Biosimilarity assessment of biopharmaceuticals for quality control

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List of Abbreviations

ADCC	Antibody-dependent cellular cytotoxicity
BHK	Baby hamster kidney
CDC	Complement-dependent cytotoxicity
CE-MS	Capillary electrophoresis-mass spectrometry
CHO	Chinese hamster ovary
CQA	Critical quality attributes
CMC	Chemistry, manufacturing and controls
CDRs	Complementarity-determining regions
CH	Heavy chain constant domain
CL	Light chain constant domain
EPO	Erythropoietin
E. coli	*Escherichia coli*
Fab	Antigen-binding fragment
FVIIa	Activated factor VII
FDA	Food and Drug Administration
Fc region	Fragment crystallizable region
FcRn	Fragment crystallizable region receptor
Fuc	Fucose
Gal	Galactose
Gla	γ-carboxyglutamate
GlcNAc	*N*-acetylglucosamin
GalNAc	*N*-acetylgalactosamin
cGMP	Current Good Manufacturing Practice
HC	Heavy chain
hFSH	Human follicle-stimulating hormone
HOS	Higher order structure
Hya	β-hydroxyaspartate
Hyn	β-hydroxyasparagine
ICH	International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use

IgG	Immunoglobulin
IdeS	Immunoglobulin-degrading enzyme from Streptococcus pyogenes
LC	Light chain
LC-MS	Liquid chromatography-mass spectrometry
mAbs	Monoclonal antibodies
MALDI-TOF/TOF-MS	Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass spectrometry
MS	Mass spectrometry
nLC-ESI-MS/MS	nano Liquid Chromatography-Electrospray Ionization-Mass Spectrometry
Neu5Ac	N-acetylneuraminic acid
Neu5Gc	N-glycolylneuraminic acid
NS0	Nonsecreting murine myeloma
NMR	Nuclear Magnetic Resonance
PTMs	Post-translational modifications
Pyro-Glu	Pyroglutamate
TNFR	Tumor necrosis factor receptor
TWIMS	Traveling wave ion-mobility spectrometry
UHPLC-QTOF-MS^{E}	Ultra-High Performance Liquid Chromatography Quadrupole Time-of-Flight Mass Spectrometry
UHPLC-IMS	Ultra-High Performance Liquid Chromatography Ion-Mobility Spectrometry
VH	Heavy chain variable domain
VL	Light chain variable domain

1 Introduction

1.1 Terminology of biopharmaceuticals

Descriptions such as 'biologic', 'biotechnology medicines' and 'products of pharmaceutical biotechnology' or 'biopharmaceutical' are all terms commonly written in the pharmaceutical literature. However, these pharmaceutical terminologies are used interchangeably but according to Walsh [1] define different terms to different subjects.

The term 'biologic' might be referred to any biotechnology-derived pharmaceutical product. However, in the pharmaceutical sector it is applied to medicinal products that are isolated from blood, as well as toxins, vaccines and allergen products. However, this strict definition does not include some traditional biotechnology-derived pharmaceutical products such as hormones, antibiotics and plant metabolites [1].

Most of the recently approved biopharmaceuticals represent drugs that are produced by biotechnological processes involving recombinant DNA. In accordance with the Merriam-Webster´s definition, "biotechnology" means "the manipulation (as through genetic engineering) of living organisms or their components to produce useful, usually commercial, products. The term "biopharmaceutical" defines "a pharmaceutical derived from biological sources and especially one produced by biotechnology". Hence, the terms "biotechnology-derived" and "biopharmaceutical" can be used interchangeably to define biologics manufactured by genetically altered living system [2].

1.2 Biopharmaceuticals

Nowadays, biopharmaceuticals represent one of the main pillars of industrial biotechnology and are the fastest growing and exciting field within the pharmaceutical sector. The term 'biopharmaceutical' was first used in the 1980s to describe a revolutionary class of protein-based drugs, manufactured by genetic engineering with modern biotechnological techniques. In 1982, Humulin®, a recombinant DNA-derived human insulin manufactured in *Escherichia coli* (*E. coli*) developed by Genentech and Eli Lilly, has received approval in the United States [3]. After reaching a production volume of 40,000 L, the era of large-scale cell culture started to appear [4]. Since highly glycosylated biopharmaceuticals like tissue plasminogen activator, erythropoietin (EPO), and factor VIII cannot be produced in *E. coli*, mammalian cells became the gold standard in modern pharmaceutical biotechnology later [5].

