2014;6(5):1229–42.
79. Liu H, Gaza-Bulseco G, Xiang T, Chumsae C. Structural effect of deglycosylation and methionine oxidation on a recombinant monoclonal antibody. Mol Immunol. 2008;45(3):701–8.
80. Wei Z, Feng J, Lin H-Y, Mullapudi S, Bishop E, Tous GI, et al. Identification of a single tryptophan residue as critical for binding activity in a humanized monoclonal antibody against respiratory syncytial virus. Anal Chem. 2007 Apr;79(7):2797–805.
81. Alam ME, Slaney TR, Wu L, Das TK, Kar S, Barnett G V., et al. Unique Impacts of Methionine Oxidation, Tryptophan Oxidation, and Asparagine Deamidation on Antibody Stability and Aggregation. J Pharm Sci. 2020;109(1):656–69. Available from: https://doi.org/10.1016/j.xphs.2019.10.051
82. Elgundi Z, Reslan M, Cruz E, Sifniotis V, Kayser V. The state-of-play and future of antibody therapeutics. Adv Drug Deliv Rev. 2017;122:2–19. Available from: http://dx.doi.org/10.1016/j.addr.2016.11.004
83. Pace AL, Wong RL, Zhang YT, Kao Y-H, Wang YJ. Asparagine deamidation dependence on buffer type, pH, and temperature. J Pharm Sci. 2013 Jun;102(6):1712–23.
84. Huang L, Lu J, Wroblewski VJ, Beals JM, Riggin RM. In vivo deamidation characterization of monoclonal antibody by LC/MS/MS. Anal Chem. 2005 Mar;77(5):1432–9.
85. Haberger M, Bomans K, Diepold K, Hook M, Gassner J, Schlothauer T, et al. Assessment of chemical modifications of sites in the CDRs of recombinant antibodies: Susceptibility vs. functionality of critical quality attributes. MAbs. 2014;6(2):327–39.
86. Doyle HA, Gee RJ, Mamula MJ. Altered immunogenicity of isoaspartate containing proteins. Autoimmunity. 2007 Mar;40(2):131–7.
87. Datta-Mannan A. Mechanisms influencing the pharmacokinetics and disposition of monoclonal antibodies and peptides. Drug Metab Dispos. 2019;47(10):1100–10.
88. Alam ME, Barnett G V, Slaney TR, Starr CG, Das TK, Tessier PM. Deamidation Can Compromise Antibody Colloidal Stability and Enhance Aggregation in a pH-Dependent Manner. Mol Pharm. 2019 May;16(5):1939–49.
89. Harris RJ. Processing of C-terminal lysine and arginine residues of proteins isolated from mammalian cell culture. J Chromatogr A. 1995 Jun;705(1):129–34.
90. Cai B, Pan H, Flynn GC. C-terminal lysine processing of human immunoglobulin G2 heavy chain in vivo. Biotechnol Bioeng. 2011 Feb;108(2):404–12.
91. Faid V, Leblanc Y, Berger M, Seifert A, Bihoreau N, Chevreux G. C-terminal lysine clipping of IgG1: impact on binding to human FcγRIIIa and neonatal Fc receptors. Eur J Pharm Sci

Off J Eur Fed Pharm Sci. 2021 Apr;159:105730.

