Evaluation of Bottom-Up and Middle-Up Liquid Chromatography Mass Spectrometry Methods for Forced Degradation Studies applied in Biosimilar Development

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Abbreviations

AAPH	2,2'-azobis(2-amidinopropane) dihydrochloride
ADC	antibody-drug-conjugate
ADCC	antibody-dependent cell-mediated cytotoxicity
ADCP	antibody-dependent cell-mediated phagocytosis
AF4	asymmetric flow field flow fractionation
Asn (N)	asparagine residue
Asp	aspartate residue
Asp-N	peptidyl-Asp metalloendopeptidase
AUC	analytical ultracentrifugation
BAC	boronate affinity chromatography
CD	circular dichroism spectroscopy
CDC	complement-dependent cytotoxicity
CDR	complementarity determining region
CE	capillary electrophoresis
CEX	cation-exchange chromatography
CH	constant domain of the heavy chain (numbered 1 to 3)
CHO cells	chinese hamster ovary cells
CID	collision-induced dissociation
cIEF	capillary isoelectric focussing
CL	constant domain of the light chain
CQA	critical quality attribute
Cys (C)	cysteine residue
CZE	capillary zone electrophoresis

DLS	dynamic light scattering
DSC	differential scanning calorimetry
DTT	dithiothreitol
E. coli	*Escherichia coli*
ECD	electron-capture dissociation
EMA	European Medicines Agency
EPO	erythropoietin
ER	endoplasmic reticulum
ESI	electrospray ionization
ETD	electron-transfer dissociation
EU	European Union
FA	formic acid
Fab	antigen-binding fragment
Fc	crystallizable fragment
FcγR	Fc-gamma receptor
Fc/2	half fragment crystallizable (same as scFc)
FcR	Fc receptor
FcRn	neonatal Fc receptor
Fd’	subunit consisting of VH and CH1 domains of the heavy chain
FDA	Food and Drug Administration
FTICR	Fourier-transform ion cyclotron resonance
Fuc	fucose
G0F, G1F, G2F	antibody N-glycans with core fucose (F) and 0, 1 or 2 terminal galactose
Gal	galactose
GlcNAc	N-acetylglucosamine

GMP	good manufacturing practice
HAMA	human anti-mouse antibody
HC	heavy chain
HDX	hydrogen-deuterium exchange
Hi	hinge region
HIC	hydrophobic interaction chromatography
HILIC	hydrophilic interaction liquid chromatography
HOS	higher order structure
ICH	International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
IdeS	immunoglobulin-degrading enzyme from *Streptococcus pyogenes*
IEF	isoelectric focusing
IEX	ion exchange chromatography
IgG	immunoglobulin G
IM-MS	ion mobility mass spectrometry
INN	international non-proprietary name
isoAsp	isoaspartate residue
ITC	isothermal titration calorimetry
LC	light chain
LC-MS	liquid chromatography coupled with mass spectrometry
LC-UV	liquid chromatography with UV detection
m/z	mass-to-charge ratio
mAb	monoclonal antibody
MALDI	matrix assisted laser desorption ionization
MAM	multi-attribute monitoring
Man	mannose

Met (M)	methionine residue
MS	mass spectrometry
MS/MS	tandem mass spectrometry
Neu5Ac, NANA	N-acetylneuraminic acid
Neu5Gc, NGNA	N-glycolylneuraminic acid
NMR	nuclear magnetic resonance
NS0	a cell line derived from nonsecreting murine myeloma
PAGE	polyacrylamide gel electrophoresis
PDI	protein disulfide isomerase
PNGase F	peptide:N-glycosidase F
PTM	post-translational modification
QC	quality control
R&D	research and development
RMP	reference medicinal product
RPLC	reversed-phase liquid chromatography
scFc	single chain Fc (same as Fc/2)
scFv	single chain variable fragment
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC	size exclusion chromatography
SLS	static light scattering
Sp2/0	B lymphocyte cell line that was isolated from the spleen of a mouse
tBHP	t-butyl hydroperoxide
TCEP	tris(2-carboxyethyl)phosphine
TFA	trifluoroacetic acid
TNF-α	tumor necrosis factor-α

TOF	time of flight
tPA	tissue plasminogen activator
Trp (W)	tryptophan residue
UV	ultraviolet radiation
VEGF	vascular endothelial growth factor
VH	variable domain of the heavy chain
VL	variable domain of the light chain
XRC	X-ray crystallography

1 Introduction and aim of the project

Monoclonal antibody (mAb) therapies have improved the perspectives of patients in various indications, most important in therapy in the fields of oncology and autoimmune diseases [1]. However, these therapies are highly expensive, burdening health systems and limiting access worldwide [2]. One solution is the development of biosimilars, which offer the same therapeutic success for the same indications for a lower price. A biosimilar is developed as a copy product of an approved and marketed reference medicinal product (RMP). For approval, regulatory agencies request the demonstration of physicochemical similarity, which relies on extensive analytical characterization [3]. One aspect of assessment is to compare the biosimilar candidate and the RMP in forced degradation studies [4]. In these studies, the drug is subjected to conditions, which mimic those that it may encounter during its life cycle, however exaggeratedly, to explore possible degradation in a shorter time frame.

The starting point of this work is the development of biosimilars for two RMP mAbs, bevacizumab, used in oncology, and infliximab, used for treatment of autoimmune diseases. These two biosimilars were developed at ProBioGen AG and characterized by liquid-chromatography with UV detection (LC-UV), including various modes of chromatographic separation. During forced degradation studies, unidentified species were detected in the form of new peaks. In this work, two different liquid-chromatography mass-spectrometry (LC-MS) based approaches were established for the identification of the new species: a middle-up approach and a bottom-up approach. The bottom-up approach yields detailed structural insights but is time-consuming, expensive, and bears the risk of introducing artificial modifications, thereby skewing the analysis [5]. The middle-up approach promises to be faster and less prone to artificial modifications [6] and, therefore, has the potential to accelerate biosimilar development, thereby reducing the costs. However, middle-up provides lower structural resolution than bottom-up experiments. This study aims to compare the two LC-MS based approaches to understand to which extent the faster middle-up approach can replace the elaborate bottom-up approach and to explore consistency, inconsistency, and complementarity between the two approaches.

2 Background

The technologies enabling the production of biologics have developed over time and continue to progress. In a narrower sense, biologics comprise drugs produced biotechnologically, usually by cultivation of cells, using recombinant DNA technologies [7, 8]. Historically and today, biologics are mostly protein drugs, such as hormones and mAbs. Other entities, including polysaccharides (e.g., heparin), different vaccine modalities, nucleotides, gene, or cellular therapies may also be included [7, 8], but will not be considered in this work.

mAbs are proteins with a complex structure, which cannot be synthesized chemically but must be produced by living cells [9]. The development of the producer cell lines, and the manufacturing process are particular for mAbs and account for the high costs of mAb therapies. Various post-translational modifications (PTMs) are found on mAbs, such as glycosylation, disulfide bonds, deamidation, or oxidation, which can influence their safety and efficacy. The occurrence of some PTMs can be investigated by forced degradation studies, in which the mAb is subjected to harsh conditions.

Access to mAb therapies is limited because of their high costs [2]. To provide broader access to these therapies, biosimilars are developed as follow-on products with reduced prices after patent expiry of RMP products. Abbreviated approval is based on the demonstration of biosimilarity, which includes extensive physicochemical characterization of the biosimilar candidate in comparison with the RMP mAb. Due to the complexity of mAbs, analytical characterization relies on various methodologies, including the analysis of charge variants, size variants, and the characterization of glycosylation heterogeneity. Mass spectrometry is a standard technique applied in such analyses [10]. All these aspects are covered in more detail in the following chapters.

2.1 Biologics

The following sections introduce the historical developments that enabled production of biologics, including milestones in the development of mAbs and biosimilars. Subsequently, an overview of today's important classes of biologics is given, with an emphasis on mAbs.

2.1.1 History

Many scientific discoveries and developments led to the progress in biologics production [11]. One of the most important milestones were the developments that constitute recombinant DNA technology in the 1980s. It enabled the production of proteins of one species in an expression

system of another species. Together with advances in the cultivation of prokaryotic and eukaryotic cells, this led to the emergence of the first biologics.

An early milestone was the approval of recombinant human insulin generated in *Escherichia coli* by the USA Food and Drug Administration (FDA) in 1982 [12]. However, bacterial systems such as *E. coli* often faced difficulties to produce soluble mammalian proteins [13]. Especially glycoproteins require enzymatic pathways and compartmentalization which are absent in prokaryotic systems. An important step to overcome these problems was the development of mammalian cell lines [11]. Among different mammalian cell lines, Chinese hamster ovary (CHO) cells became an important expression system and early milestones included the approval of tissue plasminogen activator (tPA) in 1987 and erythropoietin (EPO) in 1989.

Recombinant production was also crucial for the development of therapeutic mAbs. Antibody therapies already existed before, however, in the form of a variable and heterogenous polyclonal mixture, as they were extracted from pooled sera of many donors [14]. For antibody therapies, the invention of the hybridoma technology by Köhler and Milstein in 1975 [15] was a major breakthrough, as it allowed to generate mAbs, i.e., antibodies produced by one cell clone that bind only to one epitope of the target antigen. The first commercialized mAb for human treatment was developed by the hybridoma technology and approved in 1986 [8]. Muromonab-CD3 (trade name Orthoclone OKT3) recognizes CD3 on T-lymphocytes and depletes them. It was used during immunosuppressive treatment to prevent acute rejection after organ transplantation [16]. However, in general, the mAbs developed from mouse hybridoma were not useful for human therapy, as the murine mAb structure caused major problems: many patients recognized the mouse mAbs as foreign and generated human anti-mouse antibodies (HAMAs) [17]. In that sense, the specific application of muromonab was favourable as transplant patients received immunosuppressive treatment, preventing the generation of HAMAs. However, this was not the case for other mAbs developed at that time, which thus faced the problem of HAMAs. Moreover, mouse antibodies were not good to elicit human effector functions [17].

Therefore, using recombinant DNA technology, the proportion of mouse sequences was reduced in form of chimeric mAbs with the variable domains of mouse mAbs grafted onto human constant domains [18]. Humanized mAbs further increased humanness by grafting only the murine complementarity-determining regions (CDRs, see section **2.2.1**) into a human mAb framework. Ultimately, by engineering fully human mAbs were developed independently from

a mouse immune system. This development is reflected by approvals of the first Fab fragment (section **2.2.1**) derived from a chimeric mAb, abciximab, in 1994 (first chimeric full mAb was rituximab, 1998), the first humanized mAb daclizumab in 1997 and the first fully human mAb adalimumab in 2002, with higher approval rates of humanized and human mAbs compared to mouse and chimeric mAbs since 2003 [19].

The origin of the mAb is reflected by the naming convention for the international non-proprietary name (INN) [20]. The names are composed of a free-to-choose prefix, followed by substem A indicating the target structure, followed by substem B, indicating the origin and ending on -mab for monoclonal antibody. Thus, the designations of the origins are –(m)omab (murine, e.g., blinatumomab), –ximab (chimeric, e.g., infliximab), –zumab (humanised, e.g., bevacizumab) and –(m)umab (fully human, e.g., adalimumab). The INN nomenclature for mAbs (synonym: immunoglobulins) was revised in 2022 [21]. For new mAbs, four new stem suffixes are used: -tug for unmodified immunoglobulins, -bart for artificial immunoglobulins, -ment for immunoglobulin fragments, and -mig for multi-specific immunoglobulins. The use of the suffix -mab is not continued.

The complexity of the manufacturing leads to high prices for mAbs, as well as the investments on research and development, royalties, and marketing costs [22]. For example, the annual cost for breast cancer treatment with Herceptin (trastuzumab RMP) in 2009 was 37000 $ in the USA [23]. Responding to the burden by high prices, the EU was a pioneer in paving the regulatory pathway for biosimilars – follow-on products of marketed biologics in analogy to small molecule generics to reduce prices - with the first legislation published in 2004 [24]. In 2006, Omnitrope (based on somatropin) became the first biosimilar approved in the EU, followed by EPO and filgrastim-based biosimilars in 2007 and 2008, respectively. The first biosimilar mAb, Inflectra (based on infliximab), was approved in 2013. In the USA, the Biologics Price Competition and Innovation Act provided an analogous regulatory framework in 2010, and the first biosimilar, Zarxio (based on filgrastim), was approved in 2015 [8].

2.1.2 Classes

A recent survey gives an overview of approvals and sales of biologics in the period of January 2018 to June 2022 [8]. Most biologics were protein drugs, but nucleic acid- and genetically engineered cell-based products were also included. Newly approved biologics may be grouped into eight classes: clotting factors and other blood factors; thrombolytics, anticoagulants, and other blood-related products; hormones; growth factors; interferons, interleukins, and tumor necrosis factor; vaccines; mAb-based products and other products. mAbs constituted more than

50% of all new approvals considering RMPs and biosimilars. Focusing only on RMPs, mAbs represented 51% of all new approvals, highlighting this field's innovation and output potential. Also, in terms of sales, the share of mAbs has steadily increased from 50% of all protein-based biologics in 2011 to 77% in 2020 and to 80% in 2021 if COVID-19 vaccines were excluded in this year [8]. Since their introduction, the numbers of mAb approvals have been growing continuously (**Figure 1**).

Apart from full-length mAbs, which represent the large majority of antibody therapeutics, several other antibody-based formats are developed, with antibody-drug-conjugates (ADCs) and bispecific mAbs being the most important [19, 25, 26]. ADCs consist of a mAb conjugated to a cytotoxic drug. The mAb binds specifically to the target cells and is internalized, and the cytotoxin is released intracellularly. Bispecific mAbs are antibodies with two binding sites directed to two different antigens or two different epitopes on the same antigen. Further formats include immunoconjugates and antibody fragments. In contrast to ADCs, immunoconjugates are antibody-derived proteins fused or conjugated to any other biologically relevant entity, for example, another protein or a radioisotope. Antibody fragments comprise, for example, single-chain variable fragments (scFv) and antigen binding fragments (Fab) (see section **2.2.1** for mAb structure) [19, 25].

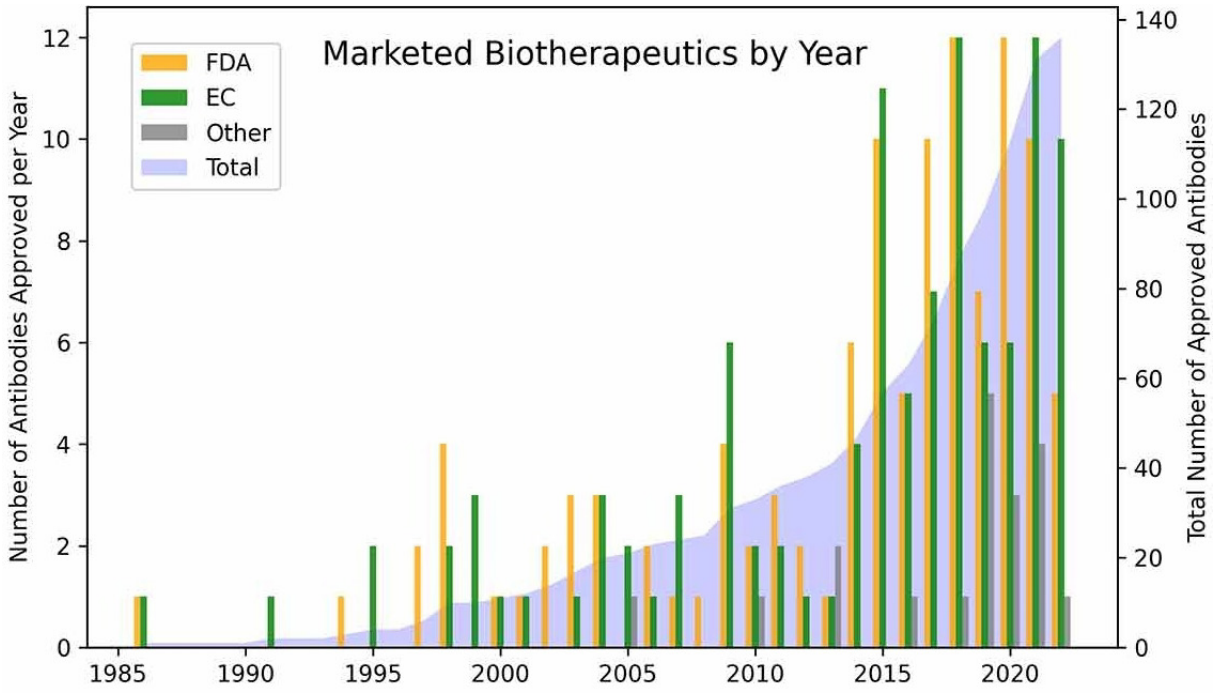

Figure 1: Numbers of monoclonal antibody-based therapeutics approved by year (bars, left x-axis) and cumulated (in blue, right x-axis). Approvals are shown for the US Food and Drug Association (FDA), the EU European Commission (EC) and other countries, which include India, Cuba, China and Japan. From [19], copyright © 2023, reprinted by permission of Informa UK Limited, trading as Taylor & Taylor & Francis Group, http://www.tandfonline.com

2.2 Therapeutic mAbs

For a better understanding of mAbs, the following sections provide background information about their structure, different development strategies, the manufacturing process, and important indications and mechanisms of action. Subsequently, typical PTMs and their effects are described, and forced degradation is introduced as part of the study of PTMs on mAbs.

2.2.1 Structure

Antibodies (in mammals) are of five isotypes designated IgA, IgD, IgE, IgG and IgM, with Ig being the abbreviation for immunoglobulin, a synonym for antibody [27]. Therapeutic mAbs are only of the IgG isotype [8]; therefore, the other isotypes are not considered here. IgGs are monomers consisting of two identical heavy chains (HCs) and two identical light chains (LCs) linked by interchain disulfide bonds [27] (**Figure 2**). Each chain is organized into several immunoglobulin domains with similar higher order structure. The domains are each about 110 amino acids long and are folded into similar compact globular structures further stabilized by intrachain disulfide bonds. The light chains have one variable domain (VL) and one constant

domain (CL). The heavy chains consist of one variable domain (VH) and three constant domains (CH1, CH2, CH3, numbered from the N-terminus). Variable domains contain sequences which are highly variable between different antibodies, whereas constant domains display almost no variability. IgG comprise four subclasses, IgG1, IgG2, IgG3, and IgG4 [27]. The subclasses differ in the disulfide bond connection between the heavy and light chains influencing the flexibility of the antibody. Most antibodies produced as biologics belong to the IgG1 subclass, however, IgG2 and IgG4 antibodies have also been developed to a lesser extent [28].

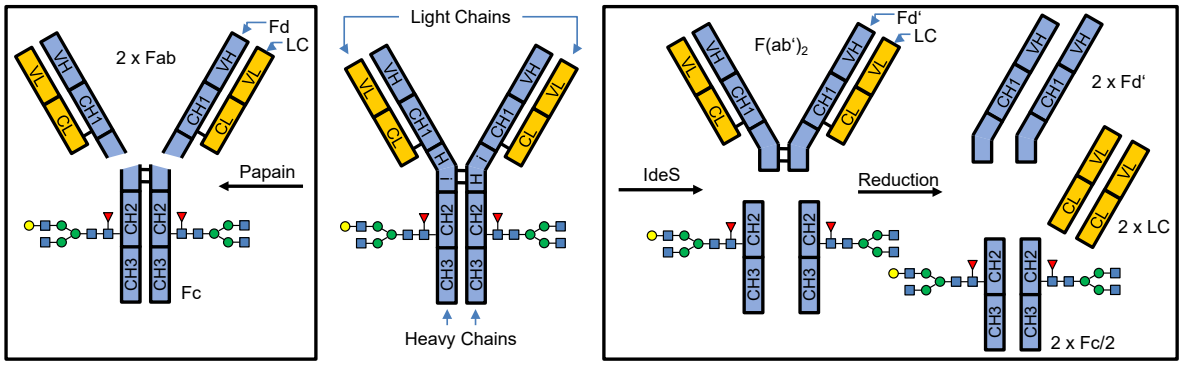

Figure 2: Schematic representation of antibody (IgG1) structure. The middle part shows an intact antibody composed of two identical heavy chains and two identical light chains. The chains are orientated with the N-terminus at the top and the C-terminus at the bottom. HC and LC are linked by intermolecular disulfide bonds (thick black lines). Each domain (VL, VH, CL, CH1-3) has one intramolecular disulfide bond (not shown), which stabilizes its globular structure. The hinge region (Hi) is less structured. The heavy chains carry N-glycans. The exemplifying glycans depicted here are G0F and G1F, which differ by the presence of a terminal galactose (blue boxes = N-acetylglucosamine, green circles = mannose, red triangle = fucose, yellow circle = galactose). The box to the right illustrates the sample preparation for middle-up analysis applied in this study (section **2.3.3.5**). Briefly, digestion by IdeS generates Fc/2 and the F(ab')$_2$. Subsequent reduction of disulfide bonds liberates the light chain (LC) and Fd'. The box on the left illustrates the Fc and the Fab fragment, which are generated by the enzyme papain (papain was not used in this study). For details, see text.

Historically, different characteristics of parts of antibodies were discovered when they were enzymatically cleaved into the N-terminal Fab fragments and the C-terminal Fc fragment, e.g. by the enzyme papain [27] (**Figure 2**). Each Fab fragment consists of one light chain (VL and CL), in conjunction with the VH and CH1 domains of one heavy chain. The name "Fab" was given due to its role in antigen binding (short for fragment antigen binding). A "F(ab)$_2$" fragment is created when the connecting inter-heavy chain disulfide bonds are retained in that fragment because of enzymatic cleavage C-terminal of the disulfide bonds, e.g. by the enzyme IdeS (Immunoglobulin-degrading enzyme from *Streptococcus pyogenes*) [10] (**Figure 2**).

Conversely, the Fc fragment encompasses the C-terminal parts of the heavy chains (CH2 and CH3 domains). It was named “Fc” as it was observed to readily crystallize (short for fragment crystallizable), reflecting the notable conservation of this region among IgGs targeting different antigens. The designation “Fc” is used when the connecting inter-heavy chain disulfide bonds are retained in this fragment, thus comprising the interconnected C-terminal halves of the heavy chains and “Fc/2” (or scFc for single chain Fc), when the two C-terminal halves of the heavy chain are separated [10].

The antigen binding capability resides in the variable domains within the Fab [27]. Specifically, these variable domains encompass three shorter segments known as CDRs, which directly interact with the antigen. In contrast, the Fc of IgG interacts with Fcγ receptors (FcγR) on immune cells and other effector molecules, such as components of the complement system, thereby eliciting immune functions. The Fc also binds to the neonatal Fc receptor (FcRn), which recycles the mAb back to the bloodstream before lysosomal degradation, increasing its half-life.

All antibodies, including IgGs, contain an N-glycan covalently attached to the heavy chain at approximately N297 [10] (**Figure 2**). The oligosaccharides are attached to asparagine (Asn, N) residues within the consensus sequence NXS/T, with X representing any amino acid except proline, in a process called N-glycosylation. The most important monosaccharides that constitute human N-glycans are mannose (Man), N-acetylglucosamine (GlcNAc), galactose (Gal), fucose (Fuc) and N-acetylneuraminic acid (Neu5Ac, NANA), which is the sialic acid most commonly found on endogenous IgG [29]. N-Glycans display a huge variety, which can generally be classified into three major types: the high-mannose, hybrid, and complex type [10]. All three types are based on a core structure of two GlcNAc, linked to a Man, from which two branches start, an α1-3 linkage to a Man and an α1-6 linkage to another Man. This core structure is further extended and again trimmed during protein export through the Golgi. mAbs carry mostly complex N-glycans with additional GlcNAc, Man, and Fuc residues. The most common N-glycans are based on a heptasaccharide (4 GlcNAc, 3 Man) of the complex type with a biantennary structure (**Figure 3A**). They vary in the terminal galactose content and the presence or absence of a core fucose, i.e., fucose linked to the Asn-bound GlcNAc. Around 20% of all antibodies contain an additional N-glycan in the Fab part of the heavy chain [30].

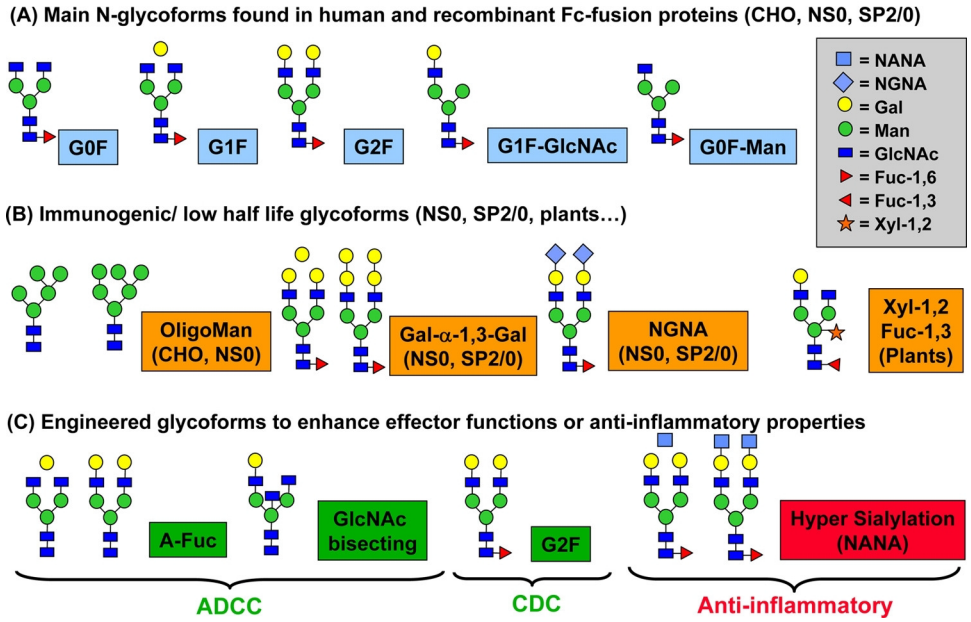

Figure 3: N-Glycans found in Fc domains of monoclonal antibodies and their effects. From [31], copyright © 2012, reprinted by permission of Informa UK Limited, trading as Taylor & Taylor & Francis Group, http://www.tandfonline.com

2.2.2 Development

Historically, the first way to produce mAbs was the hybridoma technology [11]. Briefly, a mouse is immunized with an antigen and develops B cells that produce antibodies against the antigen [27]. Different antibodies specific for different epitopes of the antigen are generated each by a different B cell clone, giving rise to a polyclonal mixture. B cells are then isolated from the animal and fused with mouse myeloma cells, which are immortal cancer B cells which themselves do not produce antibodies. The resulting fusion cells are called hybridoma cells. These combine the ability of the B cell to produce an antibody specific for the antigen with the ability of the myeloma cell to reproduce indefinitely. Only the successfully fused cells are selected, which still represent a polyclonal mixture. The hybridoma cells are isolated from each other so that every single cell gives rise to a clone, with all cells of the clone producing the same antibody, i.e., a monoclonal antibody (mAb). The different hybridoma cell clones are then screened for the best suited mAb producer and are propagated as cell lines. However, as mentioned before, the murine mAbs faced problems, as patients developed HAMAs and because activation of human immune functions by mouse mAbs was not effective.

There were different attempts to overcome these problems by generation of human hybridoma cell lines. Human B cells were fused with human myeloma cell lines and murine myeloma cells [32, 33]. Immortalization of B cells was also attempted using Epstein-Barr-Virus [34].

However, all these approaches have not succeeded because of technical difficulties and low efficiencies. Moreover, *in vitro* immunization faced problems, and it is impossible to immunize humans with an experimental antigen for ethical reasons.

Many strategies of antibody engineering are performed on the DNA sequence level based on the binding sequences derived by hybridoma and other technologies [18]. DNA recombination and the improvements in cell culture techniques allowed genes of one species to be expressed in another species. Thus, the important step is generating an antibody and then using its DNA sequence to produce it. For this purpose, the DNA sequence is usually inserted into a cell line, preferably into one that is closely related to humans [11, 18].

Today, recombinant human antibodies are generated by transgenic animals or by *in vitro* selection from large gene libraries. Transgenic animals, usually transgenic mice, carry genes for the human antibody repertoire [35]. In embryonic stem cells, the species' own genomic sequences for the light chain and for the heavy chain were replaced by the human sequences. Thus, mice producing human antibodies were bred, which can be immunized with a target antigen. The B cells are then isolated, and a cell line can be generated by hybridoma technology or by B cell cloning.

The other predominant way to generate adapted antibody sequences, specifically the binding sequences, is by *in vitro* selection. In this approach, a huge number of sequences from antibody gene libraries is screened for their binding to a target antigen. An antibody gene library can comprise more than 10^{10} different antibody sequences [36]. For *in vitro* selection, all different sequences are then tested for the best binding against a target antigen by what is known as display technologies. Among different systems, phage display is the most widely used. It involves a bacteriophage, typically the M13 phage, to express and display the binding sequences from the gene library on its surface. To select the best binder, the antigen is immobilized on a surface, such as in the cavities of a well plate [36]. In a process called panning, the antibody phage particles are incubated on the immobilized antigen, and the weak and unspecific binders are removed by washing. Thereby, antibody phages exhibiting specific binding are enriched and then eluted and used to infect *E. coli* to reproduce new binding phages. This cycle of panning and reproduction is repeated several times for an enrichment of the most specific binder. Other *in vitro* display technologies are based on entities other than phages but have in common the cycle of panning on the protein level and reproduction of the corresponding DNA [36]. These include bacterial surface display, yeast surface display, ribosome display, and mRNA display.