Köhler and Milstein developed monoclonal antibodies (mAbs) as biopharmaceuticals to treat rheumatoid arthritis and cancer in 1990s [4]. Invention of mAbs as a pharmaceutical resource

resulted not only in the production of the first therapeutical antibody, Orthoclone OKT3, of Johnson & Johnson in 1986, but also the development and approval of other mAb-based biopharmaceuticals to treat rheumatoid arthritis and cancer [4]. The first mAb (OKT3) possesses a murine structure, however, eight years later Eli Lilly launched the first chimeric antibody fragment ReoPro®, which was followed by another chimeric mAb (Fig. 1) with the name Rituxan®/Mabthera® (Roche/Biogen Idec). Afterwards, the first humanized antibody Zenapax® (Roche) was approved in 1997. Finally, Abbott introduced the technology for fully human mAb with its biotherapeutic Humira® in 2002.

The pharmaceutical industry is changing due to the clinical and commercial progress of biopharmaceuticals such as mAbs and recombinant endogenous proteins. In 2009 six products, namely Enbrel®, Rituxan®, Remicade®, Humira®, Avastin®, and Herceptin®, were the top blockbusters in the pharmaceutical market with annual sales of more than US$ 30 billion [4, 6-9]. In 2023, the biopharmaceuticals global sales market is expected to reach US$ 341.16 billion [10].

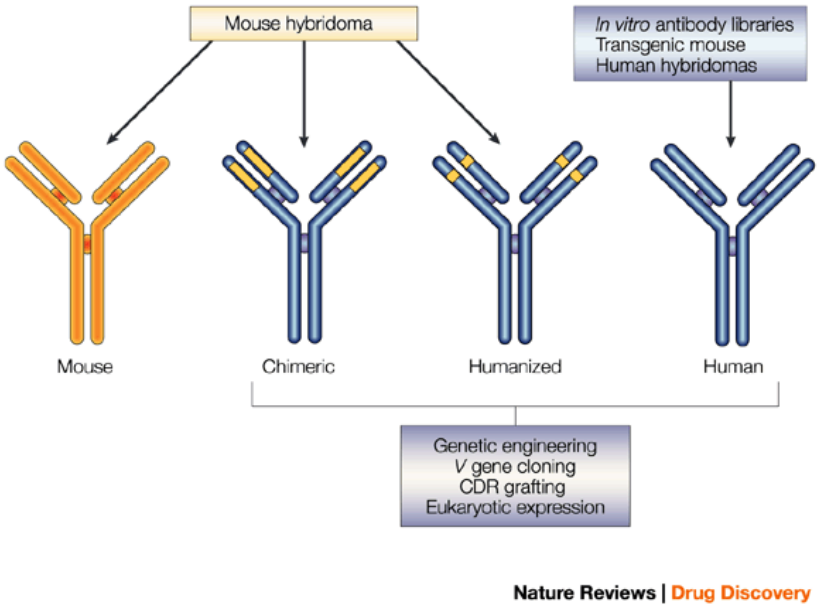


Figure 1. **Antibody engineering.** Antibody engineering starts with mouse hybridoma technology, which generates mouse mAbs. Genetic engineering enables generation of chimeric, humanized and human antibodies. Cloning of mouse variable genes into human constant-region genes revealed chimeric antibodies, whereas humanized antibodies are generated by the insertion of CDRs onto human constant and variable domain frameworks. Fully human antibodies can be produced by selection of human antibody fragments from *in vitro* libraries, and by selection from human hybridomas [11].

The classical lock-and-key model of pharmacological action is mainly described by small molecules, mostly acting as antagonists of key binding. Same mode of action can be performed with biomolecules, but its pharmacology is often more diverse. In hormone and enzyme replacement therapy, the biomolecule may play the key (ligand binding) or could react as a lock (an extracellular portion of a receptor), playing a major role in decoy therapy. Furthermore, it can even play a crucial role during enzymatic cascade processes [12].

The submitted dissertation depicts three different biopharmaceuticals called Rituximab, Etanercept and activated factor VII (FVIIa) with different modes of action. While Rituximab reacts as chimeric mAb directed against CD20 surface antigen of B lymphocytes in the Non-Hodgkin's lymphoma, Etanercept functions as a competitive inhibitor of TNFα, a major pro-inflammatory cytokine. This decoy therapy prevents TNFα from binding to its physiological receptor on the cell surface. Finally, FVIIa is used in replacement therapy, where it could directly activate the final common steps of the coagulation cascade [12].

1.3 Structure of mAbs

The predominantly marketed pharmaceutical mAb are of the immunoglobulin G1 (IgG1) type. IgG2 and IgG4 represent only a minor part of mAb-based therapeutics. Since IgG3 has a short half-life, susceptibility to proteolysis in the hinge region and extensive allotypic polymorphism, it is still not used as a pharmaceutical mAb [13]. The fragment crystallizable region (Fc) and two identical antigen-binding fragments (Fabs), which are connected via a flexible hinge region, are responsible for IgG´s function. The complementarity-determining regions (CDRs), located in the variable regions of light chain (LC) and heavy chain (HC) play a major role in antigen binding. The Fc domain protects the whole mAb from degradation, ensures a long half-life and interacts with the neonatal Fc receptor (FcRn). Furthermore, Fc domains of IgG1 and IgG3 bind to various Fcγ receptors and other components of the immune system, such as complement proteins, resulting in immune-mediated effector functions [14].