92. Song YE, Dubois H, Hoffmann M, D Eri S, Fromentin Y, Wiesner J, et al. Automated mass spectrometry multi-attribute method analyses for process development and characterization of mAbs. J Chromatogr B, Anal Technol Biomed life Sci. 2021 Mar;1166:122540.
93. Liu Z, Valente J, Lin S, Chennamsetty N, Qiu D, Bolgar M. Cyclization of N-Terminal Glutamic Acid to pyro-Glutamic Acid Impacts Monoclonal Antibody Charge Heterogeneity Despite Its Appearance as a Neutral Transformation. J Pharm Sci. 2019 Oct;108(10):3194–200.
94. Liu H, Ponniah G, Zhang H-M, Nowak C, Neill A, Gonzalez-Lopez N, et al. In vitro and in vivo modifications of recombinant and human IgG antibodies. MAbs. 2014;6(5):1145–54.
95. Liu H, May K. Disulfide bond structures of IgG molecules: structural variations, chemical modifications and possible impacts to stability and biological function. MAbs. 2012;4(1):17–23.
96. Ayalew L, D'Souza AD, Chen Y, Kim MT. Identification and quantitation of hinge cysteinylation on endogenous IgG2 antibodies from human serum. MAbs. 2020;12(1):1854923.
97. Robotham AC, Kelly JF. Detection and quantification of free sulfhydryls in monoclonal antibodies using maleimide labeling and mass spectrometry. MAbs. 2019;11(4):757–66.
98. Handlogten MW, Zhu M, Ahuja S. Glutathione and thioredoxin systems contribute to recombinant monoclonal antibody interchain disulfide bond reduction during bioprocessing. Biotechnol Bioeng. 2017;114(7):1469–77.
99. Dillon TM, Ricci MS, Vezina C, Flynn GC, Liu YD, Rehder DS, et al. Structural and functional characterization of disulfide isoforms of the human IgG2 subclass. J Biol Chem. 2008 Jun;283(23):16206–15.
100. Schuurman J, Perdok GJ, Gorter AD, Aalberse RC. The inter-heavy chain disulfide bonds of IgG4 are in equilibrium with intra-chain disulfide bonds. Mol Immunol. 2001 Jan;38(1):1–8.
101. Gu S, Wen D, Weinreb PH, Sun Y, Zhang L, Foley SF, et al. Characterization of trisulfide modification in antibodies. Anal Biochem. 2010 May;400(1):89–98.
102. Kita A, Ponniah G, Nowak C, Liu H. Characterization of Cysteinylation and Trisulfide Bonds in a Recombinant Monoclonal Antibody. Anal Chem. 2016 May;88(10):5430–7.
103. Banks DD, Gadgil HS, Pipes GD, Bondarenko P V, Hobbs V, Scavezze JL, et al. Removal of cysteinylation from an unpaired sulfhydryl in the variable region of a recombinant

monoclonal IgG1 antibody improves homogeneity, stability, and biological activity. J Pharm Sci. 2008 Feb;97(2):775–90.

104. McSherry T, McSherry J, Ozaeta P, Longenecker K, Ramsay C, Fishpaugh J, et al. Cysteinylation of a monoclonal antibody leads to its inactivation. MAbs. 2016;8(4):718–25.
105. Liu H, Ren W, Zong L, Zhang J, Wang Y. Characterization of recombinant monoclonal antibody charge variants using WCX chromatography, icIEF and LC-MS/MS. Anal Biochem. 2019;564–565:1–12. Available from: https://doi.org/10.1016/j.ab.2018.10.002
106. Xu W, Jimenez RB, Mowery R, Luo H, Cao M, Agarwal N, et al. A Quadrupole Dalton-based multi-attribute method for product characterization, process development, and quality control of therapeutic proteins. MAbs. 2017;9(7):1186–96.
107. Ratanji KD, Derrick JP, Dearman RJ, Kimber I. Immunogenicity of therapeutic proteins: influence of aggregation. J Immunotoxicol. 2014;11(2):99–109.
108. Le Basle Y, Chennell P, Tokhadze N, Astier A, Sautou V. Physicochemical Stability of Monoclonal Antibodies: A Review. J Pharm Sci. 2020;109(1):169–90.
109. Wang T, Chu L, Li W, Lawson K, Apostol I, Eris T. Application of a Quantitative LC-MS Multiattribute Method for Monitoring Site-Specific Glycan Heterogeneity on a Monoclonal Antibody Containing Two N-Linked Glycosylation Sites. Anal Chem. 2017;89(6):3562–7.
110. Yan Y, Liu AP, Wang S, Daly TJ, Li N. Ultrasensitive Characterization of Charge Heterogeneity of Therapeutic Monoclonal Antibodies Using Strong Cation Exchange Chromatography Coupled to Native Mass Spectrometry. Anal Chem. 2018;90(21):13013–20.
111. Demirdirek B, Lan W, Qiu D, Ding W, Iyer LK, Bolgar MS, 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, Anal Technol Biomed life Sci. 2019 Jan;1105:156–63.
112. Rustandi RR, Washabaugh MW, Wang Y. Applications of CE SDS gel in development of biopharmaceutical antibody-based products. Electrophoresis. 2008 Sep;29(17):3612–20.
113. Ambrogelly A, Gozo S, Katiyar A, Dellatore S, Kune Y, Bhat R, et al. Analytical comparability study of recombinant monoclonal antibody therapeutics. MAbs. 2018;10(4):513–38.
114. Lakbub JC, Shipman JT, Desaire H. Recent mass spectrometry-based techniques and considerations for disulfide bond characterization in proteins. Anal Bioanal Chem. 2018 Apr;410(10):2467–84.
115. Berkowitz SA. Role of analytical ultracentrifugation in assessing the aggregation of protein