2.2.3 Biotechnological manufacturing

A certain number of promising candidates enter the process development phase. The production process includes many steps with the goal of developing a highly controlled process to ensure stable product quality. The manufacturing process comprises upstream and downstream steps, i.e., leading to and following the mAb harvest to obtain the final mAb product.

The upstream process involves the biotechnological expression of the mAb from cell culture [11]. mAbs are almost exclusively produced in eukaryotic cells, as they provide the subcellular structures and the machinery required for correct folding and PTMs, especially important for correct glycosylation. Therefore, mammalian cells are typically used, as they are most closely related to humans. Translation is directed into the lumen of the endoplasmic reticulum (ER) by signal sequences in the LC and HC, which are subsequently removed by signal peptidases [37]. Assembly of mAbs from LC and HC takes place in the ER. Correct folding requires chaperones and correct formation of disulfide bonds requires protein disulfide isomerases (PDIs). Proteins are retained in the ER by a quality control mechanism until they are correctly folded or are otherwise exported for degradation by the proteasome. Glycosylation starts by post-translational attachment of a precursor glycan in the ER and proceeds during export through the Golgi [37]. The correctly folded and glycosylated mAbs are then secreted from the cell.

The most widely used mammalian expression system is CHO cells [11]. CHO are very similar to the human cell system, including generation of PTMs, provide good yields of the expressed protein and are robust to changes of the culture environment, thus facilitating the handling. Prior to manufacturing, a cell line must have been established with stable integration of the mAb genes by transfection using a suitable vector [11]. The producer cells are grown as suspension in bioreactors under controlled conditions. Typically, large volume stirred tank bioreactors are operated in fed-batch mode and supplied with chemically defined culture media free from components derived from animals (such as fetal calf serum, commonly used in cultivation media) to avoid risks of human pathogens in animal sera [9]. The culture process must be optimized for product quality and to maximize the yield. Important parameters, such as temperature, pH, dissolved oxygen levels, as well as the levels of nutrients and degradation products from the cell metabolism, need to be precisely controlled. For example, glycosylation is sensitive to changes of these parameters [38].

When the cells have reached maximum productivity during the late exponential or early stationary phase of cell growth, mAbs in the culture medium are separated from the cells in the harvest step. The crude mixture is purified through downstream processing [39]. Cell debris is

removed by ultrafiltration and diafiltration. Affinity chromatography by protein A (or protein G) is used to capture the mAb to separate it from other host cell proteins. As viruses can enter the production process in different ways, a virus inactivation step is necessary, often performed by low or high pH. UV light, heat, or detergents are also in use for this purpose. Clearance of inactivated virus can be achieved by nanofiltration or chromatographic separation. In so-called polishing steps, the mAb is further purified from contaminants such as host cell proteins, aggregates, leached protein A or protein G as well as residual DNA and residual virus by chromatography including cation-exchange (CEX), anion-exchange (AEX), hydrophobic interaction (HIC) and other methods [39]. Finally, ultra-/diafiltration is performed to achieve the required concentration for the formulation. The purified and formulated mAb is then filled into glass vials or syringes and stored before application. Establishing upstream and downstream processes requires extensive optimization during process development to obtain mAbs of high quality with good yield.

2.2.4 Indications and mechanism of action

The most important applications for mAbs are treatments for different cancers and autoimmune diseases [40]. A recent review article grouped mAbs according to the medical fields of their use into the following categories: oncology, haematology, dermatology, rheumatology, gastroenterology, pulmonology, orthopaedics, cardiology, ophthalmology, immunology, and nephrology [41]. While this illustrates the diverse uses of mAbs, this categorization should not be seen too rigorous, as most mAbs are approved for several indications, and usually, at least one is cancer-related. This highlights the exceptional importance of mAbs application for cancer treatment [18]. Moreover, infectious diseases have recently gained importance with the development of mAbs for the treatment of COVID-19.

mAbs exert their effects in different ways [42]. Blockade of growth factors or their receptors by mAb binding inhibits the proliferation of cells relying on the growth factor signalling or can even lead to apoptosis. Another prominent example is the neutralization of viruses, exploited in passive immunization. In addition to the direct effects of binding to their antigen, mAbs can elicit effector functions. When bound to the cell surface antigen on a target cell, cytotoxic effects such as complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC) can be triggered to kill the target cell. In CDC, the classical complement pathway is activated, leading to target cell lysis by forming a membrane attack complex. In ADCC, effector immune cells (for example, natural killer cells, macrophages) are recruited and kill the target cell.

In this study, investigations were performed on bevacizumab and infliximab and biosimilar candidates thereof.

Bevacizumab was invented by Roche under the trade name Avastin. Avastin is approved by the European Medicines Agency (EMA) for the treatment of the following indications, often in combination with other drugs: metastatic cancer of the colon or rectum, metastatic breast cancer, advanced non-small cell lung cancer under certain circumstances, advanced or metastatic kidney cancer, epithelial cancer of the ovary, cancer of the fallopian tube or the peritoneum as well as cancer of the cervix if it is persistent, recurrent, or metastatic [43]. The FDA in the USA approved Avastin in 2004, the EMA in the EU in 2005. Its patent and exclusivity expired in the USA in 2019 and in the EU in 2022 [44]. Bevacizumab binds to vascular endothelial growth factor (VEGF) and neutralizes it. VEGF is a signalling molecule that binds to VEGF receptors on endothelial cells, thereby promoting the development and outgrowth of blood vessels, i.e., angiogenesis. Tumours actively secrete VEGF and thereby increase their vascularization for supply and growth [45].

The RMP Remicade (the trade name for infliximab), produced by Johnsons & Johnson and Merck, was approved in 1998 in the USA by the FDA and in 1999 in the EU by the EMA. The patent and exclusivity expired in the EU in 2015 and in the USA in 2018 [46]. Remicade is approved by the EMA for the treatment of the following indications, often in combination with other drugs: rheumatoid arthritis, Crohn's disease, ulcerative colitis, ankylosing spondylitis, psoriatic arthritis, and psoriasis [47]. Infliximab binds to tumor necrosis factor-α (TNF-α), which is an important cytokine involved in the modulation of the immune system and the balance between healthy and inflammatory conditions [48].

2.2.5 Typical PTMs and possible effects

PTMs occur at various sites during the complete lifecycle of the mAb. PTMs are quality attributes and may be classified as critical quality attributes if they impact the biological activity (potency) and the comprehensive clinical drug profile, comprising pharmacokinetics, pharmacodynamics, safety, immunogenicity, and efficacy [49]. Therefore, they must be characterized. This section gives an overview of commonly observed PTMs of mAbs.

2.2.5.1 Glycosylation

N-Glycosylation is the most complex PTM found on mAbs [7]. While mammalian cell lines produce a limited number of complex glycans, the composition and distribution still depend on the cell line, the developmental stage, and the conditions in the bioreactor, such as temperature,

pH, and available monosaccharides. N-Glycosylation may display heterogeneity, i.e., the very same mAb is found with different glycans. These differ in the amounts of galactose, fucose, and sialic acid or in the occurrence of high-mannose-type glycans (**Figure 3**).

The exact correlation of specific glycan structures with certain effects can be difficult [50]. Glycans often need to be enriched to be used in binding assays and then compared to the starting material, which is still heterogenous and may have additional other PTMs, possibly influencing the results. Studies also differ in the level of enrichment. Experiments on receptor binding face additional difficulties, as FcγR display heterogeneity as well, and cells in assays may express different receptors, which altogether makes the evaluation of the correlation of glycan structures and effects challenging. This may, in part, be an explanation for contradictory reports. While a case-by-case examination is advised, a few trends can be observed from the literature [50].

N-Glycosylation has effects on the structure and function of the mAb. N-Glycans stabilize the CH2 domains and IgGs without glycans have a higher tendency to aggregate [51]. The Fc N-glycan also has an influence on the binding to Fc receptors and, thus, on effector functions. Non-glycosylation drastically reduces binding to most FcγR [50]. Important effector functions of glycosylated mAbs include CDC and ADCC (**Figure 3**). Some studies have indicated that higher levels of terminal galactose lead to improved binding to C1q and stronger CDC [52, 53]. For ADCC, the effects of bisecting GlcNAc, as well as of core-fucose, were discussed. However, while it may be a combinatorial effect, afucosylation seems to be more influential in enhancing ADCC [54, 55]. While high-mannose type glycans are untypical for mammalian cell culture, still low levels have been reported [56]. Certain high-mannose structures displayed increased ADCC but decreased CDC activity and increased clearance [56-58].

The influence of glycans on serum half-life has also been investigated. Recycling of mAbs by the FcRn seems to be independent of glycosylation [59]. While some studies show different clearance rates of mAbs that only differ in glycosylation [60, 61], other studies did not show differences in pharmacokinetics [62, 63]. Possible effects might involve clearance by the asialoglycoprotein receptor or the mannose receptor, which recognize and clear glycoproteins with specific glycan structures (**Figure 3**).

Notably, some sugar moieties are immunogenic, such as α-Gal and N-Glycolylneuraminic acid (Neu5Gc, also known as NGNA), which can be produced by Sp2/0 and NS0 cells, the latter also to a lesser extent in CHO cells [10] (**Figure 3**).

2.2.5.2 Disulfide bonds

Disulfide bonds are critical, as they directly affect the higher order structure, which is important for the function of the mAb [64]. Normally, all cysteine residues in mAbs participate in canonical (i.e., correctly formed) disulfide bonds, however, exceptions have been observed [65]. First, free cysteine residues may result from incomplete formation or from reduction of formed disulfide bonds. For example, formed disulfide bonds may be reduced at the harvest step during manufacturing when reducing enzymes are released [66, 67]. Free cysteine has been shown to impact antigen binding and to reduce thermal stability [68, 69]. Second, non-canonical disulfide linkages may arise from shaking and stirring and contribute to aggregate formation [70-72]. In contrast, disulfide isoforms have been observed in recombinant IgG2 and IgG4, which are not regarded as critical as they also exist *in vivo* [73-75]. In addition to agitation, non-canonical disulfide linkages are also promoted by heat, oxygen radicals, and high pH [76]. Third, disulfide bonds have been shown to degrade through the ß-elimination mechanism, which is accelerated at basic pH and high temperature [77] and which may result in the formation of a non-reducible thioether [78, 79].

2.2.5.3 Glycation

Glycation is the covalent attachment of reducing sugars to lysine residues and N-terminal primary amine groups of the light and heavy chains [80]. Glycation can result from reducing sugars such as glucose, which are added as energy source for the mAb-producing cells during cultivation. Reducing sugars can also be generated by degradation of more complex carbohydrates such as sucrose, commonly used in formulation buffers [81]. It has not been reported to impact safety or efficacy but might cause immunogenicity because it does not occur naturally on mAbs [82]. Moreover, glycation should be monitored if lysine residues are in an exposed position in the CDRs or other areas of protein-protein interaction, as they might affect binding [80, 83].

2.2.5.4 Deamidation

Deamidation of asparagine (Asn) to aspartate (Asp) or isoaspartate (isoAsp) is one of the most common PTMs encountered in mAbs [84, 85]. The reaction occurs via a succinimide intermediate, which can also be found in mAbs (**Figure 4**). The negative charge conferred by Asp and isoAsp changes the local physicochemical properties and may influence structure and stability. When Asn deamidation occurs in CDRs, it has been shown to reduce binding affinity to the target [86-88]. Therefore, Asn in CDRs is usually avoided by engineering the sequence or needs to be carefully monitored. Asn deamidation is promoted by basic pH values, which the

mAb may encounter during manufacturing [80]. For example, bases may be introduced during cell cultivation for pH regulation. During downstream processing, high pH is applied for elution during anion exchange chromatography (AEX) and pH neutralization following low pH protein A elution or low pH virus inactivation [80].

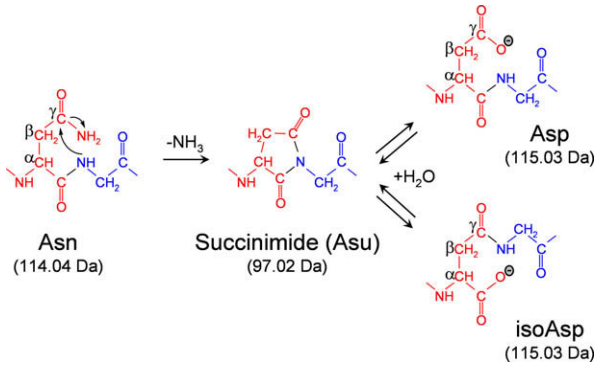

Figure 4: Reaction of asparagine deamidation and aspartic acid isomerization by the intermediate formation of succinimide with theoretical monoisotopic masses indicated. From [88], copyright © Elsevier 2009.

2.2.5.5 Oxidation

Oxidation and deamidation are the two most commonly observed chemical modifications of mAbs [84, 85] and, together with aggregation and fragmentation, constitute the major degradation pathways [80]. In this work, the focus is on oxidation of mAbs; therefore, this part describes oxidation in more detail.

Cysteine, methionine, tryptophan, histidine, and tyrosine residues are susceptible to oxidation in that order [82, 89, 90]. Cysteine may be oxidized to cysteine sulfenic acid, which is unstable under physiological conditions and may be reduced back to its thiol form by the thioredoxin–thioredoxin reductase system [91]. However, it may also be overoxidized to cysteine sulfinic acid and even to cysteine sulfonic acid. Because of their high susceptibility, biologics usually do not contain free cysteine [90], but exceptions have been reported as described in section **2.2.5.2**. Most relevant to oxidation are methionine [6, 92-96] and tryptophan residues [97-99]. Methionine can be oxidized to methionine sulfoxide and further to methionine sulfone, while oxidation of tryptophan results in various oxidation products, including oxindolealanine, (4, 5, 6, or 7)-hydroxytryptophan, dioxindolealanine, kynurenine and N-formylkynurenine [100] (**Figure 5**).

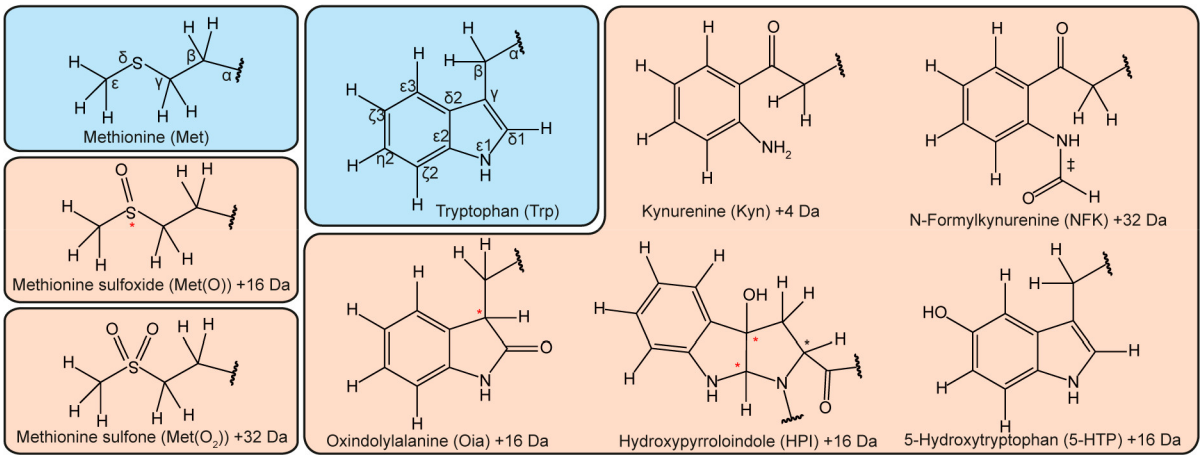

Figure 5: Oxidation products of methionine and tryptophan residues that may occur on monoclonal antibodies. Asterisks indicate chiral centres. The related mass changes are indicated. From [100], copyright © 2020 ACS Publications. Further permissions related to the material excerpted should be directed to ACS.

Oxidation can be caused by a variety of factors present during manufacturing and storage, which may act by themselves or as cofactors. Shah et al. [101] reviewed that oxidation may be caused by polysorbates [102-104], sugars [105], salts [106], peroxides [107], metals [108-110], dissolved oxygen [111], oxygen in vial headspace [112] and light [113-115]. Even buffers may play a role by stabilizing or destabilizing the protein [116].

The Fc contains methionine (Met, M) residues, which are conserved and, therefore, found in most mAbs. Taking bevacizumab as an example for the numbering of residues, these are M258 in CH2 and M434 in CH3, and in the three-dimensional structure, these residues are located close to the CH2-CH3 interface and are surface exposed [117]. In other mAbs, the exact positions of these residues may differ by a couple of amino acids because of varying lengths of CDRs and are often referred to as M252 and M428 [93]. A third methionine residue, which is buried in the CH3 domain [96], is found in some IgG1 based mAbs, including in bevacizumab (M364). Oxidation of the two surface-exposed methionine residues has been described for many mAbs, typically with higher susceptibility of M258 compared with M434, whereas the third methionine residue is less or not oxidized [92, 94, 96, 118-121].

Oxidation of the conserved methionine residues in the Fc induces structural changes and destabilization mostly located in the CH2 domain [94, 95, 122] and decreases the thermal stability [94, 95, 101, 122]. Increased aggregation was reported by one study [94] but not by others [122, 123]. The CH2-CH3 interface is also part of the binding sites of some Fc interacting proteins [101]. Oxidation reduces binding affinities with Protein A and G [120, 124, 125] and with FcRn [92, 95, 96, 101, 120, 126] and thus reduces serum half-life [96, 126]. FcγR have a

distinct binding site with none of the Fc methionine residues directly involved in binding [123, 127, 128]. While binding of most FcγR is not influenced by oxidation, small effects have been reported for FcγRIIa binding as well as effects on antibody-dependent cell-mediated phagocytosis (ADCP) [92, 129]. For ADCC activity, a minor loss was observed [101]. Moreover, decreased CDC activity was found [95]. A possible explanation may be that the conformational changes at the CH2-CH3 interface may impair IgG oligomerization, which is required for the binding of C1q of the complement system to initiate CDC.

Oxidation in the Fab region is often due to tryptophan residues (Trp), which are exposed when located in the CDRs and leads to increased aggregation and reduced antigen binding. Reduced antigen binding may result from aggregation [101] but has also been demonstrated without or with only low levels of concomitant aggregation [97, 98, 130].

2.2.5.6 Other PTMs

Some PTMs that occur frequently include N-terminal pyroglutamate formation and C-terminal lysine clipping. The N-termini of light and heavy chains of many mAbs contain a glutamic acid or a glutamine, which can undergo spontaneous cyclization to form pyroglutamate. The conserved heavy chain sequence ends in a C-terminal lysine residue. Mammalian producer cells express endogenous carboxypeptidases that cleave off the C-terminal lysine residue, but the levels of cleavage can vary between cell lines [131]. As these modifications often occur only partially, they may increase the product heterogeneity, as they contribute to charge variation and mass variation [10]. These modifications reportedly do not alter the biological activity. However, it is advised to monitor them as an indicator of a consistent production process [7]. Another modification at the N-terminus is the presence of a complete or partially truncated signal peptide of heavy chains and light chains, which should generally be removed during mAb secretion from the cell [132]. Its presence increases heterogeneity and may contribute to aggregation as it is relatively hydrophobic [65]. A recent review lists several more PTMs found on mAbs, including many with low frequency of occurrence, which were thus rated not highly relevant [82].

2.2.6 Forced degradation to study PTMs

Forced degradation is the application of stress conditions that exaggerate the conditions to which a mAb would usually be exposed [80]. Forced degradation studies are performed in drug development to elucidate potential modifications to support the mAb process and product development. Moreover, the degraded materials generated during these studies serve to qualify the analytical equipment and methods for their detection.

Forced degradation is also found in different regulatory guidance documents. ICH Q1A reads "Stress testing of the drug substance can help identify the likely degradation products, which can in turn help establish the degradation pathways and the intrinsic stability of the molecule and validate the stability indicating power of the analytical procedures used." [133]. Moreover, it is specified that the effect of temperature, humidity, oxidation, photolysis, and low and high pH must be tested on a single batch of the drug substance. ICH Q5E refers to comparability of biological products subject to changes in their manufacturing process, relevant for biosimilarity, and states that similar stability compared to the RMP should be demonstrated including data from stress conditions [134].

The most widely used agent for forced oxidation of proteins is H_2O_2 [101, 135], oxidizing mainly methionine residues [90, 136]. Recently, site-specific oxidation during H_2O_2 stress was achieved utilizing a ligand bound to the mAb [93]. Besides H_2O_2, t-butyl hydroperoxide (tBHP) is used for oxidation. Compared to H_2O_2, tBHP is bulkier and oxidizes mainly surface exposed methionine residues of mAbs. Alternatively, 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) may be used to oxidize both methionine and tryptophan residues [101].

Bevacizumab and infliximab have been subjected to various kinds of forced degradation for different purposes. Stresses included chemical oxidation, mostly with H_2O_2, exposure to light, elevated temperature, humidity, mechanical stresses (shaking, stirring, vibration), low and high pH, increased ionic strength, and others such as high concentration of guanidine. Effects on quality attributes such as size variants, aggregation, or antigen binding were studied [137-141]. Stressed samples were used to assess quantification by RPLC-UV [142], to compare stability in different formulations [143], and to evaluate the analytical quality control for compounded mAbs in a hospital [144]. Forced degradation is also applied during biosimilarity assessment, focusing on the effects on size variants, charge variants, higher order structure stability, aggregate formation, and potency [145-150]. While the studies did not investigate effects on PTMs, for bevacizumab and infliximab, levels of PTMs measured by liquid chromatography coupled with mass spectrometry (LC-MS) after forced degradation are often reported as part of biosimilar studies. Several biosimilar studies subjected infliximab to forced oxidation, thermal stress, and other stresses and assessed levels of oxidation [4, 151, 152] and deamidation [4, 152]. In two studies, bevacizumab biosimilars and RMPs were compared under forced oxidation, thermal stress, and other stresses. Zhang et al. [153] used tBHP and demonstrated levels of oxidation, whereas Cao et al. [154] used H_2O_2 but did not report details from their PTM assessment. Moreover, Coghlan et al. [76] compared disulfide bond shuffling on biosimilar and RMP versions of mAbs including bevacizumab.

2.3 Biosimilars

Analogous to the generic versions of small molecule drugs, biosimilars are developed after patents of RMP biologics expire. As mentioned before, biological drugs, especially therapeutic mAbs, are highly expensive and impose an economic burden on healthcare systems [155]. Biosimilars are marketed cheaper than RMPs because of lower development costs and can thus increase patient access to treatment.

2.3.1 Biosimilar concept

The FDA summarizes that biosimilar or biosimilarity means that "the biological product is highly similar to the reference product notwithstanding minor differences in clinically inactive components and that there are no clinically meaningful differences between the biological product and the reference product in terms of the safety, purity, and potency of the product" [156]. This concept is accepted in many countries with small differences in the regulations of each regulatory agency [157]. The requirement for the follow-up product to be similar and not identical is due to the complexity of mAbs resulting from the manufacturing process. As the manufacturing process, including the producer cell line, is proprietary, an identical copy product is impossible to achieve [3]. Even the RMP product changes over time, as the production process needs to be adapted to changing market requirements and technological conditions. The cell-based production also leads to inherent heterogeneity within the product. Importantly, approval relies largely on a comprehensive demonstration of physicochemical and functional similarity, which provides confidence that pre-clinical and clinical studies will likewise demonstrate safety and efficacy like the RMP [3]. The approval process includes the possibility of abbreviated clinical trials.

2.3.2 Development of biosimilar mAbs

The development of a biosimilar differs significantly from the development of the RMP. Briefly, the biosimilar company must establish its own manufacturing process for the biosimilar because that of the RMP is proprietary [157]. The biosimilar developer must comprehensively characterize the RMP to gain a deep understanding of its quality attributes. Several product batches of the RMP are analysed for biosimilar development as their analytical characteristics may change over time. This defines the ranges in which the quality attributes are allowed to deviate over time and constitutes the target profile for biosimilar development. The biosimilarity assessment ("comparability exercise") is supposed to demonstrate structural,

functional, and clinical similarity within these ranges [158]. Thus, analytical characterization constitutes a large part of biosimilar development.

A biosimilar may be developed with lower costs than an RMP, introducing price competition to the market [2]. The approval success rate of drug candidates, including new RMP mAb candidates, ranges from 10 to 20%, illustrating the high risks that developers of RMPs face [159, 160]. In contrast, the development of a biosimilar is targeted towards the RMP, which, as an approved biologic, has successfully overcome many risks of failure that can arise during development and has already demonstrated its benefits [82]. For example, the biosimilar's risk of instability is manageable, as the stability depends mostly on the primary structure as well as the resulting higher order structure. These characteristics are likely to be matched by the biosimilar candidate, supposed that similarity of critical quality attributes (CQA) can be reached as well. In addition to the promising developability perspective, development costs may be lower if smaller tailored pre-clinical and clinical studies are required based on the comprehensive analytical comparison.

This does not mean that biosimilar development is easy. The biosimilar product characteristics are largely influenced by the manufacturing process, which can never be replicated identical from the proprietary process of the RMP, moreover using a different producer cell line and cell clone [10]. Nevertheless, biosimilar development can be regarded as following a successful recipe.

In addition to providing cheaper treatment, the development of biosimilars has more beneficial aspects [155]. Intense analytics increases understanding of products. The risk of shortages is reduced, as biosimilars increase the available alternatives to an RMP biologic. Moreover, biosimilar development promotes innovation. Developers of RMPs may focus on new inventions and improvements in fear of price competition. The manufacturing process of a biosimilar requires higher batch-to-batch consistency, involving the latest analytical techniques.

2.3.3 Analytical characterization of RMP and biosimilar

Physicochemical characterization constitutes a large part of biosimilar comparison. ICH Q6B highlights the importance to characterize the structural heterogeneity of biologics by state-of-the-art physicochemical methodologies [161]. Quality attributes include the amino acid sequence, disulfide bonds, post-translational modifications, charge variants, aggregates and fragments, higher order structure, and biological activities [157]. The many quality attributes are investigated by an extensive range of methods. Differently modified species (as well as species-related contaminants) can be characterized by separating them based on different

physicochemical properties [10]. Investigation of the amino acid sequence and PTMs is mostly performed by liquid chromatography and by electrophoretic and mass spectrometry (MS) methods.

2.3.3.1 Liquid chromatography instrumentation

Liquid chromatography is well established for the quality control of mAbs because of its robustness and separation capability [7]. The basic setup of a liquid chromatography system consists of a sample injection system, a binary pump for the eluents, a chromatographic column, and a detector. More components may be added, making liquid chromatography a highly automized system for sample analysis. Many methods use UV or fluorescence detection; however, it can also be hyphenated to electrospray ionization (ESI)-MS [7]. Classically, the detection of proteins and peptides is achieved by a UV detector, exploiting the absorption at 205-215 nm by peptide bonds or at 280 nm by conjugated π-systems of aromatic amino acid side chains [162]. In quality control (QC) laboratories, the detection is mostly performed using UV detectors, first, because they are less expensive than a mass spectrometer and second, because they are relatively easy to use and require minimal technical expertise. A mass spectrometer which is used for protein characterization requires regular, time-consuming cleaning to maintain sensitivity, and technical problems can result in down time, because complex instrumental problems can usually only be solved by experienced technical personnel. In contrast, a broken UV light source can be easily exchanged with only little technical knowledge.

2.3.3.2 Chromatographic methods

Among the different modes of chromatography for protein analysis, reversed-phase liquid chromatography (RPLC) has the highest separation capacity [162]. Separation relies mainly on the strength of the hydrophobic interaction of hydrophobic amino acids with the alkyl chains of the stationary phase under denaturing conditions. Typical nonpolar stationary phases are n-butyl or n-octyl for proteins and n-octadecyl for peptides. Elution is achieved by increasing the proportion of the organic component (often acetonitrile) in a mixture with water. Classically, detectors for UV or fluorescence are used, however, RPLC may also easily be coupled with MS detection. RPLC is used for diverse aspects, such as evaluation of identity, purity, and integrity, determination of molecular weight and primary structure, identification of PTMs such as oxidation, as well as for quantification and purity assessment, and in release tests [7, 163]. In some cases, physicochemical changes as, for example, by PTMs, are too small to separate the

intact mAb (unmodified vs modified); therefore, the mAb is cleaved into smaller parts, as discussed in section **2.3.3.5**.

Hydrophobic interaction chromatography (HIC) also separates proteins based on hydrophobic interactions, but in contrast to RPLC, non-denaturing conditions are used [7]. The non-denaturing conditions preserve mAbs in their native state, making it suitable for further investigations. Therefore, the mAbs' surface hydrophobicity determines the retention. HIC employs stationary phases from reversed-phase columns with low carbon loading of short-chain bonded chains, such as propyl, phenyl, phenyl sepharose, or ether [7]. The separation occurs under a decreasing salt concentration gradient, utilizing pure aqueous mobile phases with salt additives. HIC may be used for separation of oxidized species [10]. Notably, HIC has emerged as the method of choice for analysing ADCs, allowing for the separation of various ADC species with differing drug-to-antibody ratios [164]. The drugs are usually highly hydrophobic, and the drug-loaded species elute in order of increasing hydrophobicity.