1.4 Biosimilarity

1.4.1 Biosimilars

A biosimilar is defined as a medicinal product that is highly similar to an already approved reference product. Apart from minor variations in clinically inactive components there should be no clinically meaningful differences between follow-on and reference medicinal product

concerning safety, purity and potency. Taken this definition in consideration, a biopharmaceutical is biosimilar to a reference biodrug if it is highly similar to the reference product in safety, purity and potency. While purity is related to some critical quality attributes (CQA) within the manufacturing process, the potency is depending on stability and efficacy of the biosimilar medicinal product [15].

Unlike generics of chemical drugs, which have clearly defined manufacturing processes and generally do not require preclinical and only abbreviated procedures in clinical trials for regulatory approval, biopharmaceuticals cannot be generic due to the biological system used for its production. Complexity of the manufacturing process of the biopharmaceutical itself and structural complexity of the molecule make an exact copy of a biopharmaceutical impossible. Therefore, follow-on products require clinical trial data to demonstrate their biosimilarity [2, 16]. Demonstration of molecular and biological similarity of biosimilars and their reference products by chemistry, manufacturing and controls (CMC) comparison is indispensable. But the totality-of-the-evidence approach (comparability data of CMC, nonclinical and clinical studies) can ensure biosimilarity and approval of a follow-on biodrug [2, 17].

1.4.2 CMC Development

The manufacturing process of follow-on medicinal products begins with the development of a cell line. As an expression system three expression systems (bacteria, yeast and mammalian cells) are usually selected in biotechnological industry. After insertion of the appropriate DNA sequence, cell screening and selection is performed to establish a desirable cell bank [18], which should be comprehensively characterized to demonstrate microbiological purity and identity of the biopharmaceuticals [19]. Establishment of a robust and scalable up-stream and down-stream process is indispensable. However, achievement of high yields requires optimization of culture media and growth conditions and efficient extraction and recovery procedures. This should be accompanied with in-process verification, which serves to increase productivity without alteration of primary sequence (amino acid sequence) and post-translational modifications (PTMs). Solubilization and refolding of insoluble biopharmaceuticals are essential for therapeutic proteins, which could aggregate during the manufacturing process. In addition, variations in cell bank and manufacturing processes might cause impurities that are different from the reference biopharmaceutical. Down-stream

processing needs to remove all possible impurities such as host-cell proteins, DNA, viruses, medium constituents and metabolic byproducts [15, 18].

Clinical and commercial use of each batch of biotechnology medicines should be tested in compliance with current good manufacturing practice (cGMP) to ensure good drug characterization with respect to identity, potency, quality, purity, and safety. Pre-defined specifications of the biopharmaceutical product need to be assured. Especially, purity is often assessed by multiple assays, which investigate product-related variants (biologically active) or product-related impurities (biologically inactive). Hence, setting up product specification is crucial for a biosimilar development within the variation of the reference biopharmaceutical for quality control and assurance [15, 20]. Every manufacturing batch should be investigated for biopharmaceutical product characterization, which include amino acid sequence, isoform profiles, product variants, heterogeneity, product and process-impurities and higher order structures (HOS).

1.4.3 Setting Specifications

Specifications of the medical product are consisting of multiple CQA, which are essential elements to draw a quality-by-design approach. ICH (International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use) Q8 (R2) on pharmaceutical development points out the following regarding CQA, distributions, and limits: "*A CQA is a physical, chemical, biological, or microbiological property or characteristic that should be within an appropriate limit, range, or distribution to ensure the desired product quality"* [21]. According to Alt et al. the assessment of CQA of drug substance is based on the impact of the quality attribute on potency, safety and immunogenicity [21, 22].

Final biopharmaceutical CQA may be influenced by manufacturing process conditions Therefore, the definition and assessment of biopharmaceutical CQA is crucial, even if they are not directly connected to drug substance or drug product process performance. Since process parameters have a substantial impact on product-related substances or product-related impurities, ICH guidance Q8(R2) and Q11 on development and manufacture of drug substances (Chemical entities and biotechnological/biological entities) state that correlation between process and drug substance's CQA should be extensively analyzed and determined during process development [21, 23].

The purpose of CQA assessment is defining the influence of a specific alteration on bioactivity, pharmacokinetics/pharmacodynamics, immunogenicity and safety in a systematic and rational manner. In biosimilarity assessment, the comparability protocol should contain a risk assessment study to demonstrate the effects of proposed changes on all product CQA. Despite the non-CQA may not impact directly the product, they must still be considered in comparability for monitoring process robustness and consistency. As mentioned in several guidelines the structure-activity relationship and the correlation between quality attributes and safety and efficacy should be taken into consideration for comparability studies [24-29].
Biosimilars and reference biopharmaceuticals have different manufacturing processes and different analytical procedures. Furthermore, different laboratories are used for the assays. Hence, the attributes of the biosimilar may not be identical to the reference biopharmaceutical. However, the specifications should include and control CQA of the reference product. The setting of specifications should be based on the applicant´s experience with the biosimilar and the results of comparability studies. Several lots of follow-on biopharmaceutical must be used in specification setting, in which the applicant should prove that the set of limits for given specifications are within the range of variability of the reference product over the shelf-life of the biopharmaceutical product, except if it is justified to be otherwise [30].