biopharmaceuticals. AAPS J. 2006 Sep;8(3):E590-605.
116. Hong P, Koza S, Bouvier ESP. Size-Exclusion Chromatography for the Analysis of Protein Biotherapeutics and their Aggregates. J Liq Chromatogr Relat Technol. 2012 Nov;35(20):2923–50.
117. Rogstad S, Faustino A, Ruth A, Keire D, Boyne M, Park J. A Retrospective Evaluation of the Use of Mass Spectrometry in FDA Biologics License Applications. J Am Soc Mass Spectrom. 2017 May;28(5):786–94.
118. Berkowitz SA, Engen JR, Mazzeo JR, Jones GB. Analytical tools for characterizing biopharmaceuticals and the implications for biosimilars. Nat Rev Drug Discov. 2012;11(7):527–40. Available from: http://dx.doi.org/10.1038/nrd3746
119. Mouchahoir T, Schiel JE, Rogers R, Heckert A, Place BJ, Ammerman A, et al. New Peak Detection Performance Metrics from the MAM Consortium Interlaboratory Study. J Am Soc Mass Spectrom. 2021;
120. Biologics Stakeholder U, Dawdy A. Enhancing Biotherapeutic Process and Product Knowledge with the Multi-Attribute Method (MAM).
121. Liu H. A high-resolution accurate mass multi-attribute method for critical quality attribute monitoring and new peak detection. Thermo Fish Sci. 2019;
122. Todoroki K, Mizuno H, Sugiyama E, Toyo'oka T. Bioanalytical methods for therapeutic monoclonal antibodies and antibody–drug conjugates: A review of recent advances and future perspectives. J Pharm Biomed Anal. 2020;179.
123. Schenerman M, Sunday B, Kozlowski S, Webber K, Gazzano-Santoro H, Mire-Sluis A. Analysis and structure characterization of monoclonal antibodies. Bioprocess Int. 2015;13(8).
124. European Medicines Agency [EMA]. ICH Topic Q 6 B Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products. 1999 [cited 2021 Mar 22]. Available from: http://www.emea.eu.int
125. Rogers RS, Abernathy M, Richardson DD, Rouse JC, Sperry JB, Swann P, et al. A View on the Importance of "Multi-Attribute Method" for Measuring Purity of Biopharmaceuticals and Improving Overall Control Strategy. AAPS J. 2018;20(1).
126. Tajiri-Tsukada M, Hashii N, Ishii-Watabe A. Establishment of a highly precise multi-attribute method for the characterization and quality control of therapeutic monoclonal antibodies. Bioengineered. 2020;11(1):984–1000. Available from: https://doi.org/10.1080/21655979.2020.1814683
127. Sarah Rogstad. Development and Assessment of the Multi-Attribute Method (MAM)

Substance (23of33) Quality – Oct. 16-17, 2019 - YouTube. [cited 2021 Mar 22]. Available from: https://www.youtube.com/watch?v=lupH_P9lcr0