Charge variants caused by PTMs can be separated into acidic variants and basic variants by ion exchange chromatography (IEX), typically cation exchange chromatography (CEX) [7, 162]. Variations in charge may have different reasons, for example oxidation, deamidation, C-terminal lysine clipping, formation of isoaspartic acid or pyroglutamate or sialic acid in the glycans. Electrostatic interactions of charged surface patches of the mAb and the opposite charges of immobilized charged components of the stationary phase mediate retention in IEX. In CEX, elution is achieved by a gradient from low pH, low salt concentration to either low pH, high salt concentration or to high pH.

In addition to the mAb monomer, the occurrence of aggregates (high molecular weight species) and fragments (low molecular weight species) needs to be investigated [7, 162]. These size variants can be separated by size exclusion chromatography (SEC). Separation is achieved by porous stationary phases. Smaller analytes enter the pores and thus take longer to travel through the column, while larger molecules progress faster. Therefore, this mode does not rely on retention by chemical interaction. Isocratic elution is usually performed with aqueous mobile phases with a low salt concentration.

Separation by hydrophilic interaction liquid chromatography (HILIC) is based on different hydrophilicity and is used to discriminate polar to highly polar analytes that would be poorly retained in RPLC [7, 162]. Applications include the analysis of released glycans, glycopeptides, and even glycoproteins. HILIC can also be coupled to MS.

Another type of chromatography employed for mAb characterization is boronate affinity chromatography (BAC), which can also discriminate between different glycoforms [7].

If a non-destructive detector is used, separated protein species can also be collected for further analysis.

2.3.3.3 Electrophoretic methods

Another important principle for protein separation is electrophoresis [7, 162]. One commonly employed size separation method is sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). SDS masks charges of the denatured protein, allowing separation based only on the molecular weight. The resolution of PAGE does not allow very exact size determination [162]. Still, it can show the distribution of monomers, dimers, and typical fragments, such as separated light chains or subunits after enzymatic digestion. SDS-PAGE is used complementary to SEC. Another gel-based method is isoelectric focusing (IEF) for the analysis of charge variants complementary to IEX. Protein bands can be cut from the gels for further investigation. As an alternative to gel electrophoresis, capillary electrophoresis (CE) is gaining more and more importance for mAb analysis because of higher automation, high separation efficiency, and fast analysis times [165]. Different modes are available, with CE-SDS as an alternative to SDS-PAGE for the determination of molecular weight and size variants and capillary isoelectric focussing (cIEF) as an alternative to gel-based IEF for analysis of charge heterogeneity [7]. Capillary zone electrophoresis (CZE) is able to separate charge variants, complementing IEX. Coupling CE with MS is also possible but is still much less widespread than liquid chromatography [165].

2.3.3.4 LC-MS-based methods

The analysis of proteins by mass spectrometry requires so-called soft ionization techniques, among which electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) have proven the most suitable [7, 10]. Both are typically used with a time of flight (TOF) mass analyser which has the advantages of high resolution and mass accuracy and a suitable *m/z* range for analysis of large molecules [166]. Another important mass analyser is the orbitrap system, and ESI-Orbitraps are likewise widespread instrument types in the biologics industry. Other mass analysers, such as Fourier-transform ion cyclotron resonance (FTICR), still offer higher resolution; however, instruments with this type of analyser are not yet widely applied in the biologics industry, due to their high costs, elaborate maintenance of their superconducting magnets and difficult coupling to liquid chromatography [7, 167, 168]. The main advantage of ESI-MS compared with MALDI-MS is that it can be directly coupled with

liquid chromatography, making it the preferred instrument type for the analysis of mAbs. Separation of a sample by liquid chromatography can also be applied before MALDI-TOF analysis; however, it must be performed offline by spotting the eluting samples onto a MALDI target plate as discrete spots. Therefore, the direct coupling of liquid chromatography with ESI-MS is advantageous, as samples can be measured continuously around the clock with an efficiency that cannot be reached by liquid chromatography with MALDI [162]. Further advantages of ESI-MS are generally a higher mass accuracy [7, 10] and a better reproducibility of the ion production [162].

Among the different liquid chromatography modes, RPLC is especially suited for direct coupling with ESI-MS, because elution does not require salts, which may cause ion-source contamination and signal suppression [162]. Among different additives, formic acid (FA) is commonly used to support the ionization of analytes. For RPLC-MS, the sample is separated by chromatography and the eluate from the column is infused into the ESI source of the mass spectrometer, where the separated analytes are sequentially desolvated, ionized and transferred by ion optics to be analysed by the respective mass analyser. MS reveals the mass of the analyte. Additional information may be gained from fragmentation of the analyte in the mass spectrometer by tandem mass spectrometry (MS/MS) experiments [162]. Techniques for the fragmentation of proteins in ESI-MS include collision-induced dissociation (CID), electron-capture dissociation (ECD) and electron-transfer dissociation (ETD).

2.3.3.5 Levels of structural characterization

When MS analysis is used, the levels of characterization are designated according to how the analytes are introduced into the mass spectrometer: top for intact mAb, middle for subunits, and bottom for protein digests. The approaches are further divided into “up” and “down” methods, with “up” referring to MS-only analysis and “down” to additional fragmentation in the gas phase by MS/MS (**Figure 6**). For historical reasons, MS analysis of peptides is always called “bottom-up”, regardless if MS/MS is performed or not [166]. Moreover, the analysis of intact mAb without MS/MS is commonly called intact analysis, whereas “top-up” is not used.

In the top-approach, the mAb is analysed intact (~150 kDa) [7, 166]. Therefore, it is also called intact mAb analysis. The top-approach doesn’t require sample preparation, except for possibly a buffer exchange, and is therefore comparatively quick and easy to perform. However, the insights gained are limited, as structural changes indicated by the observed mass changes cannot be localized. Intact mass analysis is useful for the determination of features such as the major

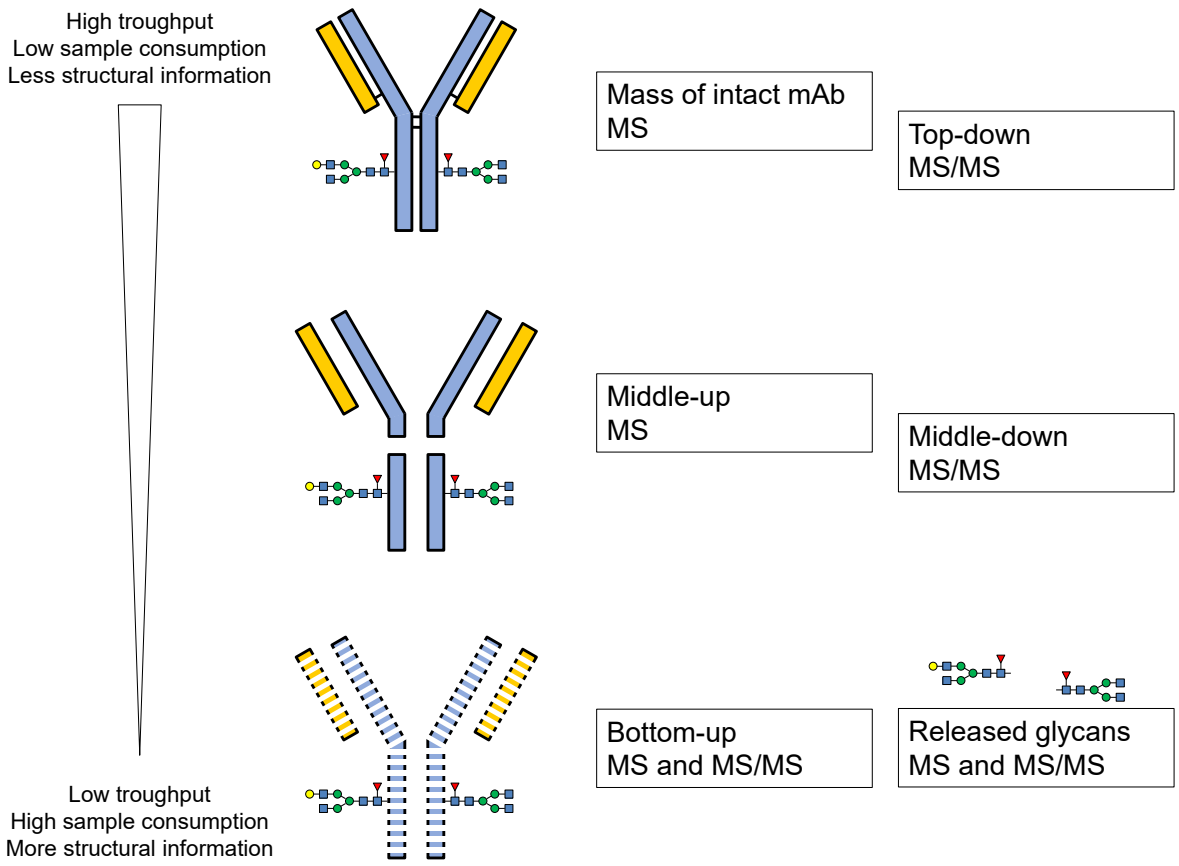

Figure 6: Overview of mass spectrometry-based approaches for structural characterization of monoclonal antibodies. Adapted from [166].

glycoforms, especially as the location of the N-glycans is usually not a question, as they reside on a conserved sequence. Amino acid substitutions are not a frequent problem, but if they occur, they are a major problem, which can be detected by intact analysis [169].

In the bottom-up approach, a protease like trypsin digests the protein into peptides. The approach is also called peptide mapping [7, 166]. Typically, in preparation for digestion, the protein is denatured from the native conformation to a denatured conformation. This is important so that otherwise buried or inaccessible parts of the protein become accessible for the next sample preparation steps. Disulfide bonds are reduced with a reducing agent such as DTT. Free thiol groups of cysteine residues are then alkylated with an alkylating agent such as iodoacetamide (IAM) to prevent reformation of disulfide bonds. If these steps have been performed in the presence of a strong denaturant such as guanidine in high concentration, it is advised to perform a buffer exchange to a less denaturing composition before enzymatic digestion. Otherwise, the protease will also be denatured and loses its function. Subsequently, the protease generates peptides, which are then analysed. Bottom-up offers the highest structural resolution. MS/MS is used to fragment the peptides, allowing to pinpoint mass changes to the amino acid on which they occur. Moreover, the fragmentation data adds more

confidence that the assignments are correct. Another application is the confirmation of the sequence on the amino acid level by MS/MS. However, bottom-up analysis also has drawbacks. Many PTMs, including asparagine deamidation and methionine oxidation are increased by factors such as increased temperature and elevated pH [5, 170-174]. As an example, peptide mapping is often performed under slightly basic pH, for optimal activity of trypsin. Moreover, areas of the mAb are exposed in the denaturing conditions that would otherwise be buried and protected. However, the basic conditions may promote deamidation. The risk of artificial modifications is especially prominent with protocols that use elevated temperatures to achieve sample preparation in a shorter time [5]. Conversely, when incubations are performed at lower temperatures, more time is required to achieve complete reduction, alkylation, and digestion. Among the different approaches, bottom-up is also the most time-consuming, most expensive, and requires the largest amount of sample.

For the middle-up approach, the mAb is separated into large subunits, hence also called subunit analysis, by reduction of disulfide bonds or by limited digestion [7, 166]. Reduction of disulfide bonds yields the LC and HC. Limited digestion under native conditions was previously performed by enzymes such as papain, pepsin, or Lys-C, which cleave first in the accessible hinge region, producing Fab, F(ab)2, Fc, and Fc/2 [166]. However, these enzymes have limited specificity and produce several truncated versions, complicating the analysis. Today, IdeS (IgG degrading enzyme of *Streptococcus pyogenes*) is the enzyme of choice for this task, as it only cleaves the mAb in the hinge region below the inter-heavy chain disulfide bonds [10]. Thereby, two Fc/2 subunits and the $F(ab)_2$ are generated. Subsequent reduction of the disulfide bonds releases the two light chains and the two Fd' subunits (**Figure 2**). Similarly, as described for bottom-up, denaturing conditions are important to achieve complete reduction of disulfide bonds. All three subunits have a mass of approximately 25 kDa. In comparison to bottom-up, structural resolution is limited to the subunits. Nevertheless, the information whether a modification occurs on the Fab (LC or Fd') or on the Fc region, may be useful. For example, in case of oxidation, it may indicate if reduced effector function results from oxidation in the Fab and thus probably from disturbed target binding or from oxidation in the Fc part and thus probably from disturbed interaction with effector molecules such as the complement system or FcγR. Moreover, middle-up sample preparation has a lower risk of artificial modifications than bottom-up, because the cleavage site is accessible under native conditions and cleavage can be performed in shorter time. The denaturing step for reduction, which exposes otherwise shielded parts is performed afterwards, hence the mAb is subjected to denaturing conditions for much

shorter time. Opposed to the typical trypsin digestion in basic environment applied for bottom-up, IdeS digestion is performed at slightly acidic pH.

In addition to the middle-up, there is also the middle-down approach. The two approaches have in common the fast and thus beneficial subunit sample preparation. In middle-down, MS/MS is employed to fragment the subunits along the peptide bond backbone to yield the amino acid sequence and post-translational modifications from the fragments. Likewise, in addition to intact mAb analysis by MS, the top-down approach employs MS/MS on the intact mAb. However, up-to-date, middle-down, and top-down techniques are still under development, and MS/MS fragmentation is usually incomplete [175].

2.3.3.6 Advantages of LC-MS

Mass spectrometry adds another dimension to the identification of protein species, the protein mass, which can be determined with high accuracy not reached by any other analytical technique [162]. MS detection also achieves higher sensitivity than UV detection (but not necessarily higher than fluorescence detection), which is important for the detection of low-abundant species. Liquid chromatography and MS complement each other. Whereas in classical LC-UV, in which analytes are identified by their retention times in comparison with a standard, mass spectrometric detection adds an additional dimension to the analytical signal, the m/z value. Thus, MS offers additional selectivity, which can be even more increased by techniques such as MS/MS. Not only does MS improve the identification of proteins by liquid chromatography. Conversely, prior separation of protein species by liquid chromatography also improves identification by MS. Different species in a mixture may yield ions with the same m/z values as the target analyte, resulting in interferences and, different species may influence each other, for example by ion suppression [176]. Moreover, modifications without mass differences, such as aspartate isomerization, L-cysteine to D-cysteine and serine racemization may be discriminated by different retention times in LC-MS [82]. Therefore, the highest selectivity is achieved if liquid chromatography separation and MS are combined.

A recent article lists 40 different PTMs (some of which can occur on different amino acids) that may be distinguished by MS according to the mass change caused by them [82]. Thus, LC-MS allows for the simultaneous measurement of different attributes by a single LC-MS run, referred to as multi-attribute monitoring (MAM). This is an advantage over classical characterization without MS, in which CQAs need to be analysed by the orthogonal use of different methods, for example, charge variants by IEX, oxidation by RPLC or HIC, and glycan profiling by HILIC [5, 177]. Especially peptide-mapping LC-MS/MS provides site-specific information on CQAs

and enables a more detailed understanding of the product [177]. Moreover, relative quantification shows the ratio of abundance of modified to total species [177].

2.3.3.7 Higher order structure characterization

ICH Q6B additionally requests characterization of the higher order structure (HOS) of proteins (secondary, tertiary, and quaternary structure) because of its pivotal role in biological functionality [161]. To this end, different biophysical methods are used [178]. Most routinely used methods can only determine a global average about the three-dimensional mAb structure, and each method is limited for the determination of specific characteristics. These include fluorescence spectroscopy, differential scanning calorimetry (DSC), circular dichroism spectroscopy (CD), and analytical ultracentrifugation (AUC) [179, 180]. For example, DSC, as well as the derivative isothermal titration calorimetry (ITC), measure thermodynamic changes that are correlated to structural changes (unfolding) caused by temperature changes. In the case of mAbs, DSC can usually discern only between the unfolding of the less stable CH2 domains and all other domains and does not yield more specific information about the three-dimensional structure. Among these routine methods, CD is most informative about HOS, as it can determine the percentages of α-helices, β-sheets, and unordered conformations, as each structural element has a unique CD spectrum. However, these are only percentage data and, therefore, still far from elucidating the real three-dimensional structure. Moreover, these methods cannot detect low abundant conformation isoforms in a mixture [181]. Still, a combination of these methods is frequently used to demonstrate that a biosimilar generates a similar outcome as the RMP regarding HOS.

Nuclear magnetic resonance (NMR) and X-ray crystallography (XRC) are important methods that offer high structural resolution [10, 164, 182]. However, using these techniques for mAbs faces several difficulties, such as the mAbs' large size, flexibility in some regions of the mAb (e.g., hinge region or CDR loops), and large amounts of sample required. XRC requires crystallisation, which is difficult for mAbs, and access to a synchrotron radiation facility. Moreover, it should be noted that XRC structures represent a solid state and not the in-solution state. NMR is limited for mAb analysis because of low natural abundance of active nuclei and the large size [10]. However, recently two-dimensional ^{1}H-^{13}C methyl NMR was used to assess the HOS of a mAb [183] and perturbations of the HOS caused by oxidation [184]. In addition to the drawbacks mentioned above, data analysis of XRC and NMR studies is complex and time-consuming, another obstacle in biosimilarity studies.

A mass spectrometry-based approach to derive information about the HOS of proteins is hydrogen-deuterium exchange (HDX) MS [182]. It works by measuring the rate at which protein backbone amide hydrogen atoms exchange with deuterium in a deuterated solvent, providing information on protein folding, conformational changes, and interactions. HDX MS was used for biosimilarity assessment of bevacizumab in conjunction with mass determination by native MS and ion mobility mass spectrometry (IM-MS) to derive information about mass, charge, and conformation [182].

Different techniques such as SDS-PAGE or CE-SDS, SEC, AUC, asymmetric flow field flow fractionation (AF4), static and dynamic light scattering (SLS & DLS), or visual inspection can characterize the presence of aggregates [162].

2.3.4 Scope of the dissertation

The aim of this study was to evaluate the middle-up and bottom-up approach for analysis of forced degradation studies in mAb biosimilar development. The mAbs investigated in this study are biosimilar versions of bevacizumab and infliximab as well as their respective RMPs Avastin and Remicade. Forced degradation studies are used to support biosimilarity assessment. In this study, forced oxidation was applied to the mAbs, because oxidation is one of the most frequently observed PTMs on mAbs and can represent a CQA. Stress testing for oxidation is also prescribed by ICH Q1A [133]. The samples were incubated with H_2O_2 as it is the most commonly used oxidative agent in forced oxidation studies of mAbs. Middle-up and bottom-up analysis had to be established. For middle-up analysis (manuscript 1), stressed samples were digested by IdeS and disulfide bonds were reduced under denaturing conditions. Samples were analysed by LC-MS, using a QTOF mass spectrometer. Middle-up was used to confirm the identity of the chromatographically separated species and to assess PTMs with focus on oxidations. Conclusions about the localization of oxidations were drawn from the positions of methionine residues in the two mAbs. Furthermore, the time course of oxidation levels was compared between the RMP and the biosimilar versions. For bottom-up analysis (manuscript 2), stressed samples were denatured, disulfide bonds were reduced and alkylated and digestion was performed by trypsin or Lys-C. Samples were analysed by LC-MS/MS on the same QTOF mass spectrometer as in manuscript 1. MS/MS was used for identification of the modified amino acids, which included methionine, cysteine, and tryptophan residues. MS data was used for relative quantification of the modifications. Biosimilarity was evaluated by analysis of the time course of oxidation. The results of bottom-up and middle-up analysis were compared regarding consistency, inconsistency, and complementarity.

3 Manuscripts

3.1 Manuscript 1

Forced Degradation Testing as Complementary Tool for Biosimilarity Assessment

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Abstract: Oxidation of monoclonal antibodies (mAbs) can impact their efficacy and may therefore represent critical quality attributes (CQA) that require evaluation. To complement classical CQA, bevacizumab and infliximab were subjected to oxidative stress by H2O2 for 24, 48, or 72 h to probe their oxidation susceptibility. For investigation, a middle-up approach was used utilizing liquid chromatography hyphenated with mass spectrometry (LC-QTOF-MS). In both mAbs, the Fc/2 subunit was completely oxidized. Additional oxidations were found in the light chain (LC) and in the Fd' subunit of infliximab, but not in bevacizumab. By direct comparison of methionine positions, the oxidized residues in infliximab were assigned to M55 in LC and M18 in Fd'. The forced oxidation approach was further exploited for comparison of respective biosimilar products. Both for bevacizumab and infliximab, comparison of post-translational modification profiles demonstrated high similarity of the unstressed RMP (RP) and the biosimilar (BS). However, for bevacizumab, comparison after forced oxidation revealed a higher susceptibility of the BS compared to the RP. It may thus be considered a useful tool for biopharmaceutical engineering, biosimilarity assessment, as well as for quality control of protein drugs.

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Article

Forced Degradation Testing as Complementary Tool for Biosimilarity Assessment

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Abstract: Oxidation of monoclonal antibodies (mAbs) can impact their efficacy and may therefore represent critical quality attributes (CQA) that require evaluation. To complement classical CQA, bevacizumab and infliximab were subjected to oxidative stress by H_2O_2 for 24, 48, or 72 h to probe their oxidation susceptibility. For investigation, a middle-up approach was used utilizing liquid chromatography hyphenated with mass spectrometry (LC-QTOF-MS). In both mAbs, the Fc/2 subunit was completely oxidized. Additional oxidations were found in the light chain (LC) and in the Fd' subunit of infliximab, but not in bevacizumab. By direct comparison of methionine positions, the oxidized residues in infliximab were assigned to M55 in LC and M18 in Fd'. The forced oxidation approach was further exploited for comparison of respective biosimilar products. Both for bevacizumab and infliximab, comparison of posttranslational modification profiles demonstrated high similarity of the unstressed reference product (RP) and the biosimilar (BS). However, for bevacizumab, comparison after forced oxidation revealed a higher susceptibility of the BS compared to the RP. It may thus be considered a useful tool for biopharmaceutical engineering, biosimilarity assessment, as well as for quality control of protein drugs.

Keywords: middle-up approach; liquid chromatography-mass spectrometry (LC-MS); QTOF-MS; biopharmaceuticals; forced stability testing; structure reactivity relationship; bevacizumab; infliximab; biosimilar

1. Introduction

As is generally the case for biopharmaceuticals, therapies with monoclonal antibodies (mAbs) are highly expensive, and therefore access is limited worldwide. When biopharmaceuticals run out of patent protection, biosimilars are developed as follow-on products [1]. For their approval, abbreviated procedures may be accepted by the authorities in analogy to the small molecule generics [2]. Physicochemical similarity plays a major role in the approval process. Critical quality attributes (CQA) have to be evaluated, which may impact pharmacological response [3].

Biosimilars offer a comparatively cheaper alternative to alleviate the burden that biopharmaceutical therapies pose to health care systems [4]. Currently, 11 biosimilar mAbs have been approved for five different blockbuster mAbs in the US and the EU [5]. Among these are biosimilars for Avastin (bevacizumab) and Remicade (infliximab).

Bevacizumab binds to vascular endothelial growth factor A (VEGF-A), preventing the activation of VEGF receptors on endothelial cells, which is necessary for their proliferation and migration. Thereby, bevacizumab inhibits angiogenesis, a process that is critical in the development of different tumors. Infliximab acts by binding to tumor necrosis factor-α (TNF-α), a cytokine that mediates inflammatory responses and modulation of the immune system. Infliximab is used for therapy of autoimmune and inflammatory diseases.

A therapeutic mAb product is a complex heterogenic mixture. While the same amino acid sequence is shared among all the mAb molecules in the mixture, different posttranslational modifications (PTM) are introduced during the production process. Its development and production therefore demand for special analytical methods [6–8].

One important PTM is oxidation, because it can have impact on the safety and efficacy of the mAb product. A close examination is mandatory in engineering and quality control of biopharmaceuticals. Thus, oxidations may be considered critical quality attributes (CQA), especially if they occur in the complementary determining regions (CDR), where they may impact the binding to the target antigen [9]. Oxidation also plays an important role if it occurs in the Fc part. Oxidations in this part can impact structure and stability [10] as well as the binding to protein A and protein G, relevant for downstream processing. Furthermore, a modified binding to neonatal Fc receptor for IgG (FcRn) may impact serum half-life [11–13]. Among several amino acids that can undergo oxidation, reactions of methionines have been extensively studied, because of their high susceptibility [14].

To investigate the susceptibility of a mAb towards oxidation, forced oxidation studies are performed in which exaggerated conditions are used to forcefully elicit oxidation. These studies are especially important in the identification of CQAs [15,16]. According to the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH), stability testing of new drug substances and products should include long-term, intermediate, and accelerated stability testing (ICH Q1A) [17]. It is recommended to investigate the effect of temperature, humidity, oxidation, and photolysis on the drug substance. For accelerated oxidation, H_2O_2 or tert-butylhydroperoxid (tBHP) are reported to specifically target methionines [14,18]. In the same studies, 2,2-azobis(2-amidinopropane) dihydrochloride (AAPH) mainly targeted tryptophanes when additional free methionine was added to the reaction mixture. However, a recent study on one mAb showed that while H_2O_2 and tBHP exclusively led to methionine oxidation in the Fc, AAPH additionally oxidized both tryptophanes and methionines, which led to considerably different functional impacts, compared to the peroxides [19]. Thus, the specificity of AAPH for tryptophanes seems less clear.

Sources of oxidation, to which a mAb product is exposed, include peroxides, polysorbates, sugars, metals, air oxygen, and light [20]. Oxidation may occur during fermentation, purification, filling, and finishing, as well as during storage. It is thus considered as a critical degradation reaction. Therefore, the products oxidation profile depends on and can vary according to different production processes.

Reversed phase liquid chromatography (RPLC) is the preferred method for investigation of mAb oxidation on the physiochemical level because of its resolution, robustness, and compatibility with mass spectrometry [6]. The changed hydrophobic nature of oxidized species allows for their separation by RPLC and mass-spectrometric detection provides high selectivity.

The mAbs are analysed by LC-MS with different levels of structural resolution, and each level offers different advantages [21]. Determination of the mass of the intact mAb (also referred to as top level) is well suited for high-throughput applications, because it requires the least sample preparation and yields general information, such as verification of the expected mass and major modifications, such as the main glycoforms. As a complementary approach for detailed structural resolution, the mAb is digested into small peptides (bottom-up level). Also called peptide mapping, this approach reveals minor and less abundant modifications and enables their localization to specific peptides. However, because of elaborate and time-consuming sample preparation to avoid the artificial introduction of PTMs, this application only allows for low throughput [22]. In a third approach (middle up level), mAbs are enzymatically digested to yield the subunits fragment crystallizable region (Fc) and

antigen-binding fragment (Fab). The subsequent reduction of disulfide bridges liberates the light chain (LC), the variable fragment of IgG heavy chain (Fd'), and the constant fragment of IgG heavy chain (Fc/2). Modifications can be localized to these three subunits. Thus, comparatively simple sample preparation and analysis make the middle-up approach a valuable complementary tool offering a compromise between high-throughput and detailed information.

The application of forced degradation with subsequent analysis on the subunit level has recently gained popularity. Among other degradation pathways such as deamidation, this approach has been used to study oxidation in mAbs, mostly elicited by chemical oxidation as well as by exposure to light [16,23–27]. Two biosimilarity studies of infliximab have included forced degradation in the biosimilarity assessment. Pisupati et al. used temperature and humidity stress [28]. However, no strong effect of oxidation was observed by peptide mapping. Kim et al. applied H_2O_2 on infliximab. By peptide mapping they showed methionine oxidations that were comparable between the reference product and the biosimilar [29]. Some studies have applied forced oxidation as well as other forced degradation treatments on bevacizumab and infliximab for analysis of aggregate formation, for binding assays and to support method validation [30–33]. However, as these studies were conducted on the intact mAbs, no structural information about PTMs introduced by forced degradation was provided.

In this study, biosimilars of bevacizumab and infliximab were subjected to forced oxidation using H_2O_2, together with their reference products, to support the biosimilar assessment. Analysis on the middle-up level was selected in order to provide detailed information about the susceptibility to oxidation of methionine residues. The results of the faster middle-up method are checked for consistency to published data obtained from the bottom-level [29].