1.4.4 Comparability Study

The scope of comparability studies is to compare the pre- and post-change products in terms of quality, safety and efficacy, as stated in guidance documents [31-34]. Some CQA would be considered comparable if they are within a predefined window, imposed either by regulators for commercial products or by the manufacturer during the earlier stage clinical development. Harmonized tripartite guidelines referred to as ICH Q5E (Comparability of Biotechnological/Biological Products Subject to Changes in their Manufacturing Process) are the most existing industry standards, which were developed for the comparability exercise of biopharmaceuticals. They were issued to support innovators of biopharmaceutical products to develop internal comparability during pre- and post-changes for their bioproducts. Here, this type of comparison will be referred to as an ‘internal’ comparison [34].
Performing a comparability study of a biopharmaceutical product after a process change from batch to batch within a company can be difficult. In addition, comparing two biopharmaceutical products from different manufacturers in order to demonstrate

biosimilarity is challenging. Since biosimilars differ from generics (small molecules), the demonstration of biosimilarity requires extensive analytical tools and non-clinical and clinical studies. Here, this type of comparability is referred to as an 'external' comparison [2, 34].

Any change conducted in an existing manufacturing process from biosimilar or reference product manufacturer may lead to unexpected variations in analytical studies. Giving an interpretation of practical significance of any alteration in their process-related and product-related impurity profiles is even more challenging. Since biosimilar development faces much more variations, the biosimilar producer will have to demonstrate an extensive analytical biosimilarity study [2].

Notwithstanding, ICH Q5E (comparability of biotechnological/biological products subject to changes in their manufacturing process) is issued for comparing pre- and post-changes of biotechnology-derived products [28], but some described scientific principles can also be applied to investigate the biosimilarity between a proposed follow-on product and its reference molecule.

Biosimilarity assessment in comparison to the comparability study requires more extensive and comprehensive data. Since the biosimilar manufacturer has a different manufacturing process than the reference product manufacturer, he will certainly have different cell lines, raw materials, equipment, processes, process controls and acceptance criteria. In February 2012, Food and Drug Administration (FDA) stated in its guidance for industry (Quality Considerations in Demonstrating Biosimilarity to a Reference Protein Product) [35] that "*The applicant should characterize, identify, and quantify impurities (product- and process-related as defined in ICH Q6B) in the proposed biosimilar product and the reference product. If comparative physicochemical analysis reveals comparable product-related impurities at similar levels between the two products, pharmacological/toxicological studies to characterize potential biological effects of specific impurities may not be necessary*".

1.5 Post-translational Modifications (PTMs)

PTMs are physiological modifications of proteins after the translation process in cells. In the case of biopharmaceuticals PTMs may also occur during the manufacturing process. As PTMs could lead to protein alterations, which may influence bioactivity and immunogenicity of biopharmaceuticals, it is crucial to analyze them and to understand their impact to the functions of biopharmaceuticals.

Figure 2 exemplarily depicts the structure and some of its possible PTMs of the mAb Rituximab. The potential PTMs found in Rituximab mAb are N-terminal glutamine (Q1), which may form N-terminal pyroglutamic acid, clipping of the C-terminal lysine, N-glycosylation and disulfide bonds [36].

To control the reproducibility of the production of the pharmaceutical protein, the manufacturer should monitor PTMs during multiple steps of the production process, control of their levels and assess their functional influence on the biopharmaceutical. The variability levels of PTMs, which are permitted in the development or commercial phase of the biopharmaceutical, can differ and are commonly defined by information concerning their impact and manufacturing history. However, the real clinical influence of alteration in the amount, level and forms of the diverse PTMs is unknown [34].