128. Vandenheede I, Sandra P, Sandra K. Multi-Attribute Analysis of Monoclonal Antibodies Using the Agilent InfinityLab 2D-LC Solution and Q-TOF MS.
129. Liu P, Zhu X, Wu W, Ludwig R, Song H, Li R, et al. Subunit mass analysis for monitoring multiple attributes of monoclonal antibodies. Rapid Commun Mass Spectrom. 2019;33(1):31–40.
130. Chen B, Lin Z, Zhu Y, Jin Y, Larson E, Xu Q, et al. Middle-Down Multi-Attribute Analysis of Antibody-Drug Conjugates with Electron Transfer Dissociation. Anal Chem. 2019 Sep;91(18):11661–9.
131. Singh SK, Narula G, Rathore AS. Should charge variants of monoclonal antibody therapeutics be considered critical quality attributes? Electrophoresis. 2016;37(17–18):2338–46.
132. Zhang Y, Guo J. Characterization and QC of biopharmaceuticals by MS-based "multi-Attribute method": Advantages and challenges. Bioanalysis. 2017;9(6):499–502.
133. Wang D, Nowak C, Mason B, Katiyar A, Liu H. Analytical artifacts in characterization of recombinant monoclonal antibody therapeutics. J Pharm Biomed Anal. 2020;183:113131. Available from: https://doi.org/10.1016/j.jpba.2020.113131
134. Lippincott J, Apostol I. Carbamylation of cysteine: a potential artifact in peptide mapping of hemoglobins in the presence of urea. Anal Biochem. 1999 Feb;267(1):57–64.
135. Ji Y, Liu M, Bachschmid MM, Costello CE, Lin C. Surfactant-Induced Artifacts during Proteomic Sample Preparation. Anal Chem. 2015 Jun;87(11):5500–4.
136. Zang L, Carlage T, Murphy D, Frenkel R, Bryngelson P, Madsen M, et al. Residual metals cause variability in methionine oxidation measurements in protein pharmaceuticals using LC-UV/MS peptide mapping. J Chromatogr B, Anal Technol Biomed life Sci. 2012 May;895–896:71–6.
137. Liu P, O'Mara BW, Warrack BM, Wu W, Huang Y, Zhang Y, et al. A tris (2-carboxyethyl) phosphine (TCEP) related cleavage on cysteine-containing proteins. J Am Soc Mass Spectrom. 2010 May;21(5):837–44.
138. Wang Z, Rejtar T, Zhou ZS, Karger BL. Desulfurization of cysteine-containing peptides resulting from sample preparation for protein characterization by mass spectrometry. Rapid Commun Mass Spectrom. 2010 Feb;24(3):267–75.
139. Boja ES, Fales HM. Overalkylation of a protein digest with iodoacetamide. Anal Chem. 2001 Aug;73(15):3576–82.

140. Gadgil HS, Bondarenko P V, Pipes GD, Dillon TM, Banks D, Abel J, et al. Identification of cysteinylation of a free cysteine in the Fab region of a recombinant monoclonal IgG1 antibody using Lys-C limited proteolysis coupled with LC/MS analysis. Anal Biochem. 2006 Aug;355(2):165–74.
141. Pristatsky P, Cohen SL, Krantz D, Acevedo J, Ionescu R, Vlasak J. Evidence for trisulfide bonds in a recombinant variant of a human IgG2 monoclonal antibody. Anal Chem. 2009 Aug;81(15):6148–55.
142. Krokhin O V, Antonovici M, Ens W, Wilkins JA, Standing KG. Deamidation of -Asn-Gly- sequences during sample preparation for proteomics: Consequences for MALDI and HPLC-MALDI analysis. Anal Chem. 2006 Sep;78(18):6645–50.
143. Gaza-Bulseco G, Li B, Bulseco A, Liu HC. Method to differentiate asn deamidation that occurred prior to and during sample preparation of a monoclonal antibody. Anal Chem. 2008 Dec;80(24):9491–8.
144. Dong Q, Liang Y, Yan X, Markey SP, Mirokhin YA, Tchekhovskoi D V, et al. The NISTmAb tryptic peptide spectral library for monoclonal antibody characterization. MAbs. 2018 Apr;10(3):354–69.
145. Kumar M, Chatterjee A, Khedkar AP, Kusumanchi M, Adhikary L. Mass spectrometric distinction of in-source and in-solution pyroglutamate and succinimide in proteins: a case study on rhG-CSF. J Am Soc Mass Spectrom. 2013 Feb;24(2):202–12.
146. Morand K, Talbo G, Mann M. Oxidation of peptides during electrospray ionization. Rapid Commun Mass Spectrom. 1993 Aug;7(8):738–43.
147. Chen M, Cook KD. Oxidation artifacts in the electrospray mass spectrometry of Abeta Peptide. Anal Chem. 2007 Mar;79(5):2031–6.
148. Sokolowska I, Mo J, Rahimi Pirkolachahi F, Mcvean C, Meijer LAT, Switzar L, et al. Implementation of a high-resolution liquid chromatography-mass spectrometry method in quality control laboratories for release and stability testing of a commercial antibody product. Anal Chem. 2020;92(3):2369–73.
149. Mire-Sluis A. Advancing the Promise of Analytics of the Future using Multi Attribute Methodology.
150. Analytical Characterization 2017 Biotherapeutics Analytical Summit. [cited 2021 Mar 21]. Available from: https://www.biotherapeuticsanalyticalsummit.com/AnalyticalCharacterization/17
151. Dirksen E, Biopharmaceuticals Christopher Yu SB. 13th Symposium on the Practical Applications of Mass Spectrometry in the Biotechnology Industry (Mass Spec 2016)