2. Materials and Methods

2.1. Chemicals and Consumables

Hydrogen peroxide (H_2O_2) solution (30% (w/w) in H_2O), tris(2-carboxyethyl)phosphine (TCEP, 0.5 M, pH 7.0 aqueous solution), and 2-(*N*-morpholino)ethanesulfonic acid monohydrate (MES monohydrate) were obtained from Sigma-Aldrich (Steinheim, Germany). Sodium azide (NaN_3) and potassium dihydrogen phosphate (KH_2PO_4) were obtained from Merck Millipore (Darmstadt, Germany). Acetonitrile (ACN) and formic acid were obtained from Fisher Scientific (Schwerte, Germany). Di-sodium hydrogen phosphate dihydrate ($Na_2HPO_4{\cdot}2H_2O$) was purchased from Bernd Kraft GmbH (Duisburg, Germany), and guanidine-HCl was from AppliChem (Darmstadt, Germany). All purchased chemicals were of highest purity. Ultrapure water was obtained using LaboStar 2-DI/UV system (SG Wasseraufbereitung und Regeneration GmbH, Barsbüttel, Germany) equipped with LC-Pak Polisher and a 0.22-µm membrane point-of-use cartridge (Millipak). Mass calibration reference masses (HP-921, HP-1821, HP-2421) were from Agilent (Waldbronn, Germany). IdeS enzyme and Vivaspin 500 ultrafiltration spin columns were purchased from Genovis (Lund, Sweden) and Sartorius (Göttingen, Germany), respectively.

Phosphate buffer of pH 6.5 (Sørensen's phosphate buffer) was prepared freshly by mixing 6.87 volumes of a 66.71 mM KH_2PO_4 stock solution with 3.13 volumes of a 66.72 mM $Na_2HPO_4{\cdot}2H_2O$ stock solution. MES buffer solution of pH 6.5 was 10 mM.

The bevacizumab reference product (RP) Avastin (lot B8502H09) from Roche (Basel, Switzerland) and the infliximab RP Remicade (lot 9RMKA60302) from Janssen Biologics B.V. (Leiden, The Netherlands) were used for this study. The biosimilar (BS) antibodies were produced at ProBioGen AG. The biosimilars were expressed in chinese hamster ovary cell culture, purified from the supernatant by protein A and eluted at a low pH. Bevacizumab biosimilar was further kept at a low pH for virus inactivation. Both BS antibodies were buffer-exchanged with the same buffer as the RP. Other purification steps that would be performed for clinical material were not applied.

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2.2. Generation of Stressed mAb Samples

Forced oxidation was performed by incubation of RP and BS at a final concentration of 0.6 µg/µL antibody, 0.05% (w/v) H_2O_2, 0.1% (w/v) NaN_3 and phosphate buffer (pH 6.5 at a final dilution of 1:4) at 37 °C for 24 h, 48 h, or 72 h after thorough vortexing (Table 1). Control samples, prepared using water instead of H_2O_2, were treated similarly. All experimental stress conditions were applied to the biopharmaceuticals as independent triplicates. After forced oxidation, mAbs were five times buffer exchanged with 400 µL MES buffer by centrifugation in an ultrafiltration column (molecular weight cut off = 10 kDa) and then diluted to 1 µg/µL in MES buffer. Protein concentration was determined by measuring the absorbance at 280 nm on a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

Table 1. Overview of experiments. The reference product (RP) and biosimilar (BS) version of two mAbs (bevacizumab and infliximab) were subjected to forced oxidation (oxi) and control (con) treatment. Each experiment was performed as independent triplicate.

mAb	Product	Duration of Forced Oxidation or Control Treatment			
		0 h	24 h	48 h	72 h
Bevacizumab	RP	con -	con oxi	con oxi	con oxi
	BS	con -	con oxi	con oxi	con oxi
Infliximab	RP	con -	con oxi	con oxi	con oxi
	BS	con -	con oxi	con oxi	con oxi

2.3. Digestion and Reduction of mAb Samples

Stressed mAb samples were digested at a final concentration of 1 µg/µL in MES buffer with IdeS at 1 unit enzyme/1 µg mAb. To yield the Fc/2, Fd', and LC subunits, 100 µg of mAb were buffer-exchanged and adjusted to a final volume of 50 µL MES buffer (β_{mAb} = 2 µg/µL). IdeS (10 µL of a stock solution, β_{stock} = 10 units/µL) was added and the final volume was adjusted to 100 µL by addition of MES buffer (β_{mAb} = 1 mg/µL; β_{IdeS} = 1 unit/µL). Digestion was achieved within 30 min at 37 °C. For reduction, 500 µL of guanidine-HCl (8 M solution) and 66 µL of TCEP solution (500 mM) were added to result in a final volume of 666 µL. Thus, the composition of the final mixture expressed in volume percent is: mAb digest (15%), 8 M guanidine-HCl (75%), and 500 mM TCEP (10%). After vortexing samples were incubated at ambient temperature for 1 h to complete reduction of the disulfide bonds.

2.4. Liquid Chromatography and Mass Spectrometry

Samples were analysed using a 1290 Infinity II UHPLC system coupled to a 6550 iFunnel Q-TOF mass spectrometer with an electrospray ionization source (Agilent, Waldbronn, Germany). Aliquots of the sample (0.75 µg) were injected onto a reversed phase C4 column (Aquity UPLC Protein BEH C4 Column, 300 Å, 1.7 µm, 2.1 mm, 100 mm, Waters, Manchester, UK) and the column temperature was maintained at 80 °C. Mobile phase A was 0.1% (*v*/*v*) formic acid in water, and mobile phase B was 0.1% (*v*/*v*) formic acid in ACN. The flow rate was 0.5 mL/min. The complete run consisted of 1 min isocratic elution (20% solvent B), followed by a 40 min gradient (20% to 32% solvent B), a subsequent purge step (quick rise to isocratic elution at 95% solvent B), and a final 5 min reequilibration step (quick decrease to isocratic elution at 20% solvent B).

Mass spectrometer settings included a mass range of *m*/*z* 600 to *m*/*z* 3,200; nebulizer: 2.8 bar, drying gas flow: 14 L/min; drying gas temperature: 290 °C; sheath gas flow: 12 L/min; sheath gas temperature:

375 °C; fragmentor voltage: 400 V. Spectra were acquired at 2 spectra/s. Internal mass calibration was achieved by continuously spraying of internal reference compounds through the reference nebulizer.

Data acquisition was controlled with the MassHunter software (Agilent, Santa Clara, CA, USA).

2.5. Data Processing

For the identification of species, an averaged raw spectrum was generated for each chromatographic peak and deconvoluted using the maximum entropy algorithm in Bioconfirm software (Agilent). Deconvoluted masses were matched to expected masses calculated based on the amino acid sequence of the antibody and variable posttranslational modifications (with ±70 ppm match tolerance).

Semiquantification was based on extracted ion chromatograms (EIC). For each species, the most intensive *m*/*z* charge state was used with an *m*/*z* tolerance of ±0.01, and the intensity (I) of the resulting EIC peak was used. The relative amount of the 3-fold oxidized (3ox) species was calculated according to:

$$a_r = \frac{I_{3ox}}{I_{0ox} + I_{3ox}}.$$

3. Results

3.1. Subunit Mass and PTMs of Unstressed Bevacizumab and Infliximab

For both biopharmaceuticals under investigation, i.e., bevacizumab and infliximab, IdeS digestion and reduction resulted in the separation of the antibody subunits as expected. The units were separated by liquid chromatography and detected in the MS as individual signals.

In biosimilar comparison, the amino acid sequence is the most basic criterion. The measured subunit masses matched with those calculated from the amino acid sequence, for the RP as well as for the BS for both mAbs. In case of both mAbs, no differences were observed between the respective RP and the BS, except that in infliximab RP, c-terminal lysine clipping was incomplete, whereas in the BS it was almost complete.

PTM profiles of the unstressed mAbs were analysed, as PTMs represent an important criterion in biosimilarity assessment. Figures 1A and 2A show the total ion chromatograms (TIC) of the 48 h control of bevacizumab BS and infliximab BS, respectively. As expected, both mAbs did not show any PTMs in the LC. The PTM profile of unstressed bevacizumab BS included Fc glycans G0F and G1F as well as small amounts of unglycosylated Fc/2 and Fd' pyroglutamate (Figure 1A). Also for infliximab BS, Fc glycans G0F and G1F and a small amount of Fd' pyroglutamate were found (Figure 2A). Low levels of oxidized Fc/2 species were found in both mAbs. Especially infliximab displayed low levels of oxidized LC and Fd' species, whereas in bevacizumab, these were almost absent. In both mAbs, a noticeable chromatographic peak eluted between the LC and the Fd'. In case of bevacizumab, this peak was identified as the undigested heavy chain. In infliximab this peak contained not only the heavy chain but also several masses that presumably represented fragments. No difference was observed between the different time points of control treatment (without H_2O_2), suggesting that the control treatment did not exert any effect on the sample. In both mAbs, analysis of the unstressed samples showed a high degree of similarity between BS and RP.

3.2. Forced Oxidation

For forced oxidation, samples were treated with H_2O_2 for 24 h, 48 h, and 72 h. Analogously, to the above-mentioned analysis of the control samples, the chromatographic profiles of the BS after forced oxidation closely resembled that of the RP.

In bevacizumab, the most abundant species of Fc/2 after forced oxidation was the 3-fold oxidized (3ox) species, while its LC and Fd' mostly remained unoxidized (Figure 1B). When infliximab was oxidized, the most abundant Fc/2 species was the 2ox species (Figure 2B). Furthermore, its LC had been completely converted with 1ox LC representing the most abundant species. Similar to the findings for

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the LC, the biggest part of infliximab Fd' was found as 1ox. Concerning these main oxidation states, no differences were observed between BS and RP of bevacizumab and infliximab.

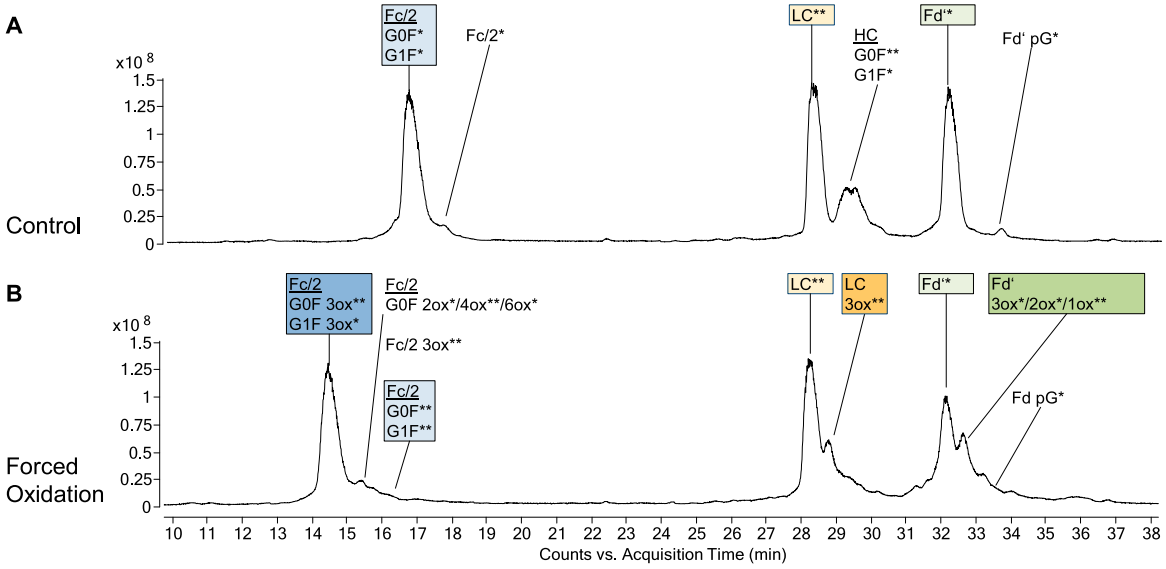

Figure 1. Species identified in biosimilar (BS) of bevacizumab after 48 h of control treatment (**A**) or forced oxidation (**B**). Annotations show the different protein species and their modifications in the chromatographic peaks as identified by their mass. The mass difference between the observed mass and the theoretical value is indicated as follows: 0 to 0.5 Da = *, 0.6 to 1.5 Da = **, 1.6 to 4.0 Da = ***. Ox, oxidations; pG, pyroglutamate modification. Coeluting species within one annotation are listed in order of spectrum intensity.

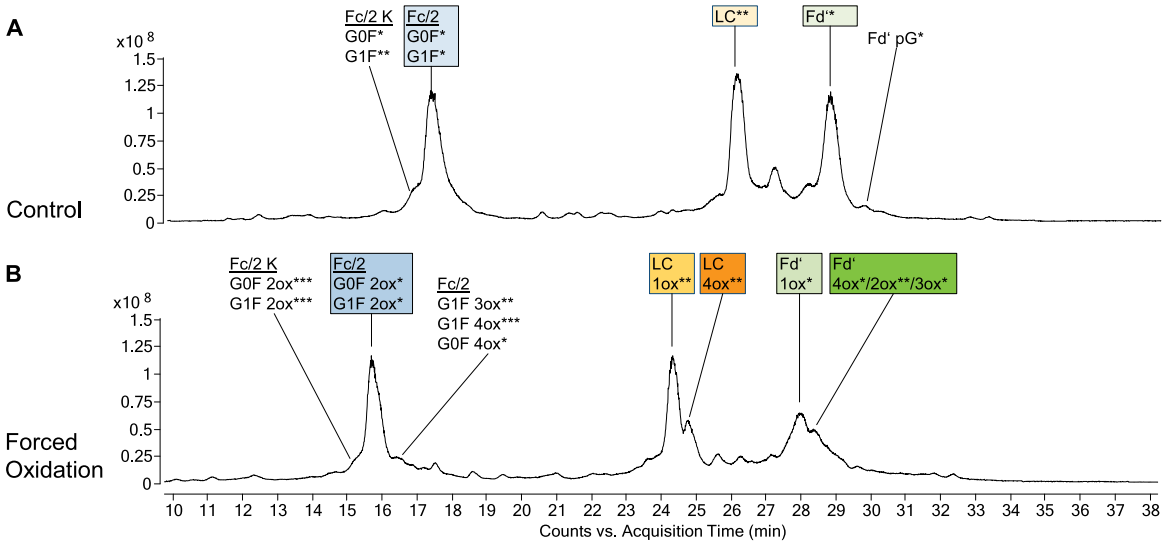

Figure 2. Species identified in biosimilar (BS) of infliximab after 48 h of control treatment (**A**) or forced oxidation (**B**). Annotations show the different protein species and their modifications in the chromatographic peaks as identified by their mass. The mass difference between the observed mass and the theoretical value is indicated as follows: 0 to 0.5 Da = *, 0.6 to 1.5 Da = **, 1.6 to 4.0 Da = ***. K, additional c-terminal lysine; Ox, oxidations; pG, pyroglutamate modification. Coeluting species within one annotation are listed in order of spectrum intensity.

In addition to the main oxidized species described above, additional species with higher oxidation states appeared. In case of the LC and Fd', these higher oxidized species eluted as peaks that were not fully separated from the main oxidized species. In bevacizumab, in addition to the main species of 3ox

Fc/2, unoxidized LC and unoxidized Fd' the next most abundant species were 4ox Fc/2, 3ox LC and 3ox Fd', respectively (Figure 1B). In infliximab, in addition to the main species of 2ox Fc/2, 1ox LC, and 1ox Fd', the next most abundant species were 3ox Fc/2, 4ox LC, and 4ox Fd', respectively (Figure 2B).

Some minor differences were found between BS and RP with regard to the oxidation states. In bevacizumab, small amounts of higher oxidation states of Fc/2 (4ox, 6ox) were only found in the BS. Likewise, in infliximab, only the BS displayed small amounts of higher oxidation states of Fc/2 (3ox, 4ox) and smaller amounts of Fd' intermediate oxidation states (2ox, 3ox) were only found in the BS, whereas these were virtually absent in the RP.

3.3. Time Course of Forced Oxidation for Comparison of BS and RP

While the Fc/2 of bevacizumab was completely converted from the unoxidized to the 3ox species, the bigger part of the LC and Fd' remained unoxidized. Analysis of the TICs of bevacizumab (as shown in Figure 1B) of the 24 h, 48 h, and 72 h forced oxidation experiment indicated an ongoing shift of LC and Fd' species from their main species (0ox) to the higher oxidation states. To elucidate and monitor the progression of the oxidation reaction, EICs were generated for the unoxidized species (LC 0ox and Fd' 0ox) and for the corresponding oxidized species (LC 3ox and Fd' 3ox as the most abundant oxidized species). The relative abundance of 3ox species increased with longer forced degradation treatment, both for the LC and the Fd', in BS as well as in the RP (Figure 3). This analysis of the forced oxidation samples showed that the ongoing conversion of both the bevacizumab LC and Fd' was stronger in the BS than in the RP.

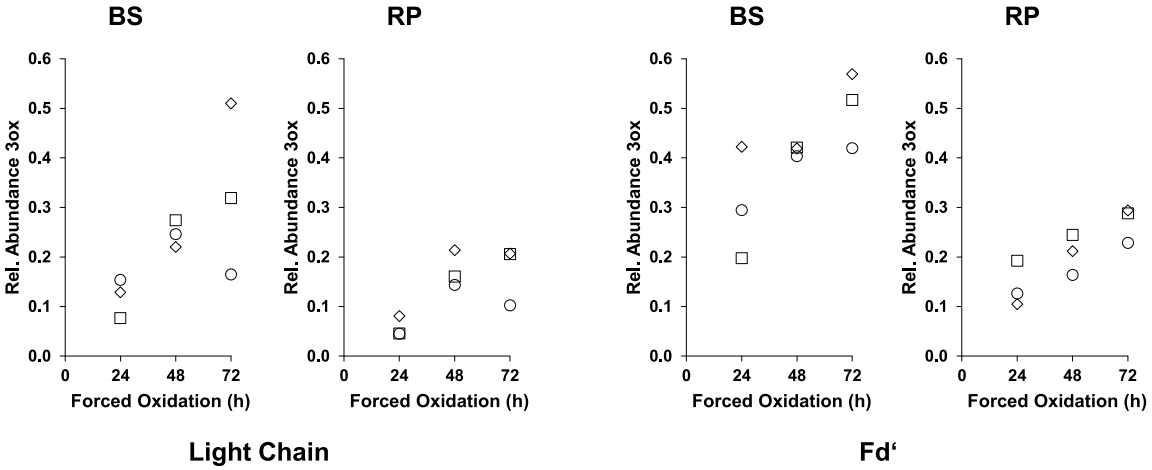

Figure 3. Time course analysis of bevacizumab biosimilar (BS) and reference product (RP) in forced oxidation samples. EICs were generated for light chain (LC) 0ox, LC 3ox, Fd' 0ox and Fd' 3ox and height of the EIC peak was measured. The rel. abundance 3ox was calculated using the equation given in Section 2.5. Square, circle, and diamond each represent one experimental repetition. Ox, oxidations.

4. Discussion

4.1. PTMs in Unstressed Samples

The PTM profiles of BS and RP were compared in unstressed samples of bevacizumab and infliximab (Figures 1A and 2A). All samples were processed on the middle-up level and thus, glycosylated Fc/2, and unmodified LC and Fd were observed as main species. RP and BS showed highly similar PTM profiles in case of both mAbs, with the exception that in infliximab, lysine clipping was found to be different for RP and BS. The presence of c-terminal lysine does not alter the biological activity, but it should be monitored as an indicator for a consistent production process [6,34]. Additional species of low abundance included N-terminal pyroglutamate in both mAbs and unglycosylated Fc/2 in bevacizumab. These results underline the value of the middle-up approach for PTM investigation.

A similar approach was also reported by Zhang et al. in another mAb study [16]. Interestingly, the unstressed infliximab samples displayed slightly higher initial levels of oxidized species than those of bevacizumab, as will be discussed later. Biosimilar comparison also showed high similarity with the RP in terms of the amount of oxidized species.

4.2. Subunit Analysis after Forced Oxidation

The results presented in our study can help in the identification of methionines that are prone to oxidation and which thus may represent potential CQA. The amount of oxidation that was predominantly observed for each subunit (here designated as most abundant species of the subunit) after forced oxidation was compared between bevacizumab and infliximab as both mAbs differ in the composition and position of methionines in the Fc and the Fab subunits (Figure 4).

Fc/2	
M258	M255
M364	-
M434	M431

light chain	
M4	-
-	M55

Fd'	
-	M18
M34	M34
M83	M85

Figure 4. Methionine (M) positions for bevacizumab (left column) and infliximab (right column).

The Fc/2 subunit harbors the two conserved methionines M252 and M428 and can additionally contain M358 (EU numbering [35], methionine positions in bevacizumab and infliximab are slightly different as shown in Figure 4). As M252 and M428 are surface exposed, they display a higher susceptibility towards oxidation [11,26,36]. Our data on bevacizumab oxidation show the most abundant species of Fc/2 as 3ox (Figure 1B), while in infliximab, the most abundant Fc/2 species was the 2ox species (Figure 2B). The methionine residues M252 and M428 are present in both mAbs, whereas bevacizumab additionally contains M358. These results suggest that all methionines of Fc/2 subunit become oxidized. Studies on other mAbs containing the same three Fc/2 methionine positions demonstrated that all three residues become highly oxidized when subjected to H_2O_2 [18,19]. When tBHP was used, M358 was found less oxidized than the two-surface exposed methionines [11,19,23]. Shah et al. compared different oxidative reagents and showed that for M358 H_2O_2 led to stronger oxidation than tBHP [19]. Kim et al. subjected infliximab to 0.1% H_2O_2 at 5 °C for up to 6 h [29]. Utilizing peptide mapping they found that M255 was highly oxidized, whereas M431 was not oxidized. In contrast, our data support the oxidation of M431 as well. Most likely the prolonged incubation time and higher temperature led to higher progression of oxidation in our study.

Concerning the Fab part, bevacizumab and infliximab behaved very differently. In bevacizumab, the LC and the Fd' remained mostly unoxidized (Figure 1B). In contrast to the results of bevacizumab, in infliximab the LC and the Fd' were completely converted to oxidized forms, with 1ox representing the most abundant form (Figure 2B).

Both mAbs contain one methionine in the LC, however at different positions, bevacizumab at M4 and infliximab at M55 (Figure 4). The finding that the bevacizumab LC was mostly unoxidized whereas the infliximab LC became mostly 1-fold oxidized, suggests that M4 is not reagent accessible, whereas M55 can be readily oxidized. Our finding of unoxidized M4 is in accordance with the results of Folzer et

al., in which the only LC methionine, M4, remained mostly unoxidized [18]. Furthermore, our finding of oxidized LC M55 in infliximab is supported by the results of peptide mapping reported by Kim et al., who showed an intermediate level of M55 oxidation in infliximab, using milder conditions for forced oxidation [29].

The two methionine residues of the Fd' subunit, M34 and M83 (M83 in bevacizumab corresponding to M85 in infliximab), are present in both mAbs. Additionally, infliximab contains M18, which is not present in bevacizumab (Figure 4). While the Fd' subunit of bevacizumab remained mostly unoxidized, that of infliximab was completely transformed with 1ox Fd' representing the most abundant oxidation state. This suggests that the 1ox Fd' subunit in infliximab was generated by oxidation of M18. This is supported by literature reports that M34 and M83 undergo only slight oxidation upon forced degradation testing, despite the location of M34 in or adjacent to heavy chain CDR1 [13,18,23,37,38]. Our finding of oxidized M18 in Fd' is also in accordance with the peptide mapping results of Kim et al, who showed that M18 was oxidized, whereas M34 and M85 were not oxidized in infliximab [29].

In summary, the comparison of bevacizumab and infliximab after forced oxidation suggested that in addition to the conserved and oxidation prone Fc/2 methionines of both mAbs, LC M55 and Fd' M18 of infliximab are highly susceptible to oxidation.

Small levels of oxidized species were found in the unstressed control samples of infliximab, but were basically absent in bevacizumab. This is probably due to the oxidation prone methionines found in infliximab LC and Fd', which may undergo slight oxidation during production or storage.

Additionally, lower amounts of higher oxidation states were found for each subunit of the stressed samples, i.e., 4ox Fc/2, 3ox LC and 3ox Fd' in bevacizumab and 3ox Fc/2, 4ox LC and 4ox Fd' in infliximab (Figures 1B and 2B). These may occur on less solvent-exposed and thus slowly oxidizing methionines [13,18,23,37]. Another explanation may be the unspecific oxidation of other amino acids like tryptophanes that has also been observed elsewhere and that might be enhanced due to the harsh conditions used within this study [14,18].

4.3. CQA Considerations

The finding of oxidation susceptible methionines in Fc and Fab indicates that these residues may represent CQA. For a full CQA assessment, structure-function relationships need to be established. However, including evidence from literature can initially help to estimate the criticality of the observed oxidations. A risk assessment based on the evaluation of the impact of oxidation on functional properties of the mAbs is strongly recommended for the establishment of acceptance criteria.

Oxidation of the conserved methionines in Fc has been extensively studied, as they are present in all therapeutic mAbs of the IgG1 class, which represents the majority of the marketed mAbs [23]. The resulting effects include decreased binding to Protein A and Protein G, which is used for mAb purification and can be used in different assays [11,12]. Also, a reduced binding to FcRn as well as to Fc-gamma receptors (FcγR) was demonstrated. Binding to FcRn contributes to the extended mAb circulation half-life while binding to FcγR is responsible for effector functions [11,19,39]. In case of bevacizumab, FcγR mediated effector functions are reported not to play a biological role, whereas for infliximab, antibody-dependent cell-mediated cytotoxicity (ADCC) may contribute in the activity in inflammatory bowel disease. Both for bevacizumab and infliximab, FcγR binding and related functional tests are part of the biosimilarity assessment [34,40]. Together, the known impact of oxidation indicates the role of the conserved Fc methionines as possible CQA.

While bevacizumab did not show strong levels of oxidations in the Fab, infliximab showed strong oxidation in both, the LC and in the Fd' subunit. Our findings suggest that in infliximab M18 of the Fd' and M55 of the LC become oxidized. As M55 is located in the CDR2, the oxidation of this residue may directly impact the target binding [38]. Oxidation of methionines as well as of trypthophanes in the CDRs can result in destabilisation of Fab, increased aggregation as well as decreased target binding [9,19,41]. Pisupati et al. performed a comparability study between infliximab and the biosimilar Remsima [28]. Both the RP and the BS were analysed after subjection to elevated

temperature and humidity stress. The stress resulted in decreased TNF neutralizing ability which could be related to amino acid modifications in the CDRs such as LC M55 and HC M34. Thus, oxidation of infliximab at M55 may represent a CQA. M18 is located outside of the CDRs [38]. Still, the impact of M18 oxidation on properties such as aggregation tendency or target binding should be examined [19]. Thus, the findings of oxidation prone methionines in the Fc of both mAbs and in the Fab of infliximab are important for further investigations, such as on binding to FcRn and FcγR, as well as target binding. Such investigations are mandatory to assess the functional implications of the detected modifications.

4.4. Different Susceptibility of Bevacizumab BS and RP during Forced Oxidation

Regarding the biosimilar comparison, we intended to use harsh oxidation conditions, because milder conditions might not be sufficient to amplify initial differences between the BS and the RP. Our results demonstrate that the use of H_2O_2, which is a strong oxidant specific for methionines, is suitable for this purpose. It has been shown that different oxidative agents lead to different effects. In a study comparing H_2O_2, tBHP and AAPH for oxidation of one mAb, the peroxides strongly and almost exclusively oxidized the three Fc methionines [19]. AAPH additionally led to a smaller extend of Fab CDR oxidation, namely to one methionine and one tryptophan. Even though these residues were oxidized only to approximately 5%, a huge effect of the AAPH oxidation on the mAbs function such as ADCC, target binding, and related target cell proliferation was observed. Therefore, the use of tBHP and AAPH is addressed to future studies to yield a broader picture of oxidative degradation. The time course comparison of the two bevacizumab mAbs under forced oxidation showed an increased relative abundance of higher oxidation states in LC (3ox) and Fd' (3ox) of the BS compared to the RP. Moreover, small amounts of higher oxidation states of Fc/2 (4ox, 6ox) were only observed in the BS. However, one should keep in mind that forced oxidation conditions over-exaggerate differences in the products. Therefore, Pisupati et al. recommended to interpret the results with caution [28]. Nevertheless, the observed results may point to differences in the unstressed products, which could initiate further oxidation. Many factors in the production process are known to contribute to oxidation, including excipients and impurities such as sugars, polysorbates, peroxides, and metals, as well as light [19,20]. The production process of a BS is always different from that of the RP. Different raw materials can be one possible reason for the observed discrepancy in oxidation levels after forced degradation. For example, the formulation of bevacizumab and infliximab contains polysorbate 20 and polysorbate 80, respectively. Polysorbates are a source of hydroperoxides which in turn can lead to oxidations in the mAb [42]. The forced oxidation studies presented here can help to identify and prevent sources of oxidation for example during screening of different excipients.

Here we applied H_2O_2 for forced oxidation to mAbs and performed analysis on the subunit level. The comparison of the different methionine positions between bevacizumab and infliximab not only allowed to localize the oxidations to the subunits, but even indicated which of the methionine positions were affected. Thus, the subunit approach reveals more relevant structural information than the analysis of intact mAbs. Peptide mapping generally provides more specific information but bears a considerable risk for artificial introduction of modifications, and hence requires comprehensive and time-consuming method optimization. The good accordance of the results presented here for infliximab with published peptide mapping results of this mAb underline the usefulness of the subunit analysis as a fast alternative for these types of study.