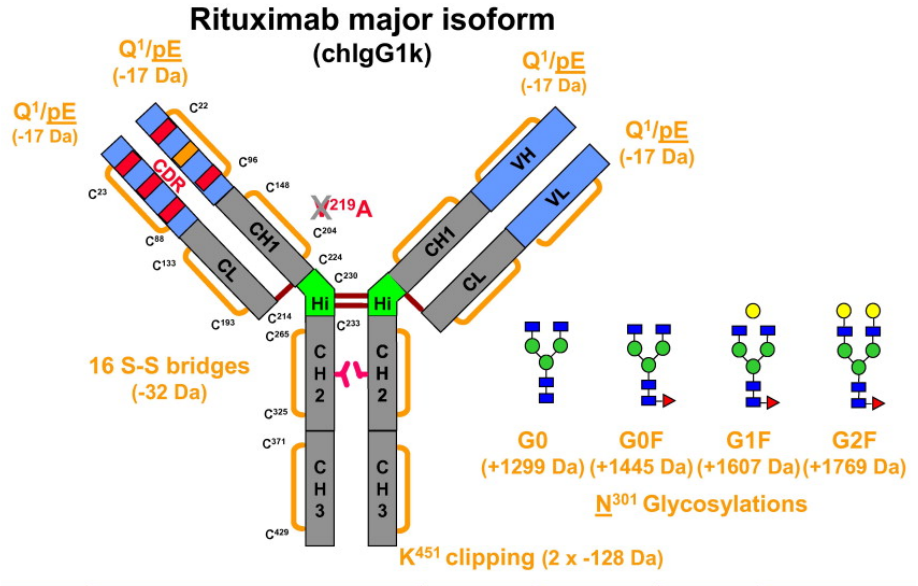


Figure 2: **Schematic structure of rituximab and its potential PTMs.** HCs and LCs are connected via one and the HCs via two disulfide bonds (S-S). The latter ones are located within a short hinge domain. 12 additional intramolecular disulfide bonds delimit six different globular domains: one variable (VL) and one constant domain for the LC (CL); and, one variable (VH) and three constant domains for the HCs (CH1, CH2 and CH3). LC and HC of rituximab have N-terminal glutamine (Q1), which may form N-terminal pyroglutamic acid (17 Da, labeled Q1/pE). Another major modification occurring in recombinant mAbs is clipping of the C-terminal lysine [36].

The evaluation of a potential biopharmaceutical requires identification of the consequences of each PTM (or altered PTM profile) on the function and safety, rather than analysis of its structure alone. Sometimes, the probable influence of changing or omitting a PTM may

already be predicted from structural analysis [37]. However, the real impact of any change in the PTM profile should be ensured via clinical studies. Acceptance of comparability protocols by FDA issued data concerning the development of biosimilar products (these may vary in PTM profile as compared with the reference product). Comparability protocols enable the introduction of alterations in post-approval phase of reference product manufacturing by application of a reduced data dossier to demonstrate comparability with the reference molecule [38].

The most familiar PTMs that biopharmaceutical industry is facing during the production and the storage are exhibited in Table 1. Each attribute is explicitly debated (in the section below) in terms of the chemical nature, and its impact on structure, stability, efficacy ogenicity and *in vivo* half-life [24].

1.5.1 N-Terminal Modifications

Pyroglutamate (pyro-Glu) is an N-terminal modification that occurs frequently in LC or HC of a mAb. The formation of pyro-Glu is caused via spontaneous cyclization of glutamine (Gln) or glutamate (Glu) side chain onto the N-terminal α–amine. Forming of a thermodynamically favored five-membered ring is thereby preferred by Gln [39-42].

The existence of N-terminal pyro-Glu or a (truncated) leader sequence is reported neither to impact the structure and function of pharmaceutical proteins nor its potency [39]. Since endogenous human IgGs are entirely modified by pyro-Glu [43], immunogenicity concerns for this modification are minimal. However, charge variants caused by both modifications may impact the inter-molecular interaction and potentially lead to aggregation.

A second N-terminal modification may occur by miscleavage of the signal peptide. N-terminal elongation or truncation resulting from miscleavage of the signal peptide is not desirable for mAb production, since it generates product heterogeneity. Untruncated or partially truncated leader sequence, which has a hydrophobic nature, may cause aggregates and remains an unknown factor concerning immunogenicity [24].

Table 1: Common PTMs of mAbs and their potential effects on stability, function, immunogenicity and pharmacokinetics/pharmacodynamics as well as their extended characterization assays [36].