Symposium Co-Chairs.
152. McCarthy D, Biologics G. USP Standards to Support Multi-Attribute Methods (MAM) and Mass Spectrometry. 2010.
153. Rogers R. Leveraging the MAM to Improve Biotherapeutic Characterization.
154. Liu P, Zhu X, Wu W, Ludwig R, Song H, Li R, et al. Subunit mass analysis for monitoring multiple attributes of monoclonal antibodies. Rapid Commun Mass Spectrom. 2019 Jan;33(1):31–40.
155. Zhang Z, Chan PK, Richardson J, Shah B. An evaluation of instrument types for mass spectrometry-based multi-attribute analysis of biotherapeutics. MAbs. 2020 Jan 1;12(1).
156. Zhou M, Gucinski AC, Boyne MT 2nd. Performance metrics for evaluating system suitability in liquid chromatography--Mass spectrometry peptide mass mapping of protein therapeutics and monoclonal antibodies. MAbs. 2015;7(6):1104–17.
157. U.S. Food and Drug Administration (FDA). Guidance for Industry: Part 11, Electronic Records, Electronic Signatures - Scope and Application. 2003;(August). Available from: https://www.fda.gov/regulatory-information/search-fda-guidance-documents/part-11-electronic-records-electronic-signatures-scope-and-application
158. European Commission. Volume 4 Good Manufacturing Practice Medicinal Products for Human and Veterinary Use Annex 11: Computerised Systems. EudraLex. 2011;4(June 2011):2005–7. Available from: https://ec.europa.eu/health/sites/default/files/files/eudralex/vol-4/annex11_01-2011_en.pdf
159. Quinn S. Using the Thermo Scientific Dionex Chromeleon 7 Chromatography Data System (CDS) to Comply with 21 CFR Part 11. WHITE Pap 80078. 2018;
160. U.S. Food and Drug Administration (FDA). Scientific Considerations in Demonstrating Biosimilarity to a Reference Product | FDA. [cited 2021 Mar 21]. Available from: https://www.fda.gov/regulatory-information/search-fda-guidance-documents/scientific-considerations-demonstrating-biosimilarity-reference-product
161. U.S. Food and Drug Administration (FDA). Quality Considerations in Demonstrating Biosimilarity of a Therapeutic Protein Product to a Reference Product Guidance for Industry. [cited 2021 Mar 21]. Available from: https://www.fda.gov/regulatory-information/search-fda-guidance-documents/quality-considerations-demonstrating-biosimilarity-therapeutic-protein-product-reference-product
162. European Medicines Agency [EMA]. Guideline on similar biological medicinal products containing biotechnology-derived proteins as active substance: quality issues (revision 1).

2012 [cited 2021 Mar 21]. Available from: https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-similar-biological-medicinal-products-containing-biotechnology-derived-proteins-active_en-0.pdf

163. Yang Y, Wang G, Song T, Lebrilla CB, Heck AJR. Resolving the micro-heterogeneity and structural integrity of monoclonal antibodies by hybrid mass spectrometric approaches. MAbs. 2017;9(4):638–45.

164. Li Y, Huang Y, Ferrant J, Lyubarskaya Y, Zhang YE, Li SP, et al. Assessing in vivo dynamics of multiple quality attributes from a therapeutic IgG4 monoclonal antibody circulating in cynomolgus monkey. MAbs. 2016 Jul;8(5):961–8.

165. Jakes C, Millán-Martín S, Carillo S, Scheffler K, Zaborowska I, Bones J. Tracking the Behavior of Monoclonal Antibody Product Quality Attributes Using a Multi-Attribute Method Workflow. J Am Soc Mass Spectrom. 2021;