Author Contributions: The study design was jointly elaborated and discussed by all coauthors. Y.F.K.D. and D.R. conceived, designed, and performed the experiments. Analytical measurements were performed by Y.F.K.D. and J.F.J. Data interpretation was done by Y.F.K.D., J.F.J., W.J., and M.K.P., K.W., V.S., and M.K.P. contributed reagents, materials, and analysis tools, supervised the investigation, analysis, and interpretation of the findings. Manuscript preparation and review was jointly done by all coauthors.

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Conflicts of Interest: Daniel Rehm, Karsten Winkler, and Volker Sandig are employees of ProBioGen AG, Berlin, Germany, a company which develops and manufactures biosimilar products. All other authors declare no conflict of interest and disclose any financial and personal relationship with people or organizations that could inappropriately influence this study.

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3.2 Manuscript 2

Comparison of middle- and bottom-up mass spectrometry in forced degradation studies of bevacizumab and infliximab

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Abstract: Monoclonal antibodies (mAbs) used as therapeutics need comprehensive characterization for appropriate quality assurance. For analysis, cost-effective methods are of high importance, especially when it comes to biosimilar development which is based on extended physicochemical characterization. The use of forced degradation to study the occurrence of modifications for analysis is well established in drug development and may be used for the evaluation of critical quality attributes (CQAs). For mAb analysis different procedures of liquid chromatography hyphenated with mass spectrometry (LC-MS) analyses are commonly applied. In this study the middle-up approach is compared to the more expensive bottom-up analysis in a forced oxidation biosimilar comparability study. Bevacizumab and infliximab as well as biosimilar candidates for the two mAbs were forcefully oxidized by H2O2 for 24, 48 and 72 h. For bottom-up, the reduced and alkylated trypsin or Lys-C digested samples were analysed by LC-MS with quadrupole time-of-flight mass analyser (LC-QTOF-MS) to detect susceptible residues. By middle-up analysis several species of every subunit (Fc/2, light chain and Fd‘) were detected which differed in the number of oxidations. For the most abundant species, results from middle-up were in line with results from bottom-up analysis, confirming the strength of middle-up analysis. However, for less abundant species of some subunits, results differed between the two approaches. In both mAbs, the Fc was extensively oxidized. In infliximab, additional extensive oxidation was found in the Fab. Assignment to specific amino acid residues was finally possible using the results from bottom-up analyses. Interestingly, the C-terminal cysteine of the light chain was partially found triply oxidized in both mAbs. The comparison of susceptibility to oxidation showed high similarity between the RMPs and their biosimilar candidates. It is suggested that the findings of middle-up experiments should be complemented by bottom-up analysis to confirm the assignments of the localization of modifications. Once the consistency of results has been established, middle-up analyses are sufficient in extended forced degradation biosimilar studies.

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Comparison of middle- and bottom-up mass spectrometry in forced degradation studies of bevacizumab and infliximab

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ABSTRACT

Monoclonal antibodies (mAbs) used as therapeutics need comprehensive characterization for appropriate quality assurance. For analysis, cost-effective methods are of high importance, especially when it comes to biosimilar development which is based on extended physicochemical characterization. The use of forced degradation to study the occurrence of modifications for analysis is well established in drug development and may be used for the evaluation of critical quality attributes (CQAs). For mAb analysis different procedures of liquid chromatography hyphenated with mass spectrometry (LC-MS) analyses are commonly applied. In this study the middle-up approach is compared to the more expensive bottom-up analysis in a forced oxidation biosimilar comparability study. Bevacizumab and infliximab as well as biosimilar candidates for the two mAbs were forcefully oxidized by H_2O_2 for 24, 48 and 72 h. For bottom-up, the reduced and alkylated trypsin or Lys-C digested samples were analysed by LC-MS with quadrupole time-of-flight mass analyser (LC-QTOF-MS) to detect susceptible residues. By middle-up analysis several species of every subunit (Fc/2, light chain and Fd') were detected which differed in the number of oxidations. For the most abundant species, results from middle-up were in line with results from bottom-up analysis, confirming the strength of middle-up analysis. However, for less abundant species of some subunits, results differed between the two approaches. In both mAbs, the Fc was extensively oxidized. In infliximab, additional extensive oxidation was found in the Fab. Assignment to specific amino acid residues was finally possible using the results from bottom-up analyses. Interestingly, the C-terminal cysteine of the light chain was partially found triply oxidized in both mAbs. The comparison of susceptibility to oxidation showed high similarity between the reference products and their biosimilar candidates. It is suggested that the findings of middle-up experiments should be complemented by bottom-up analysis to confirm the assignments of the localization of modifications. Once the consistency of results has been established, middle-up analyses are sufficient in extended forced degradation biosimilar studies.

1. Introduction

Monoclonal antibodies (mAbs) are the most important class of therapeutic proteins because of their high specificity and low side effects [1]. However, as biologics, their production is highly expensive and therefore a high burden for health systems, even in the rich countries in which they are available. One strategy to reduce costs is the development of biosimilars [2]. A biosimilar is a copy product of a marketed original biologic, that is produced once the patent of the original biologic expires. The authorization requires demonstration of physiochemical similarity and then allows for abbreviated clinical trials, thereby reducing the costs [3]. Additionally, cost-effective analytical methods are highly desired to reduce the overall development costs.

A mAb is a complex protein with many possible sites where post-translational modifications (PTMs) can be generated. Different modifications can arise during the complete life cycle of the biologics, specifically during biotechnological production, purification and storage. Modifications which lead to a degradation of the mAb and therefore have a negative impact on the safety and efficacy of the drug are classified as critical quality attributes (CQAs).

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Common modifications which have been identified as CQAs in mAbs are related to deamidation, non-enzymatic glycation, disulfide-bond formation and oxidation [4]. In drug development, potential modifications are elucidated in forced degradation studies, in which a monoclonal antibody is subjected to harsh conditions to mimic the drug's shelf-life in relatively short time. Thus, forced degradation studies are performed to support mAb process and product development [5]. They also provide data to qualify the analytical equipment for their detection and are also expected by regulatory agencies [6]. For biosimilars, similar stability compared to the reference product should be demonstrated including data from stress conditions (ICH Q5E) [7]. ICH Q1A prescribes testing the effect of temperature, humidity, oxidation and photolysis as well as low and high pH on the drug substance as part of stability data for registration application for a new drug substance [8].

Oxidation can have a negative impact on the structure [9], the thermal stability [10] and the antigen binding [11]. Moreover, oxidation may impact the binding to protein A and G, relevant for mAb purification during manufacturing, reduce serum half-life of the mAb [12–14] and may lead to aggregation [10]. Sources of oxidation include oxygen from air, dissolved oxygen as well as free radicals which can result from reaction with metals and impurities from raw materials [6]. Amino acid residues most susceptible to oxidation are cysteine, methionine and tryptophan residues (listed in decreasing likelihood) [15–17].

The most widely used agent for forced oxidation of proteins is H_2O_2 [9] oxidizing mainly methionine residues [16,18]. Recently, site-specific oxidation during H_2O_2 stress was achieved utilizing a ligand bound to the mAb [19]. Besides H_2O_2, t-butylhydroperoxid (tBHP) is used for oxidation. Compared with H_2O_2, tBHP is bulkier and oxidizes mainly surface exposed residues of mAbs. Alternatively, 2,2'-Azobis (2-amidinopropane) dihydrochloride (AAPH) may be used to oxidize both methionine and tryptophan residues [9].

As forced degradation studies generate large numbers of samples, fast analytical methods and approaches are desired to generate the required information in a cost-effective way. The available analytical toolbox for characterization of therapeutic mAbs contains many physicochemical, biochemical, and biological methods [20,21] which differ in resolution, selectivity, and sensitivity. As methods based on liquid chromatography-mass spectrometry (LC-MS) provide high values for these properties, they are well established and widely used in the analysis of therapeutic mAbs [22].

LC-MS and tandem mass spectrometry (MS/MS) methods for characterization of mAbs can be divided into three different levels as described by Zhang et al. [21]: intact or top level, middle and bottom level. The top-level approaches do not require elaborate sample preparation procedures because the intact mAb is measured. However, intact mAb analysis provides the least structural details. On the contrary, bottom-up analysis provides the highest structural resolution and pinpoints modifications to the amino acid position. However, sample preparation of the mAb is more elaborate and comprises denaturation, reduction and alkylation of disulfide bonds prior to enzymatic digestion into peptides. These procedures are relatively time and therefore cost intensive and the many steps also bear the risk of artificially introducing modifications [23]. As an option located between the top- and the bottom-approach, the middle-up approach employs limited proteolysis and subsequent reduction of the disulfide bonds to liberate the light chain, Fd' and Fc/2 subunits. Thus, sample generation is relatively fast and therefore less prone to artificial modifications. Structural resolution is lower than in peptide mapping, but mass changes indicate the type of modification and its location to one of the three subunits. Structural information can also be obtained from the middle-level by middle-down characterisation of the antibody, however the corresponding methods are currently still under development [24].

In this study, middle-up and bottom-up analysis were compared in depth focusing on forced oxidation samples of bevacizumab and infliximab as well as their biosimilar candidates. The results of the middle-up investigation have been reported previously [25]. For bottom-up, trypsin digestion was complemented by a parallel digestion with Lys-C to achieve higher sequence coverage. Sample preparation was performed closely following a peptide mapping protocol recently published by Mouchahoir and Schiel [23]. This protocol is designed to balance maximum digestion efficiency with minimum artificial modifications. H_2O_2 was used as oxidizing agent, as it is well established and most commonly applied in forced oxidation degradation studies.

We address the questions to which extent the less expensive and faster middle-up experiments by LC-MS are consistent with the more elaborate LC-MS/MS experiments in forced degradation studies and to which extent the middle-up experiment may substitute the bottom-up analysis without the loss of required information for this application.

2. Materials and methods

2.1. Materials

Sucrose (= D(+)-saccharose), hydrogen peroxide (H_2O_2) solution (30% (w/w) in H_2O), tris(hydroxymethyl)aminomethane (tris), tris (hydroxymethyl)aminomethane HCl (tris-HCl), ethylenediaminetetraacetic acid (EDTA), guanidine HCl, iodoacetamide (IAM), urea, tris(2-carboxyethyl)phosphine (TCEP, 0.5 M, pH 7.0 aqueous solution) and 2-(N-morpholino)ethanesulfonic acid monohydrate (MES monohydrate) were purchased from Sigma Aldrich (Taufkirchen, Germany). $NaH_2PO_4 \bullet H_2O$, polysorbate 20 and polysorbate 80 were purchased from Carl Roth GmbH (Karlsruhe, Germany). α,α-Trehalose dihydrate, dithiothreitol (DTT), water with 0.1% formic acid (v/v), Lys-C and Zeba Spin 7k MWCO desalting columns were purchased from Thermo Fisher Scientific (Hennigsdorf, Germany). KH_2PO_4 was purchased from Merck (Darmstadt, Germany), $Na_2HPO_4 \bullet 2\ H_2O$ was from Bernd Kraft GmbH (Duisburg, Germany) and sodium azide (NaN_3) was from ABCR (Karlsruhe, Germany). All purchased chemicals were of highest purity. Ultrapure water was obtained using LaboStar 2-DI/UV system (SGWasseraufbereitung und Regeneration GmbH, Barsbüttel, Germany) equipped with LC-Pak Polisher and a 0.22 μm membrane point-of-use cartridge (Millipak). Mass calibration reference compounds (Purine, HP-0921) were purchased from Agilent Technologies GmbH (Waldbronn, Germany). Vivaspin 500 10k ultrafiltration spin columns were purchased from Sartorius (Göttingen, Germany). Trypsin (recombinant from *Pichia pastoris*) and IdeS were purchased from Roche (Basel, Switzerland) and from Genovis (Lund, Sweden), respectively.

The reference product (RP) for bevacizumab was Avastin (lot B8502H09) from Roche (Basel, Switzerland). The RP for infliximab was Remicade (lot 9RMKA60302) from Janssen Biologics B.V. (Leiden, The Netherlands). The biosimilar (BS) candidate antibodies were produced at ProBioGen AG (Berlin, Germany).

The formulation buffer of bevacizumab was prepared by weighing 60 g α,α-trehalose dihydrate, 5.8 g sodium phosphate (monobasic, monohydrate), 1.2 g sodium phosphate (dibasic, anhydrous) and 0.4 g polysorbate 20. The buffer components were dissolved in water and diluted to a final volume of 1.0 L. The formulation buffer of infliximab was prepared by weighing 50 g sucrose, 50 mg polysorbate 80, 220 mg monobasic sodium phosphate monohydrate and 610 mg dibasic sodium phosphate dihydrate. The buffer components were dissolved in water and diluted to a final volume of 1.0 L. Phosphate buffer of pH 6.5 (Sørensen's phosphate buffer) was prepared by mixing 6.87 volumes of a 66.71 mM KH_2PO_4 stock solution with 3.13 volumes of a 66.72 mM $Na_2HPO_4 \bullet 2\ H_2O$ stock solution. MES buffer solution of pH 6.5 was 10 mM. Following the procedure described by Mouchahoir and Schiel [23], the denaturing buffer (6 mol/L guanidine HCl, 1 mmol/L EDTA in 0.1 mol/L tris, pH 7.8) and digestion buffer (1 mol/L urea in 0.1 mol/L tris, pH 7.8) were prepared with 0.1 mol/L tris buffer and adjusted to pH 7.8. All buffers for forced oxidation and peptide mapping were prepared freshly for each replication, which comprises antibody incubation between 0 and 72 h. Also proteolytic enzymes were reconstituted and frozen as aliquots newly for each replication.

2.2. Forced oxidation of mAb samples

To investigate partially oxidized species of mAbs as model for the comparison of middle-up and bottom-up mass spectrometry, the mAb originators and biosimilars were subjected to oxidizing conditions.

As described before [25], forced oxidation was achieved by incubating mAbs at a final concentration of 0.6 mg/mL antibody, 0.05% (w/v) H_2O_2, 0.1% (w/v) NaN_3 with ¼ volume of the final volume being phosphate buffer and the remaining volume filled with water. As the biosimilars were less concentrated (bevacizumab: 4.2 mg/mL and infliximab: 6.0 mg/mL) than the reference products, the reference product mAbs were diluted with the respective formulation buffer to the same concentration of the respective biosimilar prior to the oxidation procedure. Controls were prepared using water instead of H_2O_2 to control for modifications possibly resulting from other effects than the H_2O_2 treatment. For each sample, 211 µg of mAb were used. As the subsequent buffer exchange steps reduce the amount of protein, sufficient amounts of material were incubated to ensure the availability of 54 µg of protein for the digestions with trypsin and Lys-C. After incubation at 37 °C for 0, 24, 48 or 72 h, the mAb samples were buffer exchanged four times each with 400 µL of denaturing buffer in a Vivaspin ultrafiltration spin column. The ultrafiltration spin columns were selected with a 10 kDa molecular weight cut off to retain possibly detached light chains.

Protein concentration was measured by UV-Vis spectrophotometry (Nanodrop, Thermo Fisher Scientific, Hennigsdorf, Germany) using a molar absorption coefficient and molecular weight calculated with the protparam tool based on the amino acid sequence of the respective mAb [26]. The denaturing buffer was used as blank for the spectrophotometer measurements.

2.3. Sample preparation for bottom-up experiments

To avoid artificial oxidation the peptide mapping protocol of Mouchahoir and Schiel was used, which provides a detailed description of the workflow [23]. Therefore, the buffer exchanged mAb was diluted to 1 mg/mL with denaturing buffer. Reduction was performed with DTT (final concentration 5 mmol/L) for 60 min at 4 °C. Subsequently, alkylation was performed by the addition of IAM (final concentration 10 mmol/L) and incubation for 60 min at 4 °C protected from light [23]. A buffer exchange followed to provide conditions that keep the mAb in solution but allow the protease to cleave. Aliquots of 130 µL of the reduced and alkylated mAb were buffer exchanged with freshly prepared digestion buffer using a Zebaspin 7 K MWCO desalting column according to the manufacturer's instructions. Concentration of the mAb in digestion buffer was determined by Nanodrop. As blank for the spectrophotometer measurements, the digestion buffer was used. The mAb in digestion buffer was frozen at -20 °C over night. Subsequently, the mAb was thawed and 26 µg were digested with trypsin and another 26 µg of mAb were digested with Lys-C at final concentrations of 0.5 µg mAb/µL in digestion buffer for 4 h at ambient temperature. We used an enzyme to protein ratio of 1:20 (w/w). Digestion was stopped by adding 0.1% (v/v) formic acid in water at a 1:1 volume ratio. mAb samples (104 µL) were then aliquoted and stored at -80 °C until analysis.

2.4. Sample preparation for middle-up experiments

For middle-up analysis the buffer exchanged samples were diluted to 1 µg/µL in MES buffer and digested with IdeS enzyme within 30 min at 37 °C. Subsequently, 500 µL of guanidine-HCl (8 M solution) and 66 µL of TCEP solution (500 mM) were added followed by incubation at ambient temperature for 1 h to complete reduction of the disulfide bonds. Details are reported in Dyck et al. [25].

2.5. Liquid chromatography and mass spectrometry

Analysis was performed on a 1290 Infinity II UHPLC system hyphenated to a 6550 iFunnel Q-TOF mass spectrometer with an electrospray ionization source. Internal mass calibration was achieved by continuously spraying of internal reference compounds through the reference nebulizer.

Data acquisition was controlled with the MassHunter software (Agilent, Santa Clara, CA, USA). For bottom-up analysis, 2.5 µg of the digested mAbs were injected on an AdvanceBio Peptide Mapping column, 2.1 × 150 mm, 2.7 µm (Agilent Technologies GmbH, Waldbronn, Germany) heated to 60 °C. Mobile phase A was 0.1% (v/v) formic acid in water, and mobile phase B was 0.1% (v/v) formic acid in acetonitrile. The liquid chromatography was operated at a flow rate of 0.4 mL/min starting at 2% B with the eluent flow to the mass spectrometer bypassed for 1 min. Afterwards, a linear gradient of 2–10% B in 15 min and then a steeper gradient of 10–40% B in 30 min was applied. Subsequently, the solvent B content was increased to 97% in 9 min and kept at this level for 5 min. When a standard gradient terminating only in an isocratic cleaning step of 97% B was used, a markable carryover was observed. Therefore, a saw tooth gradient was employed to solve this problem, as follows: The solvent B content was decreased to 2% in 2 min and increased back to 97% in 2 min. This down and up was repeated another two times before decreasing the solvent B content to 2% in 2 min for a 5 min re-equilibration step at this level. Each sample was measured one time.

Acquisition was performed in data-dependent analysis mode with an m/z range of 100–1700 and a scan rate of 8 spectra/s in MS and an m/z range of 50–1700 and a scan rate of 3 spectra/s in MS/MS. The MS/MS isolation width was set to $m/z = 1.3$. The collision energy was adjusted according to the formula 3.6 x (m/z) / 100 – 4.8 for all ions independent of their charge state. Up to 10 precursors were selected per cycle with an absolute threshold of 2000 counts and a relative threshold of 0.001%. The option "precursor abundance based scan speed" was turned on. A target of 25000 counts/spectrum was set and the option "use MS/MS accumulation time limit" was turned on. The option "use dynamic precursor rejection" was set to No. Following instrument parameters were applied: nebulizer: 1.7 bar; drying gas flow: 11 L/min; drying gas temperature: 250 °C; sheath gas flow: 12 L/min; sheath gas temperature: 250 °C; fragmentor voltage: 170 V.

In case of middle-up analysis 0.75 µg of digested mAb were separated using an Acquity UPLC Protein BEH C4 Column (300 Å, 1.7 µm, 2.1 mm, 100 mm, Waters, Manchester, UK) at 80 °C applying a mobile phase flow of 0.5 mL/min using the same mobile phase A and B as for the bottom-up approach. Gradient elution was performed starting at 20% B (1 min hold with bypassing the MS), followed by a linear gradient of 20–32% B in 40 min, and a subsequent purge step at 95% B. Finally, re-equilibration was achieved in 5 min at 20% B. Mass spectrometric acquisition was performed at a mass range of m/z 600 to m/z 3200 applying the following parameters: nebulizer: 2.8 bar; drying gas flow: 14 L/min; drying gas temperature: 290 °C; sheath gas flow: 12 L/min; sheath gas temperature: 375 °C; fragmentor voltage: 400 V. Spectra were acquired at 2 spectra/s.

2.6. Data analysis

The acquired bottom-up data were analysed with Bioconfirm (Agilent Technologies Inc, Santa Clara, CA, USA) for peptide identification. The search criteria, all set as variable, comprised cysteine carbamidomethylation (+57.021464 Da), cysteine oxidation (+15.994915 Da), methionine oxidation (+15.994915 Da), tryptophan oxidation (+15.994915 Da) as well as multiples of these and loss of lysine at the C-terminus of the protein (−128.094963 Da).

Figures of extracted ion chromatograms (EICs) were created with Agilent MassHunter Qualitative Analysis. The peak areas of EICs were analysed in Agilent MassHunter Quantitative Analysis for relative quantification. EICs were generated for the unoxidized and for the

oxidized form of a peptide at the retention time at which the respective form had been identified by MS/MS in at least one sample. The *m/z* of the monoisotopic peak of the most intense charge state was used with a search window of ± 50 ppm or as indicated in the figures. In case of low signal intensity, *m/z* values of additional isotope peaks were included for the EIC. To mitigate variations in peak area, the peak areas of each peptide form were divided by the peak area of the ALPAPIEK peptide to calculate the relative peak area. ALPAPIEK is an intense peptide present both in the trypsin and Lys-C digests of bevacizumab and of infliximab. This is relevant for the presentation of peptides of which only the unoxidized form was identified. For peptides, of which both forms were identified, the relative level of oxidation was calculated as:

$$\%\ oxidation = \frac{rel.peak\ area_{oxidized\ peptide}}{rel.peak\ \ area_{unoxidized\ peptide} + rel.peak\ area_{oxidized\ peptide}} \times 100\ \% \quad (1)$$

According to the % oxidation findings, residues were termed "weakly oxidized" (≤1%), "partially oxidized" (5–30%) and "completely oxidized" (100%).

A

bevacizumab light chain

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L1-18               L19-42                                          L62-103
DIQMTQSPSS LSASVGDRVT ITCSASQDIS NYLNWYQQKP GKAPKVLIYF TSSLHSGVPS RFSGSGSGTD FTLTISSLQP   80
                                                                    L146-149
EDFATYYCQQ YSTVPWTFGQ GTKVEIKRTV AAPSVFIFPP SDEQLKSGTA SVVCLLNNFY PREAKVQWKV DNALQSGNSQ  160
                                                  L208-214(Lys-C)
ESVTEQDSKD STYSLSSTLT LSKADYEKHK VYACEVTHQG LSSPVTKSFN RGEC                              214
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bevacizumab heavy chain

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                     H20-38                      H44-65                              H77
EVQLVESGGG LVQPGGSLRL SCAASGYTFT NYGMNWVRQA PGKGLEWVGW INTYTGEPTY AADFKRRFTF SLDTSKSTAY   80
-87                  H99-127                                                     H154
LQMNSLRAED TAVYYCAKYP HYYGSSHWYF DVWGQGTLVT VSSASTKGPS VFPLAPSSKS TSGGTAALGC LVKDYFPEPV  160
-216
TVSWNSGALT SGVHTFPAVL QSSGLYSLSS VVTVPSSSLG TQTYICNVNH KPSNTKVDKK VEPKSCDKTH TCPPCPAPEL  240
               H255-261                    H281-294                    H308-323
LGGPSVFLFP PKPKDTLMIS RTPEVTCVVV DVSHEDPEVK FNWYVDGVEV HNAKTKPREE QYNSTYRVVS VLTVLHQDWL  320
                           H347-366(Lys-C)             H377-398
NGKEYKCKVS NKALPAPIEK TISKAKGQPR EPQVYTLPPS REEMTKNQVS LTCLVKGFYP SDIAVEWESN GQPENNYKTT  400
                         H423-445
PPVLDSDGSF FLYSKLTVDK SRWQQGNVFS CSVMHEALHN HYTQKSLSLS PGK                              453
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B