Attributes	Potential Impact	Extended Characterization Assays
N-terminal modifications • Pyro-Glu • non-cleaved signal sequence • Truncation	N-terminal modifications generate charge variants. They are considered to be low risk to comparability because of lack of impact on efficacy and are not expected to impact safety.	LC-MS or CE-MS analysis of • intact mAb • reduced mAb • IdeS digested antibodywith/without reduction • peptide mapping
C-terminal modifications • Truncation of C-terminal lysine • Amidation	C-terminal modifications generate charge variants. They are considered to be low risk to comparability because of their low percentage and lack of impact on efficacy and are not expected to impact safety.	
Glycation	Glycation is a common modification leading to the generation of acidic species. Glycation in complementarity-determining regions (CDRs) can potentially decrease potency. Glycation in general increases mAb propensity towards aggregation.	
Fc-glycosylation • Sialic acid • α1,3-Gal • Terminal Gal • Absence of core-fucosylation • High mannose	• *N*-Glycolylneuraminic acid (Neu5Gc) is immunogenic • α1,3-Gal on Fab oligosaccharides is immunogenic • The presence of galactose enhances complement-dependent cytotoxicity (CDC) and possible shorter half-life if exposed. • The absence of core-fucose enhances antibody-dependent cell mediated cytotoxicity (ADCC) • mAbs with high mannose show enhanced ADCC and shorter half-life	1) LC-MS or CE-MS analysis of • intact mAb • reduced mAb • IdeS digested antibody with/without reduction • peptide mapping 2) Hydrophilic interaction chromatography with fluorescence and MS detection
Asn deamidation	Deamidation in CDR can potentially decrease potency.	LC-MS or CE-MS analysis of peptide mapping
Asp isomerization	Isomerization in CDR can potentially decrease potency.	
Succinimide	Succinimide in CDR can potentially decrease potency.	
Met and Trp Oxidation	Oxidation in CDR can potentially decrease potency. A substantial amount of oxidation around FcRn binding site can potentially decrease its binding affinity and result a in shorter half-life.	
Cysteine-related variants • Disulfide isoforms • Free cysteine • Trisulfide bond • Thioether, D-cysteine, cysteinylation Glycation	IgG2 disulfide bond isoforms may impact potency. Higher amounts of free cysteines decrease mAb thermal stability and trigger formation of covalent aggregates. Other modifications such as thioether, D-cysteine, and cysteinylation are considered low risk because of their low levels or natural presence in humans.	1) Disulfide bond confirmation: non-reduced peptide mapping 2) Free cysteine: labeling with Ellman's reagent (DTMB)
Fragments	Fragments are considered as low risk because of their low levels.	1) Ion mobility mass spectrometry 2) Asymmetrical flow field-flow fractionation
Aggregates	Aggregation can potentially cause immunogenicity and loss of efficacy. It is a high-risk factor for comparability.	

1.5.2 C-Terminal Modifications

Truncation of C-terminal Lys and C-terminal amidation are the main C-terminal modifications. IgGs are initially produced bearing two terminal Lys residues. Cell culture conditions influence the cleavage of C-terminal Lys from recombinant mAbs, which leads to a final product with either zero, one or two C-terminal Lys residues. This outcome can be readily analysed by charge-based methods such as cation-exchange chromatography, since Lys residues carry positive charges [40].

The three-dimensional structures of antibody fractions with either zero, one or two C-terminal Lys residues were comparable as shown by hydrogen-deuterium exchange [44] or differential scanning calorimetry [45]. Furthermore, C-terminal Lys and C-terminal amidation modifications are located at the end of HC and distant from the antigen, neonatal Fc receptor (FcRn), and Fcγ receptor binding sites, and was reported to not influence mAb´s stability, function, or antigen binding [39, 46]. However, a recent work points out that truncation of C-terminal Lys is essential for an effective C1q binding and complement-dependent cytotoxicity (CDC) [47]. However, it is rapidly enzymatically removed from therapeutic mAbs in the bloodstream of humans [48], hence, a biological function of C-terminal Lys remains unclear. This is in line with Jiang et al., who demonstrated that mutant proteins lacking C-terminal Lys have no effect on potency, bio-availability, and pharmacokinetics, but may have decreased thermal stability in some specific mAbs [24, 49].

1.5.3 Deamidation

Deamidation of Asn is one of the most common PTM in pharmaceutical proteins, which can spontaneously occur at neutral to basic pH and high temperature. This facilitates the generation of a 3-to-1 ratio of isoaspartate (isoAsp) to Asp [50]. This PTM is favored by some up-stream and down-stream processing steps under stress conditions in accelerated stability studies and post administration by injection in human and animal subjects. Deamidation of Asn in the CDRs of a mAb may decrease the antigen binding affinity [51-53]. However, this modification occurs mainly in the constant domains of LC and HC of mAbs [39, 54-57]. A huge effort should be accomplished by bioengineering and assessment studies (in-process analytic) to avoid the deamidation risk of CDRs.

The impact of this modification is determined by localization of the particular Asn residues and the resulting products. Convertion of Asn to Asp or isoAsp leads to negative charges, which influence the structure and stability of biopharmaceuticals. Furthermore, formation of

isoAsp may contribute to increased structural changes compared to Asp through the insertion of a methylene group to the peptide backbone. Asn modification to Asp increases, whereas formation of isoAsp decreased Fab thermal stability [52]. Not only loss of potency but also concerns about immunogenicity is rising due to occurrence of deamidated products, especially with respect to isoAsp [24, 58].

1.5.4 Oxidation

Several amino acid residues are prone to be oxidated. The most common oxidation of recombinant protein takes place at Met [59-62] and Trp [63, 64] residues. In human IgG, two Met residues located close to the CH (heavy chain constant domain) 2-CH3 domain interface, including parts of the FcRn binding site, have been confirmed in their tendency to oxidation [59, 65, 66].