infliximab light chain

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                           L25-39                    L50-61        L62-107
DILLTQSPAI LSVSPGERVS FSCRASQFVG SSIHWYQQRT NGSPRLLIKY ASESMSGIPS RFSGSGSGTD FTLSINTVES   80
                                                                  L146-149
EDIADYYCQQ SHSWPFTFGS GTNLEVKRTV AAPSVFIFPP SDEQLKSGTA SVVCLLNNFY PREAKVQWKV DNALQSGNSQ  160
                                                  L208-214(Lys-C)
ESVTEQDSKD STYSLSSTLT LSKADYEKHK VYACEVTHQG LSSPVTKSFN RGEC                              214
```

infliximab heavy chain

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   H4-19            H20-38                      H44-52                              H79
EVKLEESGGG LVQPGGSMKL SCVASGFIFS NHWMNWVRQS PEKGLEWVAE IRSKSINSAT HYAESVKGRF TISRDDSKSA   80
-89                 H101-124                                                    H151-213
VYLQMTDLRT EDTGVYYCSR NYYGSTYDYW GQGTTLTVSS ASTKGPSVFP LAPSSKSTSG GTAALGCLVK DYFPEPVTVS  160

WNSGALTSGV HTFPAVLQSS GLYSLSSVVT VPSSSLGTQT YICNVNHKPS NTKVDKKVEP KSCDKTHTCP PCPAPELLGG  240
            H252-258                    H278-291                    H305-320
PSVFLFPPKP KDTLMISRTP EVTCVVVDVS HEDPEVKFNW YVDGVEVHNA KTKPREEQYN STYRVVSVLT VLHQDWLNGK  320
                                                  H374-395
EYKCKVSNKA LPAPIEKTIS KAKGQPREPQ VYTLPPSRDE LTKNQVSLTC LVKGFYPSDI AVEWESNGQP ENNYKTTPPV  400
                    H420-442
LDSDGSFFLY SKLTVDKSRW QQGNVFSCSV MHEALHNHYT QKSLSLSPGK                                  450
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Fig. 1. Amino acid sequences of bevacizumab (A) and infliximab (B). Highlighted in bold are the residues under investigation for oxidation (cysteine, C, in black, methionine, M, in blue and tryptophan, W, in green) and the cleavage sites of trypsin (after lysine, K, in red and arginine, R, in violet). The peptides of interest (shown in Fig. 3 and Fig. S 1) are underlined. Crossed out "K" represents lysine followed by proline, which results in a non-cleavage site for trypsin.

3. Results

3.1. Identification of oxidized amino acids

Bevacizumab and infliximab were incubated with H_2O_2 and analysed utilizing the bottom-up level to identify amino acid residues prone to oxidation. As primarily susceptible residues cysteine (Cys, C), methionine (Met, M) and tryptophan (Trp, W) were specifically targeted during data evaluation. Samples were digested with trypsin resulting in a sequence coverage of 88% on average. If combined with the information from additional Lys-C digestion, an average sequence coverage of 95% was achieved. The oxidizable amino acids as well as the cleavage sites of the two digestive enzymes are illustrated for the sequences of the two antibodies in Fig. 1. In addition to the extended sequence coverage, Lys-C digestion also allowed to detect one important oxidation site, which was not achieved after trypsin digestion, as further explained below.

3.2. Oxidation of cysteine residues

Interestingly, two cysteine residues appeared to be triply oxidized in both mAbs. One was located in the light chain peptide SFNRGEC, while the other one was located in the Fd' part of the heavy chain in the hinge region (THTCPPCPAPELLGGPSVFLFPPKPK). While all other cysteine residues were found completely converted to the carbamidomethylated form, indicating successful alkylation during sample preparation, the above-mentioned two cysteine residues appeared partially as the carbamidomethylated form and partially as the triply oxidized form. For C214 of the light chain peptide SFNRGEC, carbamidomethylation as well as oxidation was confirmed by MS/MS (Fig. 2A). The % oxidation was based on the extracted ion chromatograms (EICs) (Fig. 2B) and was calculated according to Eq. (1) reported in Section 2.6 (data analysis) with the triply oxidized form representing the oxidized peptide and the carbamidomethylated form representing the unoxidized peptide. It was found to be partially oxidized (Fig. 3 and Fig. S 1). The heavy chain

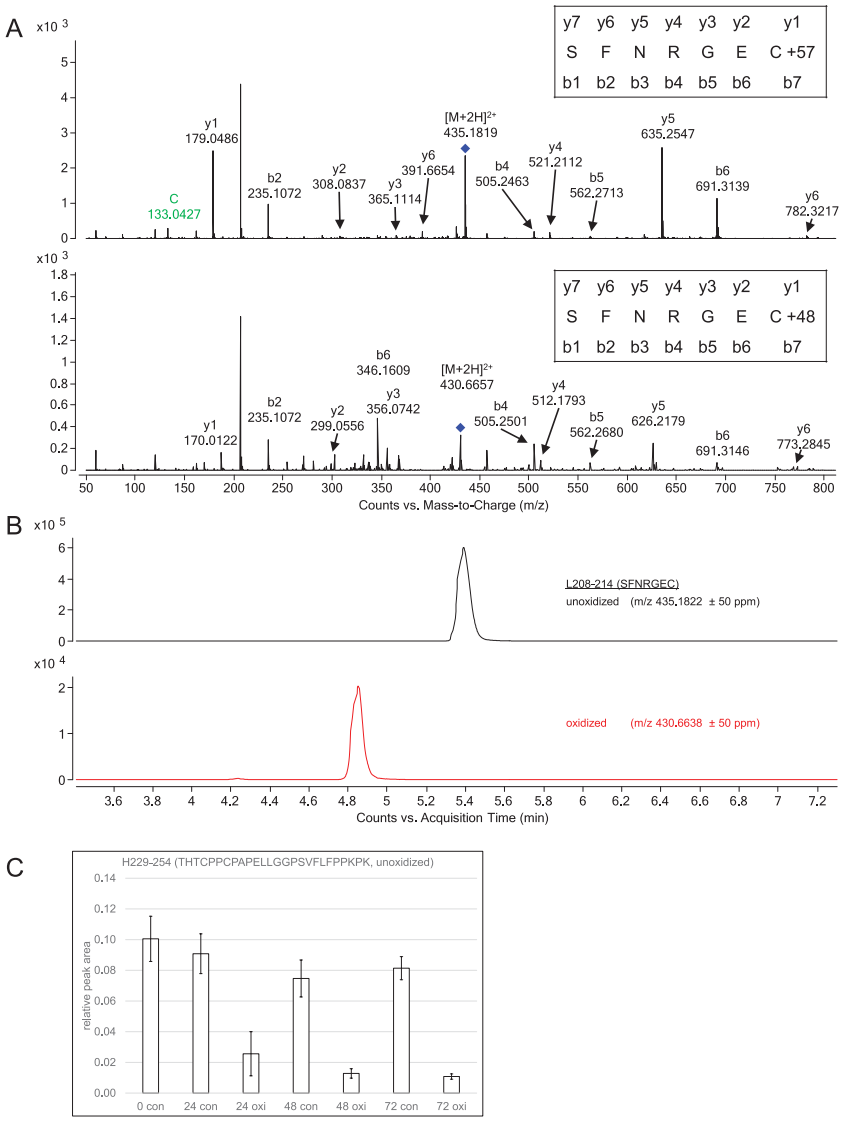

Fig. 2. Oxidation of cysteine residues resulting from forced oxidation. For the light chain peptide L208–214 (SFNRGEC) containing C214 MS/MS spectra of the alkylated (top) and triply oxidized (bottom) form (A) and extracted ion chromatograms of the respective precursor ion (B) are shown. This peptide is found both in bevacizumab and in infliximab. For the heavy chain hinge region peptide THTCPPCPAPELLGGPSVFLFPPKPK (H229–254 in bevacizumab and H226–251 in infliximab), only the unoxidized form could be unambiguously identified by MS/MS. The unoxidized form decreases after H_2O_2 treatment (C). Samples were incubated with H_2O_2 (oxi) or H_2O (con) for 0–72 h. Bars represent mean ± standard deviation of three replications. The data shown are from bevacizumab treated with H_2O_2 for 72 h. Note the different scale of the axis of the ordinate in B.

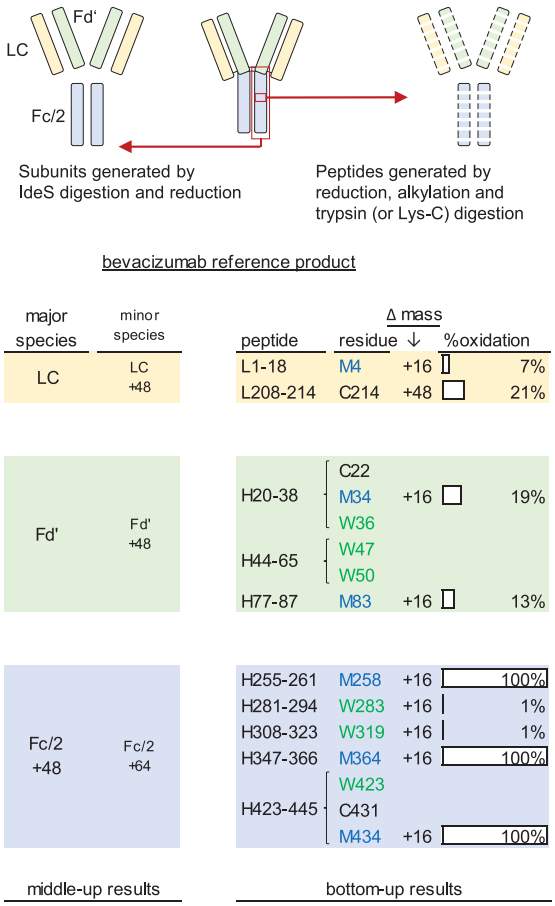

Fig. 3. Comparison of the results of forced oxidation analysis between the middle-up (left) and the bottom-up (right) approach for the bevacizumab reference product. The coloured cartoon illustrates that in middle-up analysis large subunits (LC, Fd', Fc/2) are generated, whereas in bottom-up analysis small peptides are generated. On the left side, previous middle-up results show the major (most abundant) and minor (second most abundant) species with the observed mass changes in Da indicated by a "+ ". On the right side, bottom-up results after 72 h forced oxidation show those cysteine (C), methionine (M) and tryptophan (W) residues which are oxidized ("residue") and their location in the peptide analysed ("peptide"), as well as the mass changes in Da indicated by a + ("Δ mass"). Non-oxidized C, M and W which are located in the same peptide are also shown. Vertical bars represent the % oxidation of peptides (mean of three replications) with the unoxidized and oxidized form identified by MS/MS. Bevacizumab H44–65 is shown for comparison with infliximab H44–52.

hinge region peptide THTCPPCPAPELLGGPSVFLFPPKPK contains two cysteine residues, C232 and C235 in bevacizumab and C229 and C232 in infliximab. The MS/MS spectra of the triply oxidized form were not unambiguous and therefore this oxidation is not finally confirmed. Moreover, the final assignment is complicated as these two cysteine residues are located adjacently to several proline residues. However, the unoxidized form of the peptide decreased after H_2O_2 treatment, indicating that it might indeed become oxidized (Fig. 2C).

3.3. Bevacizumab

In bevacizumab, nine oxidation sites were identified with varying degrees of oxidation (Fig. 3). Identification of the unoxidized and the oxidized form of a peptide by MS/MS spectra in at least one of the samples was the requirement to be included in the % oxidation calculation. In the light chain, M4 and C214 were partially oxidized. In the heavy chain, M34 and M83 were partially oxidized and M258, M364 and M434 were completely oxidized already after 24 h after incubation with H_2O_2. As described above, the triply oxidized form of the hinge region cysteine was not unambiguously confirmed. Additionally, W283 and W319 were weakly oxidized. For the majority of tryptophan residues, an oxidized form was not identified by MS/MS (Fig. 1 and Fig. 3 and Fig. S 1).

The tryptic peptide containing unoxidized M364 (H362–366, EEMTK) of bevacizumab eluted very early and the oxidized form of the peptide could not be confirmed by MS/MS. However, MS spectra with the expected isotopic pattern indicated the presence of the oxidized EEMTK peptide at the very beginning of the eluent infusion into the mass spectrometer. As the hydrophobicity is reduced in oxidized peptides, they usually elute earlier in reversed phase chromatography. Thus, it seems that the oxidized EEMTK peptide even eluted right before the switch of the eluent flow from waste to the mass spectrometer, with the tail of the peak still detected by MS (Fig. 4, top). Therefore, the forced oxidation samples were additionally digested with Lys-C (Fig. 4, bottom). The digest obtained from Lys-C incubation yielded the longer H347–366 (GQPREPQVYTLPPSREEMTK) which was used for relative quantification of M364 oxidation.

While the oxidized form of many tryptophan residues was not identified by MS/MS, H44–65 (containing W47 and W50) as well as H99–127 (containing W108 and W113) showed a decrease of their unoxidized form during forced oxidation (Fig. 5A and B). This suggests that these peptides might indeed become oxidized, but oxidized forms remained undetected.

3.4. Infliximab

Ten oxidation sites were identified in infliximab, including the cysteine residue C214 of the light chain as described above. The triply

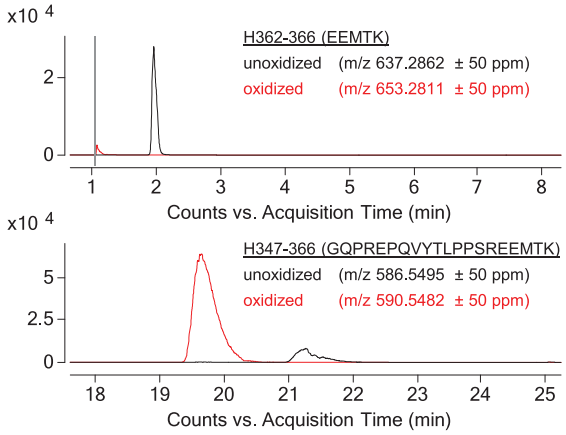

Fig. 4. Extracted ion chromatograms of peptides containing potential oxidation site M364 in the heavy chain of bevacizumab. A separate Lys-C digest was performed in addition to trypsin, as the oxidized form of the tryptic peptide containing M364 eluted too early to be properly detected. Extracted ion chromatograms show the elution of the trypsin generated peptide H362–366 (EEMTK) and the Lys-C generated peptide H347–366 (GQPREPQVYTLPPSREEMTK) of the same 24 h H_2O_2 treated sample. The 24 h time point is chosen here, because later the unoxidized form is mostly not present anymore. Extracted ion chromatograms of the unoxidized (black) and the oxidized form (red) are overlaid. Among the species shown in this figure, the supposed oxidized EEMTK (top, red trace) was the only one that was not confirmed by MS/MS data. The vertical grey line in the upper chromatogram indicates when the eluent is directed into the mass spectrometer.

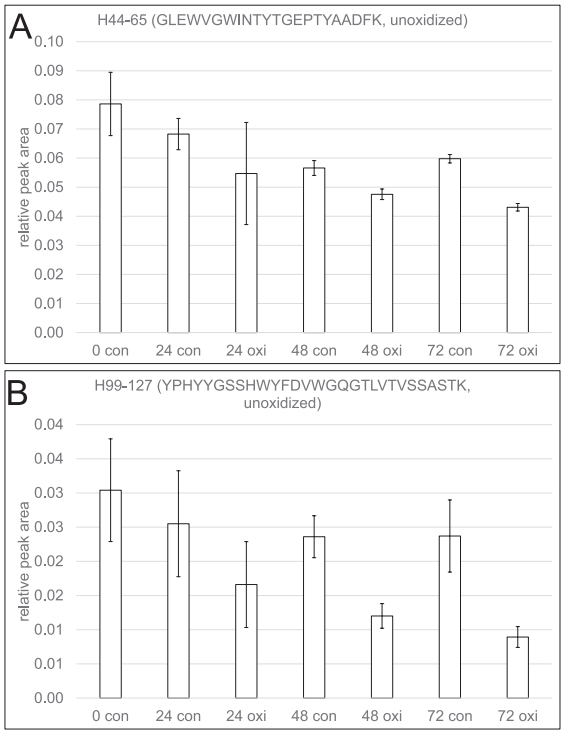

Fig. 5. Relative peak areas of unoxidized forms of peptides H44–65 (A) and H99–127 (B) from bevacizumab after different incubation times. The unoxidized forms decrease after H_2O_2 treatment. Samples were incubated with H_2O_2 (oxi) or H_2O (con) for 0–72 h. In these peptides the % oxidation could not be determined because the oxidized form was not identified by MS/MS. Bars represent mean ± standard deviation of three replications.

oxidized cysteine of the hinge region again lacked unambiguous identification. The methionine and tryptophan residues in infliximab are partially located in similar positions compared to bevacizumab, while some are in different locations. The light chain contains a methionine in a different position compared to bevacizumab. Opposed to M4 of the light chain of bevacizumab, the M55 of infliximab light chain was completely oxidized (Fig. S 1). In the heavy chain, M18 of infliximab, which is not present in bevacizumab, was completely oxidized, as well as M255 and M431, which are in analogous positions to those of bevacizumab (M258, M434). Partial oxidation occurred in M34 and in M85, positions similar to those in bevacizumab. Likewise, the tryptophan residues W280 and W316 were only weakly oxidized as in bevacizumab. Additionally, W47 was weakly oxidized in infliximab but not in bevacizumab.

The MS/MS spectra used for identification of unoxidized and oxidized W47 of the infliximab peptide H44–52 (GLEWVAIER) are shown in Fig. 6, together with the extracted ion chromatograms (EICs) used for relative quantification of oxidation. The presence of several peaks in the EICs of the oxidized peptide has been observed before and may be explained by oxidation of the tryptophan indole ring at multiple positions [27]. As described before, corresponding tryptophan residues in bevacizumab (W47 or W50) were not found oxidized (Fig. 3 and Fig. S 1). The direct comparison between the two mAbs shows one possible explanation, as the unoxidized bevacizumab peptide H44–65 (GLEWVGWINTYTGEPTYAADFK) was detected at lower abundance than the unoxidized infliximab peptide H44–52 (GLEWVAIER) (Fig. 6C). The oxidized form of the infliximab peptide H44–52 was detected with only 2044 counts, which is just above the threshold of 2000 counts to trigger MS/MS fragmentation. Supposed that the bevacizumab peptide shows equally weak oxidation levels, the oxidized form might not be abundant enough to fulfil the requirements of 2000 counts.

3.5. Time course of oxidation

Under the forced oxidation treatment applied here, most of the oxidation occurred already after 24 h. In both mAbs, oxidation usually continued to increase slightly after 24 h for residues, which were not already completely oxidized at that time, as illustrated in Fig. 7 for the methionine residues of bevacizumab. It was also the case for the weakly oxidized tryptophan residues W283 and W319 in bevacizumab and W280 and W316 in infliximab, as well as for C214 in both mAbs. Similar findings were also observed using the middle-up approach [25]. However, applying middle-up methodology no precise location of the oxidation sites was possible. Moreover, the differential kinetics of oxidation, as observed for example for M34 (in peptide H20–38) and for M83 (in peptide H77–87), which are both located in the Fd' of bevacizumab (Fig. 7), can only be observed by bottom-up, whereas middle-up can only display the sum of these individual kinetics. In the control samples, oxidation levels did not increase over time. However, low levels of oxidation were also detected in the controls (Fig. 7).

3.6. Biosimilar assessment

In addition to the reference products, the susceptibility of biosimilar candidates of bevacizumab and infliximab was tested by forced oxidation. The levels of oxidation after 72 h of H_2O_2 treatment were highly similar between the reference products and the biosimilar candidates (Fig. 3 and Fig. S 1). Also, when followed over time, the degree of oxidation of each oxidation site was highly similar between the reference product and the biosimilar candidates as displayed in Fig. 8 for M85 of infliximab as example.

4. Discussion

The forced oxidation conditions as well as the subsequent bottom-up analysis were reproducible as shown by the very similar % oxidation results between the replicates of the reference products and the biosimilar candidates, illustrated in Fig. 3 and Fig. S 1 as well as in Fig. 8. Control samples were used to exclude significant generation of artefacts. Oxidation was already detected in the control samples for the Fc subunit with both analytical approaches which is consistent to previously described results e.g. for the NISTmAb [4,28].

In summary, the oxidized form of all methionine residues was identified, and the relative amount of the oxidation determined. For tryptophan residues other than W283 and W319 in bevacizumab and W47, W280 and W316 in infliximab relative quantification of oxidation was not performed as no oxidized form was identified by MS/MS. With the exception of partially oxidized C214 of the light chain (and possibly one cysteine of the heavy chain hinge region), all other cysteine residues were only detected as completely alkylated non-oxidized species as expected.

4.1. Oxidation of cysteine residues

Methionine and cysteine residues became oxidized by the H_2O_2 treatment, whereas tryptophan was barely affected. The finding of oxidized cysteine was not expected. As cysteine residues with a free thiol group are highly susceptible to oxidation, biopharmaceuticals usually do not contain free cysteine residues [16]. Reports of cysteine oxidation in mAbs are rare and the finding of oxidation has usually not been substantiated with data [15,29]. The triply oxidized C214 of the light chain, together with the possibly triply oxidized cysteine of the heavy chain hinge region, constitutes an exception from all other cysteine residues, which in contrast were found completely alkylated in

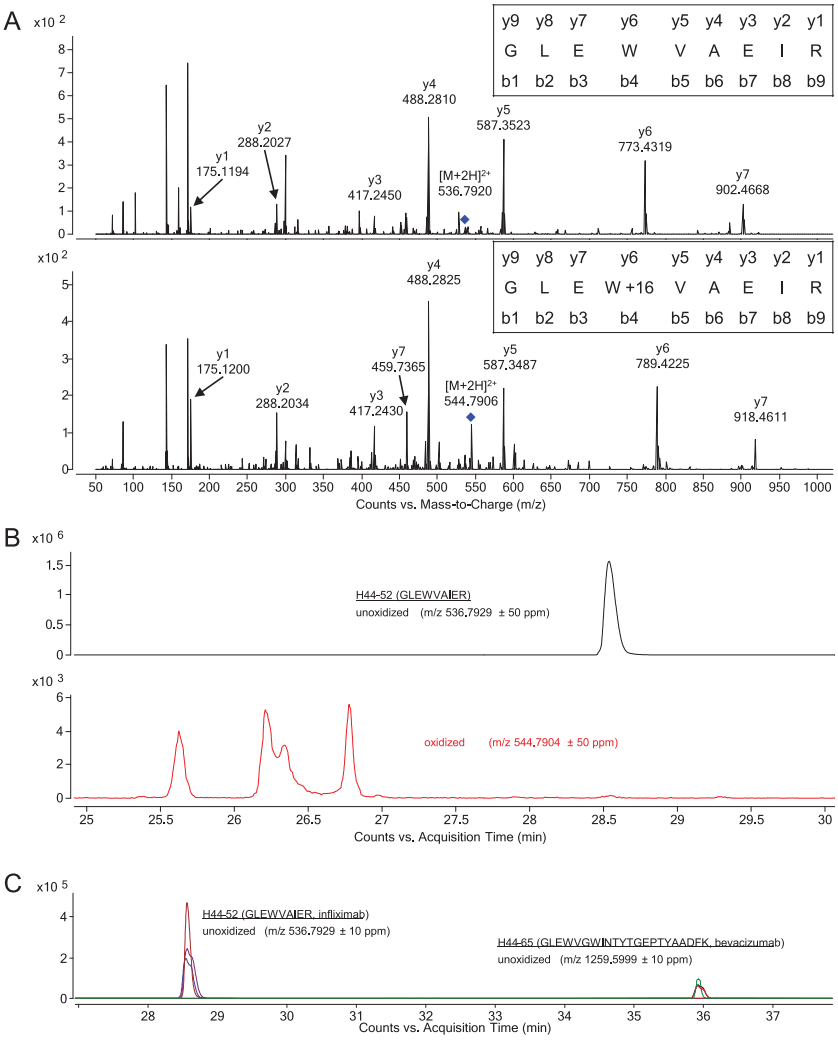

Fig. 6. MS/MS spectra of the unoxidized (top) and oxidized (bottom) H44–52 peptide (GLEWVAEIR) of infliximab (A) and respective extracted ion chromatograms of the precursor ions (B). Infliximab was treated with H_2O_2 for 72 h. The oxidized form elutes as several peaks. Note the different scale of the axis of the ordinate in B. Overlaid extracted ion chromatograms of the unoxidized forms of the infliximab peptide GLEWVAEIR and the bevacizumab peptide GLEWVGWINTYTGEPTYAADFK (H44–65) are shown each for three replications (C).

bottom-up analysis. A possible reason for this exception may be, that the fraction of triply oxidized C214 originates from mAb molecules, in which the light chain had been separated from the heavy chain, either before the H_2O_2 incubation or even as a result of the harsh treatment itself. In this scenario, the thiol group of the cysteine of the separated light chains would be highly prone to oxidation, whereas those cystine residues covalently bound by disulfide bonds are much less susceptible [15–17]. Hence, while C214 was found as partially oxidized (16–23%), this percentage might not represent the susceptibility of this residue towards oxidation but might actually reflect the proportion of separated light chain molecules among the intact mAb molecules. Further investigations on the presence of separate light chain molecules before and after forced oxidation treatment are necessary for better interpretation of these findings. During enzymatic digestion, the Lys-C peptide harbouring C214 (L208–214, SFNRGEC) would be generated on one hand from intact mAbs, in which C214 as part of the intermolecular disulfide bond was not susceptible to oxidation and therefore would result in the carbamidomethylated form after alkylation. On the other hand, the peptide would simultaneously be generated from separated light chains, which might have become completely oxidized during H_2O_2 incubation, preventing their alkylation. Investigation of two tryptic peptides of the protein Prx after forced oxidation and subsequent alkylation also showed a cysteine residue both in the triply oxidized as well as in the carbamidomethylated form [30]. The same consideration as for C214 of the light chain applies for the possibly oxidized cysteine of the hinge region. As the corresponding oxidation was found for the light chain, it is reasonable to assume the symmetric oxidation of C226 in bevacizumab (C223 in infliximab) as well. The hinge region peptide H225–228 (H222–225 in infliximab) SCDK which contains C226 (C223 in infliximab) was not identified in any form, neither unmodified nor alkylated nor oxidized. However, the adjacent hinge region peptide H229–254 (226–251 in infliximab) THTCPPCPAPELLGGPSVFLFPPKPK was modified with a + 48 Da increase found by MS, but the MS/MS spectra of this large peptide in bottom-up analysis were inconclusive. This modification may be due to oxidation, as three oxidations (3 ×16 Da) match the observed MS mass and also because the unoxidized form of the peptide decreased after H_2O_2 treatment. Possibly it could result from oxidized cysteine, as the peptide contains two cysteine residues, C232 and C235 (C229 and C232 in infliximab). Aberrant susceptibility of cysteine residues in the hinge region may be related to low molecular weight species found in mAb products such as half antibody, which is formed as a result of inter-heavy and heavy chain disulfide bonds scrambling into

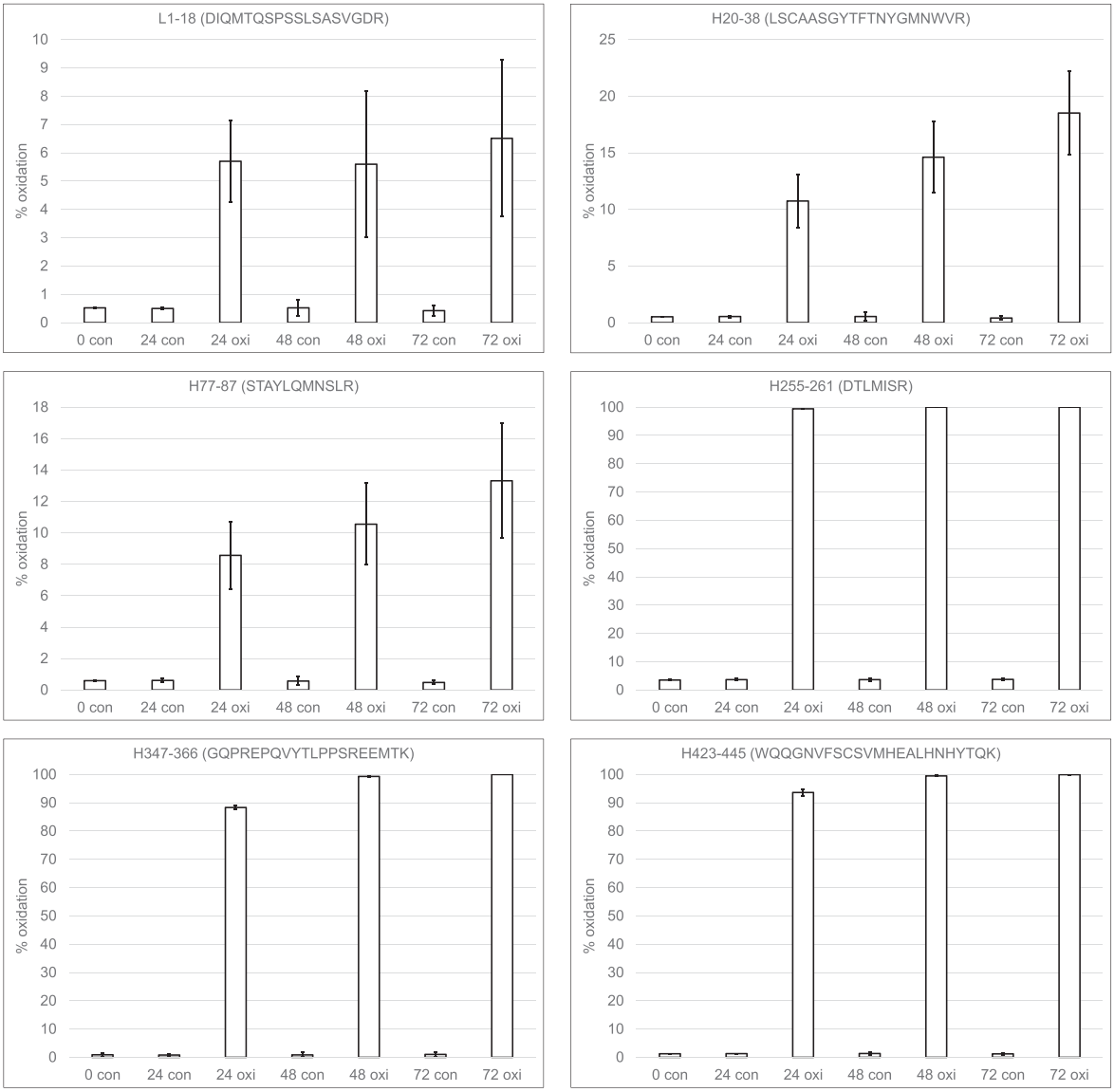

Fig. 7. Time dependance of oxidation for selected peptides. Most oxidation takes place during the first 24 h under the conditions applied. Samples were incubated with H_2O_2 (oxi) or H_2O (con) for 0–72 h. The % oxidation of the peptides containing methionine residues of the bevacizumab reference product are shown. Bars represent mean ± standard deviation of three replications.

intra-heavy chain disulfide bonds [31]. Generation of different peptides by other proteases may help to further fill gaps in the sequence coverage [32].

4.2. Practical aspects of middle-up and of bottom-up analysis

In the previous middle-up experiments [25], bevacizumab and infliximab were subjected to forced oxidation applying exactly the same conditions as in the study reported here. Middle-up analysis has several advantages compared to bottom-up analysis. The first is speed: IdeS digestion and reduction took less than 2 h and one LC-MS run took 50 min (including reequilibration), whereas for bottom-up, reduction, alkylation and digestion took about 6 h and one measurement by LC-MS took 79 min (including column purification and reequilibration). The data from subunits is simpler and hence data analysis is also faster than for peptide digests. A second advantage is the lower sample amount required for sample preparation. The bottom-up method that we used has been optimized to reduce artificial modifications and for high reproducibility however requires relatively large amounts of sample [23]. It employs a buffer exchange step between denaturation, reduction, alkylation and the subsequent digestion, in which a part of the sample is lost and therefore 135 µg of sample were used to recover enough material to allow parallel digestion with trypsin and Lys-C. If digestion with only one enzyme is required, still 60–70 µg are needed. Of note, it was observed that at lower amounts of mAb loaded on the Zebaspin buffer exchange column an excessive loss of protein occurred. For middle-up in contrast, only 12 µg were digested and reduced.

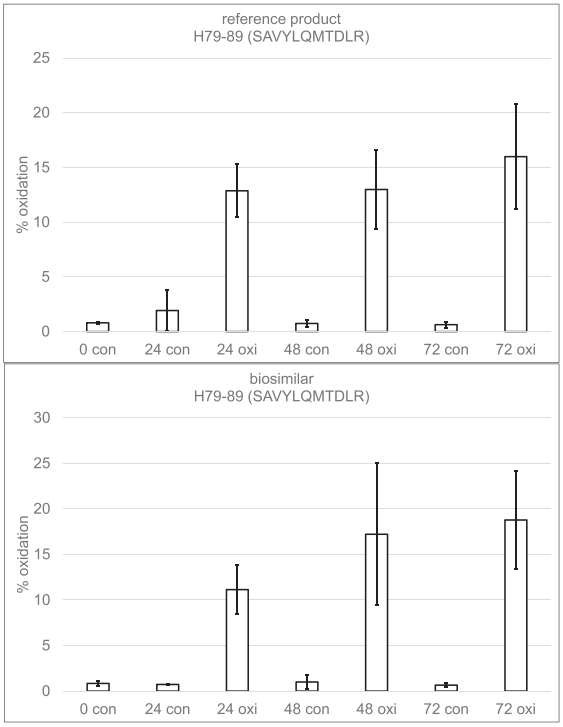

Fig. 8. Comparison of the extent of oxidation of reference product (top) and the biosimilar candidate (bottom). Similar time course under forced oxidation supports the biosimilarity. Samples were incubated with H_2O_2 (oxi) or H_2O (con) for 0–72 h. The % oxidation of the peptide containing M85 in infliximab (H79–89) is shown. Bars represent mean ± standard deviation of three replications.

4.3. Comparison of middle-up vs bottom-up results

Zhang et al. and Sokolowska et al. reported highly similar results for middle-up and bottom-up experiments for non-disclosed mAbs [33,34]. However, concerning oxidations, these studies only focused on methionine residues. In this study, the comparison of the results from middle-up with those from bottom-up analysis showed consistencies but also differences. The major species (most abundant form) of the subunits of the two mAbs that were found by middle-up were in line with the bottom-up results. The finding of subunits with an unoxidized major species by middle-up (LC and Fd' of bevacizumab) was confirmed by the bottom-up results which showed that residues located in these subunits were only partially oxidized. Also, in those cases, in which the major subunit species were found oxidized one time (LC and Fd' of infliximab), two times (Fc/2 of infliximab) or three times (Fc/2 of bevacizumab), the number of oxidations was in line with the number of strongly oxidized residues in the respective subunits found by bottom-up. Middle-up analysis reveals, how many oxidations occur together on a subunit, whereas bottom-up analysis yields the information of how many oxidations occur in a peptide. In a peptide, depending on its length, often only one oxidation is found, however also several oxidations are possible, as discussed below.

In regard of the minor species (second most abundant species) only some of the middle-up results were in line with the bottom-up results. For the light chain (LC) of bevacizumab, a minor species with three oxidations was in line with the finding of a partially three times oxidized cysteine. The LC of infliximab displayed a minor species with four oxidations, that was in line with the partially three times oxidized cysteine in conjunction with a completely oxidized methionine. However, for both mAbs in case of minor oxidized species of Fd' and Fc/2, discrepancies were observed between the middle-up and the bottom-up results. The number of oxidations found on the Fc/2 by middle-up analysis exceeded the number of completely oxidized residues found by bottom-up by one, both in bevacizumab and in infliximab. These higher oxidation states can be explained by the concomitant occurrence of the completely oxidized residues together with the weakly oxidized tryptophan residues. Taking bevacizumab as example, the four times oxidized Fc/2 can be explained by the three completely oxidized methionine residues together with one of the weakly oxidized tryptophan residues (Fig. 3). Higher mass shifts observed by middle-up analysis, such as the one assigned as four oxidations (+64 Da) need to be considered more carefully, as they may likewise be explained by different other combinations of modifications as well. Therefore, middle-up results should be corroborated by bottom-up analysis.

A discrepancy was also observed for the minor species of Fd' in middle-up analysis of both mAbs when compared with bottom-up results. One possibility to explain the fourfold oxidation on the Fd' unit of infliximab would be the common occurrence of the four oxidized residues found by bottom-up. This shows the advantage of middle-up analysis to determine whether modifications on one subunit occur together or independently of each other. Another possible explanation is the triply oxidized cysteine of the heavy chain hinge region, which however was not confirmed by MS/MS. A similar consideration applies to the minor Fd' species of bevacizumab found with three oxidations: a three times oxidized cysteine would be an explanation, but likewise this was not confirmed. The two partially oxidized residues are not enough to explain the three times oxidized Fd', however, as described in the results, two additional peptides from the Fd' might undergo oxidation, as a decrease of their unoxidized form was observed during forced oxidation (Fig. 5A and B). Digestion of the mAb with other proteases than trypsin and Lys-C may help to generate peptides to complement the data set [32].

The reason for the discrepancies remains an open question. The comparison of middle-up and bottom-up results allows to identify such discrepancies in order to investigate whether artefacts or wrong assignments are responsible for overestimation of modifications or if detection of modified residues has been missed by the bottom-up analysis. In general, discrepancies between middle-up and bottom-up which mainly result from the lack of MS/MS information should be considered in detail, before making a decision on the optimum analytical method for forced degradation studies. One solution to this problem is middle-down analysis, i.e. the mass spectrometric analysis of large subunits using MS/MS to gain additional information by fragmentation. However, as described above even for larger tryptic peptides MS/MS identification of all modification sites is challenging and needs further instrumental improvement [24]. Uncertainty is also higher in middle-up analysis because resolution is lower than in bottom-up analysis and species with slightly different masses might in worst case not be discriminated.

4.4. Biosimilar vs reference product

The results from the biosimilar candidates were highly similar to those of the reference products, both with middle-up and with bottom-up analysis. In bottom-up, also the time course of oxidation did not reveal markable differences (Fig. 7). Forced oxidation and subsequent sample preparation were replicated three times for biosimilars and reference products. Taken together, the oxidation levels after H_2O_2 treatment as seen for example for M34 or M85 of the HC (Fig. 3 and Fig. S 1) were very consistent, which underlines the reproducibility of the forced oxidation and the sample preparation method.

4.5. Bevacizumab vs infliximab

Bottom-up analysis showed complete oxidation of M55 in the LC and M18 of the Fd' of infliximab, but only partial oxidation in the LC and Fd' of bevacizumab. While middle-up can not provide this level of structural detail, it clearly demonstrated the higher susceptibility of the LC and Fd' of infliximab compared to bevacizumab, as the major species were found each with one oxidation.

4.6. Evaluation of the oxidizability of bevacizumab and infliximab