Structural changes especially in the CH2 domain are the main consequences of Met residues oxidation, which decreases IgG thermal stability [59, 65, 66] and enhances aggregation tendency [65]. Although oxidation does not influence Fcγ receptor affinity [62], oxidation of Met residues impacts CDC activity [59], *in vivo* half-life [67] and binding affinity to FcRn [62]. Trp oxidation in the CDR decreases thermal stability of IgG, enhances tendency towards aggregation and diminishes antigen binding [64, 68]. In addition, it induces yellow coloration as a result of kynurenine formation.

1.5.5 Glycation

Glycation occurs under physiological conditions as a non-enzymatic reaction between a reducing sugar and the N-terminal amine group or primary amines of Lys side chains within biopharmaceuticals [69, 70]. It may arise under stress conditions during accelerated formulation studies [71], storage [72, 73] and administration [72], and even during blood circulation [74]. It has been demonstrated that glycation of Lys residues has no impact on potency or pharmacokinetics [69, 75, 76]. However, some studies point out that glycation accelerates aggregation and impacts coloration of biopharmaceuticals [71, 77]. Thus, a case-by-case study should be performed to investigate the effects of glycation, which could be related to its site (e.g. whether it is located within the CDRs or not) or maybe molecule-dependent [24].

1.5.6 γ-Carboxylation and β-Hydroxylation

A small number of proteins are γ-carboxylated or β-hydroxylated, especially those proteins involved in blood coagulation [37, 78]. Specific carboxylase and hydroxylase enzymes carry out both modifications. Glu residues are converted to γ-carboxyglutamate (Glu → Gla) and Asp residues to β-hydroxyaspartate (Asp → Hya) or Asn residues to β-hydroxyasparagine (Asn → Hyn). These PTMs could be detected by high-performance liquid chromatography analysis of protein hydrolysates [79] and mass spectrometry [80].

γ-Carboxylation is required for calcium ion binding, activation of protein C and protein S of the anticoagulant system and plays a crucial role for the function of blood factors VII, IX and X. β-Hydroxylation is also meaningful for blood factors VII and IX as well as activated protein C. In case of native blood factor VIIa, which is a 406–amino acid glycoprotein, ten potential γ-carboxylation sites and a single potential β-hydroxylation site exits. Some studies demonstrated that both modifications are essential to maintain bioactivity of activated protein C [37].

1.5.7 Glycosylation

About 1-2% of the human genome encodes glycoproteins that are N- or O-glycosylated. However, glycosylation is one of the main and most complex PTM on cell membranes and secreted eukaryotic proteins [81]. About 30% of marketed biopharmaceuticals are glycoproteins. Taking into consideration the glycosylation issue, manufacturing of pharmaceutical glycoproteins in eukaryotic cell systems such as chinese hamster ovary (CHO), nonsecreting murine myeloma (NS0) or baby hamster kidney (BHK) cells are required. Carbohydrates bind co-translationally at specific Asp (N-linked) or serine/threonine (O-linked) residues. The consensus sequence Asn-X-Ser/Thr is crucial for N-glycans. Glycosylation is a heterogenous PTM, so multiple glycoprotein species are commonly generated [82]. Attachment of several different oligosaccharides at O- and N-glycosylation sites (microheteogeneity) and the incomplete occupation of glycan binding sites (macroheterogeneity) result in a huge number of variations. This structural diversity is substantially discussed regarding its functional significance [37]. Incomplete enzymatic trimming of the attached core sugar side chains leads to inhomogeneity of monosaccharide sequences. Therefore, a reproducible and consistent batch-to-batch glycan pattern remains a serious challenge for all manufacturers within the biopharmaceutical industry. Glycosylation

may affect secretion, activity, immunogenicity and stability of several biotherapeutics in multiple therapeutic classes [37].

1.5.7.1 N-Glycosylation

N-glycans are attached by a β-glycosidic bond via an *N*-acetylglucosamine (GlcNAc) residue to the nitrogen atom of an Asn residue in the polypeptide [83-86]. In biopharmaceuticals, three types of N-glycans (Fig. 3), namely high-mannose, complex and hybrid [83, 86] may be present. All of them share an identical core structure, which comprises two GlcNAc and three Man residues. In case of high-mannose type N-glycans additional Man structures are added to the pentasaccharide core, while in the complex-type N-glycans various combinations of galactose (Gal), *N*-acetylneuraminic acid (Neu5Ac, sialic acid), GlcNAc and fucose (Fuc) are added to the terminal Man residues of the core structure.