The susceptibility to oxidation of the methionine residues of both mAbs is in accordance with literature reports [25]. An important question is whether the observed oxidations need to be classified as CQAs. This requires the establishment of structure-function relationships. Nevertheless, for an initial assessment, knowledge from literature can help in the evaluation of criticality. Oxidation of the conserved Fc methionine residues can reduce the binding to Protein A and G, relevant for the purification of the mAb [12,13] as well as the binding to FcRn, important for the circulation half-life [14]. Oxidation of these methionine residues may therefore constitute CQAs. Infliximab showed extensive susceptibility for oxidation in the Fab at the LC M55 and the HC M18 in addition to the Fc oxidation. M55 is located in the complementarity-determining region 2 (CDR2) [35]. Oxidations in the CDRs have been reported to increase aggregation, to destabilize the Fab and to impact the target binding. While often caused by oxidized tryptophan, also methionine residues can be involved [9,11,36,37]. In a biosimilar study, the candidate Remsima was compared to the reference infliximab product Remicade after forced degradation by elevated temperature and humidity. Decreased neutralization of the target tumor necrosis factor was observed, which was attributed to different modifications in the CDRs including LC M55 (and HC M34) [38]. Therefore, oxidation of M55 in infliximab may represent a CQA and should be closely monitored. Even though M18 is not located in the CDRs [35], possible effects of oxidation on aggregation or target binding should be investigated. The oxidation of cysteine residues in both mAbs was unexpected. It might result from separation of the light chain from the heavy chain during the H_2O_2 treatment. Therefore, for future studies, the forced oxidation conditions may need to be adapted, for example by reducing the incubation temperature.

4.7. Middle-up in view of regulatory guidelines

Studies on the effects of oxidations are required by regulatory agencies and data can be obtained in an accelerated way by forced degradation (ICH Q1A) [8]. Especially for the demonstration of biosimilarity, stress studies should be included (ICH Q5E) [7]. These studies generate large numbers of samples. Thus, the middle-up analysis is beneficial because it is faster and less prone to artificial introduction of modifications in the sample preparation step than the classical bottom-up analysis. Moreover, it requires less amount of sample which is crucial when the amount of mAb is limited during the development stage when extended forced degradation tests need to be performed of the same product batch (ICH Q1A, FDA Guidance for industry: INDs for phase II and III studies) [8,39].

5. Conclusion

As outlined above, middle-up analysis is faster and requires less amount of sample and is therefore cheaper. Moreover, it can help to determine, if modifications occur together or independently of each other and is less prone to artificial modifications during sample preparation. However, unlike with bottom-up analysis, the assignments of a modified species in the middle-up spectra lack the MS/MS verification and have a larger measurement error resulting from the lower mass resolution of these spectra. Moreover, cases of higher mass shifts, such as of + 64 Da in the bevacizumab Fc/2 minor species, which were assigned as four oxidations, might as well be explained by a combination of different other modifications. Bottom-up can also reveal different kinetics at various sites during time-resolved forced degradation studies, whereas these differences are not detected by middle-up.

Therefore, it is recommended to confirm the middle-up results by bottom-up analysis. Once consistency of the data has been established, middle-up offers a faster and cheaper alternative which may be used as stand-alone as long as no new and unexplained results occur, for example in extended forced degradation studies or in routine analyses.

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CRediT authorship contribution statement

Yan Felix Karl Dyck: Methodology, Validation, Formal analysis, Investigation, Visualization, Writing - Original Draft, **Daniel Rehm:** Methodology, Writing - Review & Editing, **Karsten Winkler:** Conceptualization, Supervision, Resources, **Volker Sandig:** Conceptualization, Supervision, Resources, **Wolfgang Jabs:** Conceptualization, Methodology, Data Curation, Validation, Supervision, Project administration, Writing - Review & Editing, **Maria Kristina Parr:** Conceptualization, Methodology, Validation, Resources, Supervision, Project administration, Funding acquisition, Writing - Review & Editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests, Karsten Winkler reports a relationship with ProBioGen AG that includes: employment and equity or stocks. Volker Sandig reports a relationship with ProBioGen AG that includes: employment and equity or stocks. Yan Dyck reports equipment, drugs, or supplies were provided by ProBioGen AG. Daniel Rehm reports financial support and equipment, drugs, or supplies were provided by ProBioGen AG. Daniel Rehm reports a relationship with ProBioGen AG that includes: employment.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jpba.2023.115596.

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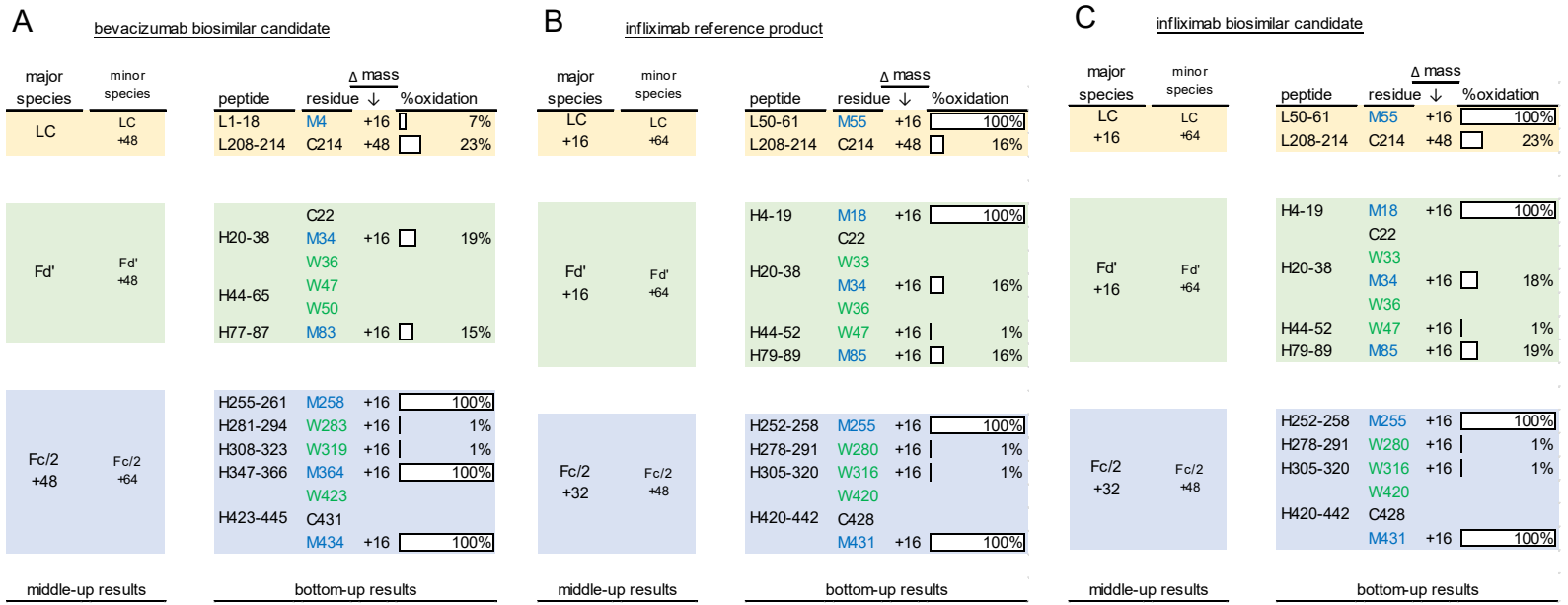

Fig. S1. Comparison of the results of forced oxidation analysis between the middle-up (left) and the bottom-up (right) approach. (A) bevacizumab biosimilar, (B) infliximab reference product, (C) infliximab biosimilar. On the left side, previous middle-up results show the major (most abundant) and minor (second most abundant) species with the observed mass changes in Da indicated by a "+". On the right side, bottom-up results after 72 h forced oxidation show those cysteine (C), methionine (M) and tryptophan (W) residues which are oxidized ("residue") and their location in the peptide analysed ("peptide"), as well as the mass changes in Da indicated by a + ("Δ mass"). Non-oxidized C, M and W which are located in the same peptide are also shown. Vertical bars represent the % oxidation of peptides (mean of three replications) with the unoxidized and oxidized form identified by MS/MS. Bevacizumab H44-65 is shown for comparison with infliximab H44-52.

4 Declaration of own contribution

In the following, the author´s contribution to the individual publications, which are used in this cumulative work, are disclosed:

Manuscript 1

- Conception and design of the experiments for the quantitation of oxidations in monoclonal antibodies by middle-up analysis with co-workers
- Execution of experiments, development of the method with co-workers
- Analysis of data
- Manuscript writing with support of co-workers

Manuscript 2

- Conception and design of the experiments for the quantitation of oxidations by bottom-up analysis in monoclonal antibodies with co-workers
- Development of the method, execution of experiments
- Analysis of data
- Manuscript writing with support of co-workers

5 Discussion

Biosimilars are developed as lower priced copy-products of expensive RMP mAbs and may increase patient access by lower development costs as well as by price competition [2]. Their development relies strongly on extensive analytical characterization for comparison of the biosimilar candidate with the RMP [3]. To reduce the development costs, fast and cost-effective analytical methods are needed. LC-MS is an established method for characterization of PTMs. The bottom-up approach is the gold standard for this application [167]. The newer middle-up approach promises to be a fast and cost-effective alternative but provides less structural resolution. In this work, both approaches were established in the PharmaMS lab at Freie Universität Berlin and used to identify previously unknown species after forced degradation. The capabilities of both methods are assessed in forced degradation studies using the common biosimilar targets bevacizumab und infliximab as model therapeutics.

5.1 Identification and localization of PTMs by middle-up analysis

Both approaches allowed to identify modifications caused by the forced oxidation treatment. However, the results of both methods differ in their level of confidence. In general, bottom-up provides higher confidence for PTM assignments because compared to middle-up, mass accuracy is higher and because MS/MS provides additional structural information. Without the structural information from MS/MS, middle-up assignments based on higher mass shifts such +64 Da (middle-up: minor species of bevacizumab Fc/2 and infliximab LC and Fd' in **Figure 7A&B**), which was interpreted as four oxidations (each +16 Da), need to be considered with caution, as they may also be explained by combinations of other modifications.

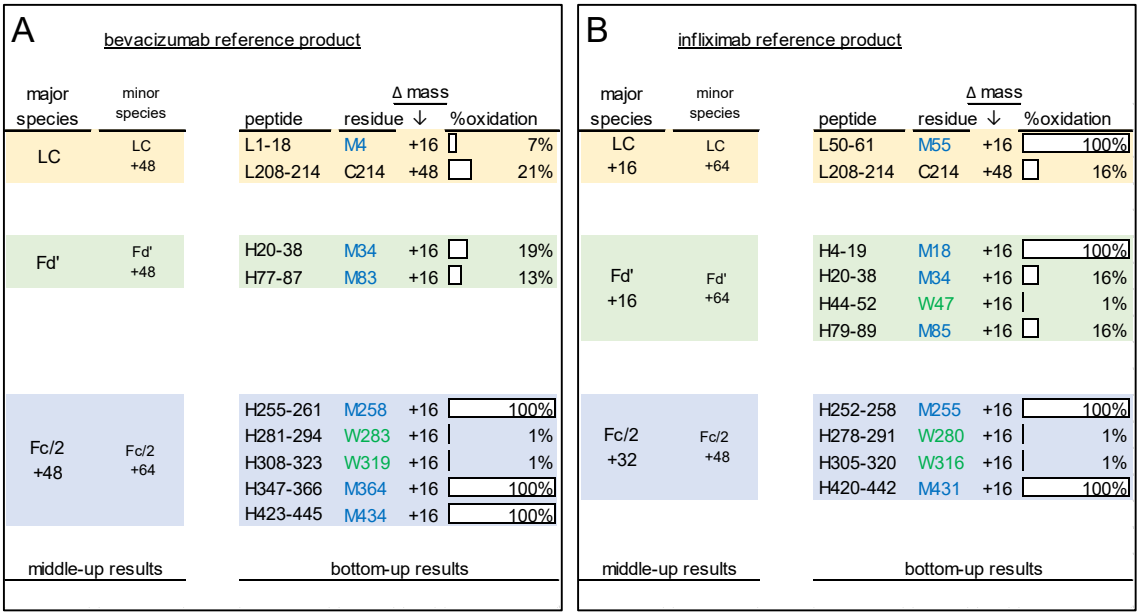

Figure 7: Middle-up and bottom-up results from forced oxidation analysis of bevacizumab (A) and infliximab (B) after 72 h. In (A) and (B), middle-up results are shown on the left side and bottom-up results on the right side. The middle-up results show the major (most abundant) and minor (second most abundant) species of each subunit with the observed mass changes in Da indicated by a "+". The bottom-up results show those cysteine (C), methionine (M) and tryptophan (W) residues which are oxidized ("residue") and their location in the peptide analysed ("peptide"), as well as the mass changes in Da indicated by a + ("Δ mass"). A mass change of +16 Da represents one oxidation, +48 Da represents three oxidations. Only those peptides are shown for which the unoxidized and oxidized form was identified by MS/MS. Vertical bars represent the % oxidation of peptides (mean of three replications). For both mAbs, results of the respective reference medicinal product are shown. Results of the respective biosimilar candidates were highly similar. The figure is modified from Fig. 3 and Fig. S1 (which also shows the results of the biosimilar candidates) from manuscript 2. The non-oxidized C, M and W located in the same peptides were shown in the figures from manuscript 2 but were removed here to focus only on the oxidized residues.

It is important to understand the localization of the PTM, as it makes a difference, for example, if an oxidation or a deamidation is found in the CDRs, where it may impact the target binding and may thus represent a CQA, while in other position, it may have no detrimental impact [97]. Bottom-up analysis localized oxidations to individual peptides and allowed to pinpoint them to the exact amino acid position by MS/MS. Middle-up detected mass changes on subunits but lacked further localization of the modified amino acid. In contrast to bottom-up, which unambiguously showed the position of oxidation, the assignments of oxidations to positions based on middle-up results in manuscript 1 were made under the assumption, that the oxidations affected methionine residues (Met, M). This assumption was reasonable, as forced oxidation was performed using H_2O_2 under conditions that are known to oxidize primarily Met [90, 101, 136]. When the number of oxidations and the number of Met were equal, the assignment was

straightforward. For example, after forced oxidation, the LC of bevacizumab was mostly not oxidized, and therefore it was concluded that the only Met, M4, is not susceptible to oxidation (middle-up: major species of LC in **Figure 7A**). In infliximab, the LC was mostly one time oxidized, leading to the conclusion that the only Met, M55, is susceptible to oxidation (middle-up: major species of LC in **Figure 7B**). However, for the Fd' of infliximab, which contains three Met, only one oxidation was detected (middle-up: major species of Fd' in **Figure 7B**). Therefore, with this middle-up result from infliximab alone, it is impossible to know which of the three Met is affected. In this case, the assignment required the direct comparison of Met positions between bevacizumab and infliximab as well as the comparison of the number of oxidations detected (middle-up: major species of Fd' in **Figure 7A&B**). Two of the three Met of infliximab's Fd' (M34 and M85) also exist in similar positions in bevacizumab, but the third Met, M18, has no correspondence in bevacizumab. From this, it was concluded that M18 must be the residue which is prone to oxidation. Thus, one limitation of the middle-up approach was that at least two mAbs with different Met positions needed to be analysed in parallel to be able to deduce all positions of oxidized Met on the middle-up level. Additional difficulties with interpretation of subunit results arose when in some subunits, less abundant species were found with higher mass shifts, e.g., +64 Da on the Fc/2 of bevacizumab (middle-up: minor species of Fc/2 in **Figure 7A**). In this case, the number of four oxidations (each +16 Da) exceeded the number of Met (three: M258, M364, M434) present in the subunit. Based on the middle-up data, these higher oxidation states could not be unambiguously explained.

Bottom-up analysis was used to confirm the assignments made by middle-up analysis and to identify further oxidations that were not explained by middle-up. The middle-up assumption that all detected oxidations would result from Met oxidation turned out to be too simplistic, as bottom-up demonstrated that also cysteine residues (Cys) and tryptophan residues (Trp) were affected, though to a lesser degree (bottom-up in **Figure 7A&B**). Moreover, when several oxidations were detected on a subunit, as in the aforementioned example (+64 Da), middle-up could not elucidate whether the oxidations were located on different residues or if double or triple oxidation was involved, which may occur on Met and Cys, respectively. An example is the triply oxidized C214 in the LC of bevacizumab and infliximab found by bottom-up analysis (**Figure 7A&B)**.

However, an important requirement for bottom-up to be informative is that complete or at least near-complete sequence coverage must be reached to provide confidence that no critical region remains undetected. In the bottom-up experiments (manuscript 2), sequence coverage was incomplete. Nevertheless, all Met containing peptides were found. Conversely, middle-up

analysis has the advantage that all regions are included in the subunits and therefore all PTMs add to the overall mass change. This advantage may be illustrated by the finding of the triply oxidized C214 by bottom-up. Initially we had focussed the search for oxidation in the bottom-up data to Met, as they are known to be susceptible to H_2O_2 treatment. However, the bottom-up results of oxidized Met could not explain the less abundant subunit species with higher numbers of oxidation detected by middle-up. The search was thus extended to Trp, as Trp oxidation has also been reported on mAbs [97, 99, 185]. As the low levels of Trp oxidation could also not help to fully explain the middle-up observation, the search was further extended to include Cys, even though oxidation of Cys is normally not expected on mAbs [90]. The finding of oxidized Trp and Cys helped to explain the subunit species with higher numbers of oxidation. Without the unexplainable middle-up results, we may not have extended the bottom-up analysis to Trp and Cys and thus have missed the oxidation of these residues.

5.2 Quantification of PTMs by middle-up analysis

Both approaches allowed quantification of oxidations. Bottom-up showed the relative oxidation levels of residues on individual peptides. Middle-up analysis detected subunits with different numbers of oxidations, each amenable to relative quantification, as shown exemplarily in manuscript 1 for the major oxidized species of LC and Fd' of bevacizumab. In the middle-up analysis, after forced oxidation, the most abundant species of the LC was the unoxidized form (middle-up: major species of LC in **Figure 7A**). The second most abundant species was the triply oxidized form (middle-up: minor species of LC in **Figure 7A**). To calculate the % oxidation of a subunit, the sum of all oxidized forms must be set in relation to the sum of all forms of the subunit (unoxidized and oxidized). In the case of the LC of bevacizumab, no other oxidation states (such as singly or doubly oxidized) were found. The relative abundance of the triply oxidized form was therefore calculated as the triply oxidized form in relation to all forms of the LC (i.e., the sum of the unoxidized and the triply oxidized form). The triply oxidized LC is explained by the finding of the triply oxidized Cys (C214) in bottom-up analysis.

In the middle-up study, the time course of oxidation of the LC and the Fd' indicated a higher susceptibility of the bevacizumab biosimilar candidate compared to the RMP. After 72 h of forced oxidation, the relative abundance of the LC's triply oxidized form was higher in the biosimilar candidate (mean = 33%, manuscript 1) than in the RMP (mean = 17%, manuscript 1). In contrast, the levels of triple oxidation of the peptide C214, determined by the bottom-up analysis, were highly similar between the biosimilar candidate (mean = 21%, bottom up in supplementary in manuscript 2) and the RMP (mean = 23%, bottom-up in **Figure 7A**). The

reason for this discrepancy between the middle-up and bottom-up results is not known. One factor that differed between the two sample preparations was the reducing agent. For middle-up, tris(2-carboxyethyl)phosphine (TCEP) was used, whereas for bottom-up, DTT (dithiothreitol) was used. TCEP has been reported to reduce oxidized Met [186]. It could be speculated that differential oxidation levels on cysteine residues may also be caused by TCEP, possibly because of conformational differences between the biosimilar candidate and the RMP. However, this does not seem plausible, as no difference was found between oxidations of Met.

The correlation between oxidation levels of the LC of bevacizumab determined by middle-up and bottom-up analysis was in a range that is in accordance with that of other reports [6, 121]. However, the relative oxidation levels on the middle-up level need to be determined more comprehensively also for the other subunits, including more oxidation states, to fully evaluate how well the two approaches compare.

Given the higher risk for bottom-up analysis that artificial modifications may be introduced during sample preparation, the bottom-up results should also be compared with the middle-up data to check for possible artefacts. A protocol for bottom-up sample preparation that was optimized to minimize artificial modifications [5] was used in manuscript 2 to mitigate this risk.

Middle-up has the advantage that it may represent modification levels in better agreement with the proteoforms of the sample. In addition, modifications may change the ionization efficiency of the molecule, which introduces a bias in the relative quantification [187]. In a small peptide, this effect is more pronounced than in a large subunit.

5.3 Unique advantages of middle-up analysis

Middle-up analysis shows whether modifications occur together on one subunit, thereby providing structural information that cannot be achieved by the bottom-up approach alone. For example, bottom-up analysis of infliximab demonstrated oxidations on three Met and on one Trp that are all located in the Fd' region (bottom-up: Fd’ in **Figure 7B**). M18 was completely (100%), M34 and M85 were partially (16-19%) and W47 was weakly (1%) oxidized. Thus, while all infliximab molecules carried an oxidized M18, bottom-up could not show whether the partially and weakly oxidized residues occur together or not. Middle-up analysis demonstrated four oxidations, indicating that oxidation of these four residues occurs together (middle-up: minor species of Fd’ in **Figure 7B**). An alternative explanation of the four oxidations may involve putative triple oxidation of a Cys in the Fd' region together with one of the Met or Trp oxidations. However, Cys oxidation on the Fd' could not be shown unambiguously.

Also, other studies have exploited middle-up analysis to investigate the concomitant occurrence of modifications. For example, the combination of peptide mapping and subunit analysis revealed that oxidation of two Met on the Fc of a mAb occurred together when induced by light exposure [188]. However, after tBHP treatment, only one oxidation was found, which was distributed randomly to either one of the two Met. Similar observations from subunit analysis and peptide mapping were made on other mAbs as well [6, 118, 121]. Subunit analysis also proved useful in a study on the structure-function relationships of symmetrically and asymmetrically modified antibodies [189].

5.4 Oxidation susceptibility of Met, Trp and Cys in bevacizumab and infliximab compared with literature

Reports on bevacizumab and infliximab that perform PTM identification are often found in analytical biosimilar studies and employ mostly bottom-up analysis [4, 76, 147-149, 151, 152, 154, 190-192]. These include identification and relative quantification of attributes such as C-terminal lysine, disulfide bonds, oxidation and deamidation, sometimes in stability studies and after forced degradation. Glycan profiles have been assessed as released glycans [148, 154, 190-192], in form of glycopeptides [4, 192] as well as on the intact level [149, 153].

The susceptibility of Met to oxidation reported in the manuscripts is similar to literature reports that also performed forced oxidation. Such reports are usually part of biosimilar studies and focus on Met oxidation after forced oxidation. In bevacizumab, after 24 h forced oxidation by H_2O_2, the conserved Met of the Fc were oxidized to different degrees: M364 (88%), M434 (94%) and M258 (99%). This order of susceptibility is consistent with that found in forced oxidation studies on other mAbs [120]. After 72 h of forced oxidation incubation, M258, M364 and M434 were completely oxidized in the bottom-up study (bottom-up: Fc/2 in **Figure 7A**). In general, the oxidation levels reported in other studies are lower, probably because other studies perform forced degradation at lower temperature (4 or 25 °C) and with shorter incubation time. Zhang et al. reported oxidation levels for bevacizumab of up to 42% (M258) and 15% (M434) after the maximum duration of 48 h oxidation, but did not show M364 oxidation [153]. The lower oxidation levels may be due to the use of another oxidizing agent, tBHP, as well as different incubation conditions (temperature, duration). It has been reported that tBHP oxidizes only the surface exposed Met, i.e., M258 and M434, whereas the more buried M364 is not affected [118], because of tBHP's more bulky structure, compared to H_2O_2 [101]. These reasons may also explain why Zhang et al. may not have found oxidation on those Met, which were detected in the bottom-up study on bevacizumab with partial oxidation (LC

M4, Fd' M34 and M83, see bottom-up in **Figure 7A**). M34 and M83 are present both in bevacizumab and infliximab and the finding of only partial oxidation of M34 and M83 (13-19%) is in line with low susceptibility reported for corresponding Met positions in other mAbs [95, 118, 136, 193].

In infliximab, the conserved M255 and M431 of the Fc were also completely oxidized after 72 h of forced oxidation (bottom-up: Fc/2 in **Figure 7B**). Infliximab has no Met corresponding to the M364 in bevacizumab. Moreover, M55 in the LC and M18 in the Fd' were completely oxidized (bottom-up: LC and Fd' in **Figure 7B**). Qualitatively, this finding is in accordance with other studies, which however report lower levels of oxidation, presumably because of forced oxidation performed under lower temperature and shorter duration. Legrand et al. also showed infliximab's oxidation levels of M55 in the LC, M18 in the Fd' and M255 and M431 in the Fc, ranging approximately between 60 and 90% after the maximum duration of 48 h [152]. Another study forcedly oxidized infliximab for only 6 h at 5 °C and reported oxidation of M55 in the LC, M18 in the Fd' and M255 in the Fc [151]. Oxidation levels ranged approximately between 5 and 20% after 6 h. Opposed to the bottom-up study, Kim et al. did not find oxidation on M431, presumably because of the milder forced oxidation conditions. M34 and M85 of the Fd' were found only partially oxidized (13-19%) (bottom-up: Fd' in **Figure 7B**). As mentioned above, M34 and M85 reportedly have low susceptibility of oxidation. Accordingly, also Legrand et al. and Kim et al. did not show oxidation of these Met [151, 152].

Oxidation of tryptophan residues is usually not observed after H_2O_2 treatment that was applied in these studies [90, 136]. Yet, a few tryptophan residues were weakly oxidized (up to 1%, bottom-up in **Figure 7A&B**), presumably because of the harsher forced oxidation conditions compared to other studies. Cysteine oxidation in mAbs has also been rarely reported and the finding of oxidation has usually not been substantiated with data [89, 194]. Cysteine residues are normally involved in disulfide bonds, but free cysteine residues that are highly susceptible to oxidation may be formed by different mechanisms (section **2.2.5.2**). In manuscript 2 it was speculated that oxidation of cysteine residues may be due to separated LC and HC, containing free cysteine residues. Separation of LC and HC after the same harsh forced oxidation conditions had been observed by size variant analysis using SEC (personal communication by Daniel Rehm, ProBioGen AG). One solution to perform forced oxidation while preserving the mAbs' integrity was to lower the incubation temperature.

5.5 Application of middle-up analysis for biologics industries

The suitability of middle-up analysis depends on the analytical task. Possible applications in the biologics industry include the research and development (R&D) department and the quality control (QC) department.

For R&D, middle-up analysis first of all represents an additional tool in the analytical toolbox. If an analytical question requires more detailed information than provided by intact analysis and if middle-up can provide this information, then it can be a fast alternative to bottom-up analysis. For example, the middle-up investigation (manuscript 1) demonstrated that the Fc of bevacizumab and infliximab and the Fab region (LC and Fd') of infliximab, but not bevacizumab, were extensively oxidized, providing a global picture. It also offers a fast alternative to classical analytical methods which rely on UV detection, because it can be used for multi-attribute monitoring, thereby replacing several other analytical methods. The middle-up study (manuscript 1) demonstrated the simultaneous identification of oxidation, C-terminal lysine clipping, N-terminal pyroglutamate and major glycoforms. Moreover, during middle-up sample preparation, in addition to fully reduced Fc/2, LC and Fd', additional peaks could be identified as variants with one and two intact disulfide bonds, indicating incomplete reduction. The incomplete reduction was then completed by additional incubation with the reducing agent. For detection of less abundant glycoforms, additional analyses may be necessary. In contrast, characterization with UV detection would require CEX for analysis of lysine clipping and pyroglutamate and BAC or HILIC for characterization of glycoforms, in addition to RPLC for oxidation. Moreover, the identity of the mAbs was confirmed by correct masses of their subunits. Multi-attribute monitoring is typically performed by bottom-up analysis [158, 195], but middle-up analysis has been suggested for this purpose as well [196]. However, as mentioned before, the interpretation of larger mass changes caused by several PTMs on a subunit may become difficult, because potentially different combinations of PTMs could account for these changes.

R&D needs a deep understanding of product characteristics. Therefore, for PTM analysis, bottom-up is the method of choice. However, R&D also often needs to analyse large numbers of samples, for example when mAbs of different clones are compared during cell line development or when forced degradation studies are performed[6]. This requires fast analytical methods. Thus, middle-up analysis may provide an alternative that is faster than bottom-up analysis but more informative than intact analysis. For analysis on the subunit level, LC-MS is needed, because modified and unmodified subunits may not always be separated sufficiently

by LC-UV. Insufficient chromatographic separation was a difficulty in the middle-up study, but the mass spectral data allowed to identify unmodified and modified subunits.

While R&D has a higher degree of flexibility concerning the choice of analytical methods, QC is performed in a highly regulated environment under regulations of good manufacturing practice (GMP). Just as R&D, QC needs analytical methods which can reliably identify CQAs. CQAs must be defined beforehand according to their impact on safety and efficacy by appropriate studies. If occurrence of PTMs such as oxidation or deamidation on a subunit (without further sublocalization) can be classified as a CQA, then middle-up analysis may be utilized to monitor if the CQA is within the specified range. For example, infliximab has only one Met in the LC (M55), which is positioned in CDR2. Its oxidation may therefore represent a CQA. During the forced oxidation study, this Met residue was highly susceptible to oxidation (bottom-up: LC M55 in **Figure 7B**). Therefore, an oxidation detected on the LC of infliximab is most likely due to oxidation of M55. The oxidation of Cys (C214) reported in manuscript 2 can be discriminated by middle-up analysis from the Met oxidation (+16 Da), because it appeared only in the triply oxidized form (+48 Da) (bottom-up: LC C214 in **Figure 7A&B)**. Oxidation of Cys in mAbs also represents a CQA, as it is caused by degradation of disulfide bonds. Thus, oxidation on LC M55 and C214 can be detected, and relative levels can be determined by middle-up analysis. For those PTMs which are CQAs, and which can be detected unambiguously and quantified by middle-up, this approach offers a fast alternative to bottom-up analysis.

In contrast, if the CQA is for example deamidation of an asparagine residue (Asn) in the CDR, middle-up may not be a suitable method, as the LC and Fd' (which harbour the CDRs) contain several other asparagine residues, which may not be CQAs. Thus, the information from middle-up analysis about the occurrence of one or several deamidations on the respective subunit may not be sufficient because the modification cannot be pinpointed to the critical asparagine in the CDR.

It should be noted that while analysis with LC-MS allows identification and relative quantification of PTMs, its acceptance in companies producing biologics is still hampered: on one hand by high costs for an MS instrument and on the other hand by the fact that MS instruments in case of malfunction may face longer downtimes, which is less of a problem for classical analytical methods relying on UV or fluorescence detection. However, the advantages of an MS instrument should be noted as well. Once an MS instrument has been acquired, it not only allows mAb characterization related to the primary structure and PTMs, importantly

including glycan characterization on different levels. It also allows to perform experiments such as HDX-MS for characterization of the higher order structure, an important application for R&D.

5.6 Practical aspects of middle-up analysis

Compared to bottom-up, the middle-up approach was indeed faster in terms of sample preparation (less than 2 h vs 6 h), and of subsequent LC-MS runtime (50 min vs 79 min). Data analysis was also faster, as the number of subunits in middle-up analysis is lower than the number of peptides in bottom-up analysis, including modified species in both cases. Data analysis was very time-consuming in both cases and the author cannot quantify the many weeks spent on each type of data; therefore, this is a personal account from these studies. Moreover, middle-up sample preparation is less prone to artificially introduce modifications, which is important for the analysis of PTMs [5, 118]. Furthermore, middle-up sample preparation required significantly less sample (12 µg vs 60-70 µg), which is another important factor, when sample amounts are limited during mAb development. The higher sample consumption in the bottom-up sample preparation was due to an additional buffer exchange step, in which protein was lost. However, this step was necessary, as omitting this step during method establishment led to lower sequence coverage. As a consequence, middle-up analysis is also less expensive than bottom-up analysis, because a more efficient process reduces costs by allowing workers to complete tasks more quickly, freeing up their time for other responsibilities. And the lower sample consumption helps to save valuable material.

5.7 Limitations of the study

The study presented here has limitations resulting from the study design of the forced oxidation, of the middle-up analysis and of the bottom-up analysis.

A drawback of the generation of stressed samples is that it was performed separately for the middle-up and for the bottom-up study. While attention was paid to repeat the second forced oxidation (for the bottom-up analyses) as accurately as possible, changes may have happened. For example, the H_2O_2 used for forced oxidation was not the same. The concentration of H_2O_2 solution is not stable over longer time and therefore the effective concentration may have been different between the two repetitions. The oxidation levels between the triply oxidized LC from the middle-up study and the triply oxidized C214 from the bottom-up study were still in good accordance, indicating no notable difference in the H_2O_2 concentration. Nevertheless, it is

recommended for similar studies to generate the stressed material one time and to conduct all analyses from aliquots thereof. Moreover, the forced degradation conditions may have been too harsh, indicated by the possible detachment of the LC from a part of the mAbs as reported in manuscript 2. For the generation of oxidized mAb samples that serve for the comparison of middle-up and bottom-up, this may not be a problem, as long as the material is equal for both types of analyses. However, it may raise concerns about the reported susceptibility of residues when compared with reports that are conducted under less harsh conditions. For example, if the LC was completely separated from the rest of the mAb, this may lead to conformational changes as well as to aggregation when otherwise buried hydrophobic residues become exposed. Especially if functional tests are to be conducted on the stressed mAb, care should be taken to ensure the structural integrity. Therefore, the integrity should be assessed by size indicating methods such as SEC, non-reduced SDS-PAGE or CE-SDS. One way to achieve oxidation and to preserve the integrity of the mAb may be to perform the forced oxidation incubation at lower temperature. While the concentration of H_2O_2 (0.05% w/v) were within the ranges reported for forced oxidation studies (0.001% to 0.5%) [135], the concentration may also be further decreased.

Another aspect to improve is the incomplete sequence coverage of the bottom-up study. The sequence coverage achieved with trypsin was 88% on average and 95% when combined with the data from Lys-C digestion. However, it would be desirable to achieve 100% sequence coverage, so that no critical regions remain undetected [5]. For example, the Cys-containing peptide SCDK of the HC (with C226, position in bevacizumab) was not found. This peptide is of special interest because the Cys participates in the disulfide bond with the LC. As the corresponding LC Cys (C214) was found oxidized, it is desirable to know if the HC C226 is likewise oxidized. In the study about the optimized peptide mapping method by Mouchahoir et al. [5], that was adapted for manuscript 2, the SCDK peptide, together with other small peptides, was among the very earliest eluting peptides. In the bottom-up study, the initial eluent flow from the liquid chromatography to the mass spectrometer was bypassed, to avoid that salts contained in the sample contaminate the mass spectrometer. During this step SCDK as well as other early eluting peptides may be lost by early elution because it is comparatively short. The study by Mouchahoir et al. shows that SCDK can be found [5]. However, if there was a possible oxidized form of this peptide, it may elute even earlier and be lost, as was the case for the short EEMTK peptide described in manuscript 2. It may be worth to test if salts can be removed by a desalting step before sample injection and then infuse the complete eluent flow into the mass spectrometer without bypassing. Also, the use of a digestion enzyme with a specificity different

from that of trypsin and Lys-C (employed in manuscript 2) may generate a larger peptide that would possibly elute later. For example, Asp-N (peptidyl-Asp metalloendopeptidase) cleaves N-terminal to aspartate and generates a peptide containing C226 (position in bevacizumab) which is longer (DKKVEPKSC) and may be detectable due to later elution. Another peptide which was not detected in bottom-up analysis in any of the samples was H299-307 (EEQYNSTYR) that contains the consensus sequence for N-glycan attachment and supposedly carries the N-linked glycan. Possible reasons that this peptide was not identified include the fragmentation of glycans during the applied collision-induced dissociation (CID) during MS/MS, with the resulting fragment masses possibly not recognized by the software for data analysis [197]. Another reason is the existence of different glycoforms, mainly G0F and G1F, as confirmed by the middle-up analyses. This heterogeneity leads to the fact that each glycopeptide is less abundant than if it consisted of one unmodified peptide. One strategy to overcome this problem is to remove the glycan by endoglycosidases such as PNGase F (peptide:N-glycosidase F). The glycans would then need to be analysed separately.

In general, parameters that may be optimized to improve the sequence coverage include the chromatography as well as settings of the mass spectrometer. For example, a shallower LC gradient may lead to improved separation of co-eluting peptides. Co-elution of peptides may pose a problem, as the number of MS/MS experiments per time unit is limited. For example, out of several co-eluting peptides, only a limited number is selected for MS/MS identification when infused into the mass spectrometer, while some co-eluting peptides meanwhile cannot be subjected to MS/MS [198]. Also, the MS/MS settings may be optimized to improve peptide identification. The bottom-up analyses (manuscript 2) were operated in the data-dependent acquisition mode, which means that precursors for fragmentation were selected according to defined criteria. For example, the 10 most intensive precursors were selected during one cycle of MS/MS. For peptides requiring more MS/MS data for identification, the settings could be adjusted and optimised, ideally without compromising the identification of possible coeluting peptides. When sequence coverage is incomplete in bottom-up analysis, middle-up can be used to confirm that the amino acid composition is as expected by the mass.

Apart from improvements of the sequence coverage, pure LC-MS runs would be preferable in addition to the LC-MS/MS runs performed here. While pure LC-MS lacks the MS/MS identification, the scan rate on the peptide masses is higher, and thereby the peak shape is better defined, which is a prerequisite for relative quantification by peak areas [198]. Especially for peptides of low abundance, the peak shape cannot be covered well because the frequency of MS acquisition is reduced when many MS/MS experiments must be performed to collect the

required fragmentation data. Performing additional pure LC-MS measurements was difficult though, due to time limitations at the multi-user mass spectrometer.

A challenge in relative quantification of modified peptides is the dynamic range in which it can be performed. mAb digests contain a large variety of peptides that are detected with different signal intensity which depends on their ionization efficiency. Furthermore, the ionization may be influenced for example by ion suppression by coeluting peptides [176]. On one hand, peptides with strong signals should not reach detector saturation, because quantification would be compromised. On the other hand, peptides with PTMs may be of low abundance and their intensity may be below the detection limit. During the bottom-up measurements, the amount of sample was adjusted so that the strongest signals were below detector saturation. Additional measurements could be performed with increased amounts of sample, so that pairs of unmodified and modified peptides, which exhibit weaker signal intensity, may reach the dynamic range. For example, in manuscript 2, low levels of Trp oxidation were demonstrated for the infliximab peptide GLEWVAIER, with intensity of the oxidized form only slightly above the threshold for MS/MS. Detection of a possibly low abundant oxidized form of the bevacizumab peptide GLEWVGWINTYTGEPTYAADFK, which contains Trp in similar positions, may have been obstructed by too low signal intensity of the oxidized form.

Also, the middle-up experiments could be improved. The column temperature was set to 80 °C, which may generate artificial modifications, when subunits are exposed to this high temperature [199]. Concerns about artificial modifications resulting from high column temperature were ruled out, because similar temperatures have also been used by others during subunit analysis, who did not report on-column degradation [118, 200]. Moreover, the levels of oxidation detected in unstressed control samples were comparable to reports from literature [167, 201]. However, since temperatures as low as 60 °C do not significantly impact the recovery of subunits from the column, as shown for bevacizumab as well as for other mAbs [202], such lower temperatures are preferable to reduce the risk of on-column degradation. Furthermore, the chromatographic separation of the middle-up study (manuscript 1) was not optimal. Separation of oxidized and unoxidized species was better, though not optimal, for the less hydrophobic Fc/2 but worse for the more hydrophobic Fd'. The incomplete separation complicated the analysis. One strategy is to use trifluoroacetic acid (TFA) as acidic modifier instead of formic acid (FA), as it improves the peak separation [203]. One problem with TFA is that it leads to ion suppression. However, TFA has been used at low concentrations for protein analyses by LC-MS, balancing the effects of improved peak separation and ion suppression [203]. Another problem of TFA is that it is highly persistent once introduced to an MS system.

Thus, it was not an option to use TFA on the LC-QTOF of PharmaMS at the Freie Universität Berlin, which is a multi-user system used both for the analysis of large molecules and small molecules. Therefore, the use of low concentrations of TFA can only be an option on devices that are completely dedicated to protein analysis.

For the LC-MS measurements, it would be beneficial to evaluate system suitability for important parameters, including metrics such as the mass accuracy, the quality of MS/MS spectra, sensitivity, mass resolution, detection limit and the accuracy of relative quantification, to better understand limitations of the measurements [198].

5.8 Conclusions and outlook

Middle-up analysis shows modifications on the mAbs subunits and thus provides lower structural resolution than bottom-up analysis, which pinpoints modifications to individual amino acid positions. In some cases, the localization of modifications may be deduced from the amino acid sequence, which was demonstrated by the middle-up assignment of oxidations to specific methionine residues (manuscript 1). The correctness of the assignment was confirmed by bottom-up analysis and both approaches were compared (manuscript 2). The insights from middle-up analysis were limited to the most abundant oxidized species and less abundant species could only be explained using bottom-up data. Moreover, uncertainty of the results is higher compared to bottom-up, as middle-up results often deal with a combination of PTMs. Furthermore, middle-up analysis provides lower mass accuracy and lacks confirmation of modifications by MS/MS. Therefore, results from middle-up experiments should be complemented once by bottom-up analysis to confirm correct assignments, for example during forced degradation studies. When the same forced degradation is tested subsequently, for example to compare stability of a mAb in different formulations, the faster middle-up analysis may be sufficient, as long as no new species are detected, which cannot be unambiguously assigned on the middle-up level.

The positions of susceptible Met in bevacizumab and infliximab determined in this study was in line with results from the literature. Relative levels of oxidation were often lower in other reports, presumably because of milder forced oxidation. In this study, the results from relative quantification of oxidation were not in accordance between middle-up and bottom-up analysis. However, this comparison was performed only on a small subset of oxidized species. For a better conclusion, the comparison of oxidation levels should therefore be further extended, with

the suggestion to include at least all most abundant and second most abundant oxidized species, comprising the largest part of oxidized species from middle-up analysis.

For future forced oxidation studies, it is recommended to also apply less harsh conditions, e.g., by lower temperature incubation, or to choose earlier time points, to better capture the time course of oxidation. Moreover, the conditions applied here may impact the integrity of the mAbs. The oxidation of Cys is normally not reported in mAbs and may result from separation of the LC from the HC. Further suggestions to improve the LC-MS measurements for middle-up and bottom-up analysis have been made in the discussion section of this work.

The comparison between middle-up and bottom-up analysis may also be interesting for investigation of other PTMs to understand, to which extent middle-up and bottom-up analysis may substitute or complement each other. During the course of experiments that were used for the manuscripts, initially also samples from forced deamidation and glycation had been analysed before focusing completely on oxidation. Middle-up in our hands seemed not capable to resolve the small mass change (1 Da) between Asn and Asp or isoAsp. Mass data from forced deamidation and control samples were highly similar and did not indicate systematic differences between the samples. In contrast, middle-up was able to demonstrate glycation in forced glycation samples. While the investigation of deamidation by middle-up was less promising, the comparison of middle-up and bottom-up may be extended to forced glycation studies.

As an interesting perspective, the LC-MS based middle-down analysis may reach maturity in the future. Middle-down analysis is based on the same fast sample preparation as middle-up analysis, but additionally provides fragmentation data. Therefore, middle-down may become another alternative to middle-up analysis complemented by bottom-up and it will be interesting to evaluate how the middle-down approach compares to the reported approaches.

6 Summary

The characterization of post-translational modifications (PTMs) is an important part of therapeutic monoclonal antibody (mAb) quality assurance. Modification by oxidation is one of the most commonly observed degradation pathways in mAbs [84]. The present work utilizes two liquid chromatography-mass spectrometry (LC-MS) based methods for investigation of PTMs with a focus on oxidation. The two approaches are compared to understand to which extent the faster and less artefact-prone middle-up analysis can replace the elaborate bottom-up analysis. The reference medicinal products (RMPs) and biosimilar candidates of the two mAbs bevacizumab and infliximab were subjected to forced oxidation by H_2O_2 to investigate their susceptibility to oxidation.

In a first step, middle-up analysis was established. PTM analysis of unstressed samples, including major glycoforms, demonstrated high similarity between the biosimilar candidates and the RMPs of bevacizumab and infliximab. Treatment with H_2O_2 generated different species of the subunits with different numbers of oxidations. The most abundant species caused by forced oxidation could be assigned to single oxidations of methionine residues (Met, M). Extensive oxidation of the Fc/2 could be attributed to M258, M364 and M434 in bevacizumab and to M255 and M431 in infliximab. Extensive oxidation was also observed in the light chain (LC) and in the Fd' subunit of infliximab, but not in bevacizumab. By comparison of the most abundant species' number of oxidations and the positions of Met between the two mAbs, the oxidations in infliximab were assigned to M55 in the LC and M18 in Fd'. However, less abundant species of Fc/2, LC and Fd' with higher oxidation numbers could not be explained. Despite high similarity of unstressed samples, forced oxidation demonstrated a higher susceptibility of the bevacizumab biosimilar candidate compared with the RMP.

In a second step, bottom-up analysis was established and used to confirm the exact location of oxidations. In addition to singly oxidized Met, singly oxidized tryptophan (Trp) and triply oxidized cysteine (Cys) residues were found. The middle-up assignments of the most abundant species to oxidations on Met residues were confirmed by the bottom-up results, demonstrating that those Met that had been assigned by middle-up were completely oxidized. Bottom-up analysis could explain the unexplained less abundant species of Fc/2, LC and Fd' with higher oxidation numbers by the simultaneous occurrence of completely oxidized Met together with other Met that were only partially oxidized and partially oxidized Cys and Trp. In contrast to the middle-up results, no notable difference was observed in the oxidation susceptibility of the bevacizumab biosimilar candidate compared with the RMP. This discrepancy between middle-

up and bottom-up results should be addressed in further studies. The bottom-up experiments were more time-consuming, sample-consuming, and expensive than the middle-up analyses. In conclusion, it is recommended that consistency of the results from the two approaches should be confirmed. If consistency can be established, the middle-up approach can be employed in forced degradation biosimilar studies, with the benefits of lower sample-consumption, faster analysis and lower expenses compared to bottom-up.

7 Zusammenfassung

Die Charakterisierung von posttranslationalen Modifikationen (PTMs) ist ein wichtiger Teil der Qualitätskontrolle von therapeutischen monoklonalen Antikörpern (mAbs). Einer der am häufigsten beobachteten Degradationswege in mAbs ist die Modifikation durch Oxidation [84]. Die vorliegende Arbeit untersucht PTMs mit Fokus auf Oxidation unter Verwendung von zwei Ansätzen, die auf Flüssigchromatographie mit Massenspektrometrie-Kopplung (LC-MS) basieren. Die beiden Ansätze werden verglichen, um zu verstehen, inwieweit die schnellere und weniger für Artefaktbildung anfällige Middle-Up Analytik die aufwändigere Bottom-Up Analytik ersetzen kann. Die Referenzarzneimittel (RMPs) und Biosimilar-Kandidaten der beiden mAbs Bevacizumab und Infliximab wurden einer forcierten Oxidation durch H_2O_2 ausgesetzt, um ihre Oxidationsanfälligkeit zu untersuchen.

In einem ersten Schritt wurde die Middle-Up Analytik etabliert. Die Untersuchung der PTMs von ungestressten Proben, einschließlich der Hauptglykoformen, zeigte eine hohe Ähnlichkeit zwischen den Biosimilar-Kandidaten und den RMPs von Bevacizumab und Infliximab. Die Behandlung mit H_2O_2 erzeugte verschiedene Spezies der Untereinheiten mit unterschiedlicher Anzahl an Oxidationen. Die häufigsten Spezies, die durch forcierte Oxidation verursacht wurden, konnten Methionin-Resten (Met, M) mit Einzeloxidationen zugeordnet werden. Eine ausgeprägte Oxidation des Fc/2 konnte M258, M364 und M434 in Bevacizumab und M255 und M431 in Infliximab zugeordnet werden. Eine ausgeprägte Oxidation wurde auch in der leichten Kette (LC) und in der Fd'-Untereinheit von Infliximab, jedoch nicht in Bevacizumab, beobachtet. Durch den Vergleich der Anzahl der Oxidationen der häufigsten Spezies und der Positionen von Met zwischen den beiden mAbs wurden die Oxidationen in Infliximab M55 in der LC und M18 im Fd' zugeordnet. Weniger häufig vorkommende Spezies von Fc/2, LC und Fd' mit höherer Anzahl an Oxidationen konnten jedoch nicht erklärt werden. Trotz hoher Ähnlichkeit der ungestressten Proben zeigte die forcierte Oxidation eine höhere Anfälligkeit des Bevacizumab-Biosimilars im Vergleich zum RMP.

In einem zweiten Schritt wurde die Bottom-Up Analytik etabliert und verwendet, um die genaue Position der Oxidationen zu bestätigen. Neben einzeln oxidierten Met wurden auch einzeln oxidierte Tryptophan (Trp)- sowie dreifach oxidierte Cystein (Cys)-Reste gefunden. Die Middle-Up Zuordnungen der häufigsten Spezies zu Oxidationen an Met-Resten wurden durch die Bottom-Up Ergebnisse bestätigt: die per Middle-Up Analytik zugeordneten Met-Reste waren vollständig oxidiert. Die Bottom-Up Analytik konnte die ungeklärten, weniger häufig vorkommenden Spezies von Fc/2, LC und Fd' mit höherer Anzahl an Oxidationen durch das

gleichzeitige Auftreten von vollständig oxidierten Met zusammen mit anderen Met, die nur teilweise oxidiert waren, und teilweise oxidierten Cys und Trp erklären. Im Gegensatz zu den Middle-Up Ergebnissen wurde kein signifikanter Unterschied in der Oxidationsanfälligkeit des Bevacizumab-Biosimilars im Vergleich zum RMP beobachtet. Diese Diskrepanz zwischen Middle-Up und Bottom-Up Ergebnissen sollte in weiteren Studien untersucht werden. Die Bottom-Up Experimente waren zeitaufwändiger, verbrauchten eine größere Menge an Probe und waren teurer als die Middle-Up Analysen. Abschließend wird empfohlen, die Konsistenz der Ergebnisse der beiden Ansätze zu bestätigen. Wenn die Konsistenz bestätigt werden kann, kann der Middle-Up Ansatz in forcierten Degradationsstudien zur Biosimilaruntersuchung eingesetzt werden, mit den Vorteilen eines geringeren Probenverbrauchs, schnellerer Analytik und geringerer Kosten im Vergleich zu Bottom-Up.

8 References

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9 Publications

9.1 Publications in scientific peer-reviewed journals

Dyck YFK, Rehm D, Joseph JF, Winkler K, Sandig V, Jabs W, Parr MK. Forced Degradation Testing as Complementary Tool for Biosimilarity Assessment. Bioengineering (Basel) 6 (2019)

https://doi.org/10.3390/bioengineering6030062

Dyck YFK, Rehm D, Joseph JF, Winkler K, Sandig V, Jabs W, Parr MK. Comparison of middle- and bottom-up mass spectrometry in forced degradation studies of bevacizumab and infliximab. Journal of Pharmaceutical and Biomedical Analysis 235(2023)

https://doi.org/10.1016/j.jpba.2023.115596

9.2 Oral presentations

The speaker is emphasized by underlining.

Dyck Y, Rehm D, Joseph JF, Winkler K, Parr MK and Jabs W. Mass Spectrometry Assisted Development of Liquid Chromatography Based Methods for Biosimilar Development and Quality Control. Berliner Hochschule für Technik – Research Day 2018

Parr MK, Dyck Y. Extended analytical workflow for biosimilar development. Glyconet 2019

Dyck Y, Jabs W and Parr MK. LC-MS characterization of therapeutic antibodies - comparison of middle-up and bottom-up approach. St. Nicholas Mass Spectrometry Symposium in Berlin 2022

9.3 Poster presentations

Dyck Y, Rehm D, Joseph JF, Winkler K, Parr MK and Jabs W. Forced Degradation Testing in Biosimilar Development and Quality Control Utilizing Liquid Chromatography Mass Spectrometry. 11th Scientific Symposium of Deutsche Pharmazeutische Gesellschaft Landesgruppe Berlin-Brandenburg for Junior Scientists 2018 Poster P10

Dyck Y, Rehm D, Joseph JF, Winkler K, Parr MK and Jabs W. Mass Spectrometry Assisted Development of Liquid Chromatography Based Methods for Biosimilar Development and Quality Control. Berliner Hochschule für Technik – Research Day 2018 (Tagungsband)

Dyck Y, Rehm D, Joseph JF, Winkler K, Parr MK and Jabs W. Forced Degradation for Biosimilarity Assessment. Glyconet 2019

Dyck Y, Rehm D, Winkler K, Sandig V, Jabs W and Parr MK. Identification of susceptible residues in a monoclonal antibody after forced degradation by peptide mapping. 12th Scientific Symposium of Deutsche Pharmazeutische Gesellschaft Landesgruppe Berlin-Brandenburg for Junior Scientists 2022 Poster P10

10 Declaration of independence

I hereby affirm that I have completed the presented cumulative dissertation independently and without unauthorised assistance. No aids other than those listed in the text were used in the writing of the dissertation.

A doctoral procedure has never been completed at any other university or applied to another department.

Yan Dyck

11 List of figures