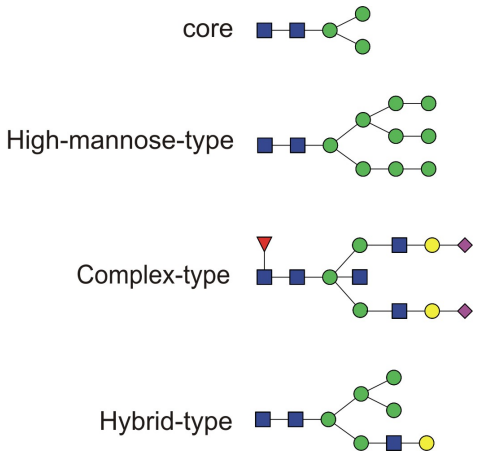


Figure 3: **Structures of types of N-glycans.** The core structure of all N-glycans consists of three mannose and two GlcNAc units, to which additional mannose units are added in case of the high-mannose N-glycans. In complex-type N-glycans GlcNAc, Gal, Fuc and sialic acids are added. The hybrid-type possesses characteristics of the two other types. Blue square represents GlcNAc, green circle Man, yellow circle Gal, red triangle Fuc and pink diamond Neu5Ac.

The nomenclature of glycans is given according to the number of GlcNAc bound to the terminal Man units of the core, such as mono, bi-, tri- or tetraantennary N-glycans [84, 85]. Galactosylated N-glycans result from additional binding of Gal or N-acetylgalactosamine (GalNAc) residues to those GlcNAc units. The reducing GlcNAc at the core structure as well as antennary GlcNAc and Gal may further be fucosylated. Furthermore, sialic acids bound to

the Gal structure expand the structural diversity of complex N-glycans. The hybrid-type subgroup merges characteristics of high-mannose- and complex-type N-glycans [86]. Complex-type N-glycans may bear a bisecting GlcNAc residue, which is added to the β1-4-linked Man of the core structure. The resulting carbohydrate structure is depending on cell type and its development stage, the protein acceptor, and the availability of monosaccharides [87].

1.5.7.2 O-Glycosylation

In contrast to N-glycans, O-glycans are α-glycosidically linked via a GalNAc monomer to the oxygen atom of Ser or Thr and may influence the shape of the protein [88, 89]. The biosynthesis of O-glycans is a post-translational process and no general consensus sequence has been identified so far [90, 91]. The most common O-glycosidic bond is the mucin-type GalNAc-α-Ser/Thr. Mucins are glycosylated proteins with a high molecular mass (> 200 kDa), which have a large proportion of about 50-80 % of carbohydrates. Different core structures of O-GalNAc-linked mucin-type glycans are available (Table 2). Further O-glycans such as O-linked Fuc and O-linked Man are also found in nature [92].

Table 2: Core structures of mucin-type O-glycans. Their structure has a GalNAc unit that is α-linked to Ser or Thr in common.

***core*-type**	**Structure**
core 1	Gal(β1-3)GalNAc-α-Ser/Thr
core 2	GlcNAc(β1-6)[Galβ1-3]GalNAc-α-Ser/Thr
core 3	GlcNAc(β1-3)GalNAc-α-Ser/Thr
core 4	GlcNAc(β1-6)[GlcNAc(β1-3)]GalNAc-α-Ser/Thr
core 5	GalNAc(α1-3)GalNAc-α-Ser/Thr
core 6	GlcNAc(β1-6)GalNAc-α-Ser/Thr
core 7	GalNAc(α1-6)GalNAc-α-Ser/Thr
core 8	Gal(α1-3)GalNAc-α-Ser/Thr

1.5.7.3 Functional impact of glycans on recombinant glycoproteins

Glycosylation of the CH2 in the Fc region is essential to uphold IgG structure and stability [93, 94] and to modulate Fc-mediated biological functions in specific cases [95-98]. The main heterogeneity of mAbs is caused by N-glycosylation, which increases the variation within the levels of sialylation, galactosylation, fucosylation and high-mannose type of glycans. Additional glycosylation of Fab rarely occurs in recombinant mAbs and is

commonly characterized by more complex and heavily sialylated N-glycans compared to Fc N-glycans [24].

Aglycosylation of mAbs occurs in low but constant amount during manufacturing, which may result in substantial conformational variations, reduced stability or almost failure of Fc effector-triggered biological functions such as antibody-dependent cellular cytotoxicity (ADCC) and CDC [94, 98-100]. However, clinical trials revealed comparable half-life for aglycosylated and glycosylated mAbs [99].

Five to nine Man are usually attached to the core GlcNAc of high-mannose N-glycans. High-mannose type N-glycans are available at approximately 0.1% in human endogenous IgG [101], but they could increase up to 10% in level in mAbs [102]. Slight conformational alteration at the glycosylation site was found by comparing mAbs of high-mannose and complex N-glycans [102-104]. High-mannose structures in mAbs decrease thermal stability but do not influence long term stability [104] or the tendency towards aggregation by thermal stress [105]. High-mannose glycans have no impact on antigen binding [106]. However, they lead to reduced binding of type I [107, 108] and type II [109] Fcγ receptors, and to deficiency in C1q binding and complement activation [106-110]. Finally, the presence of high-mannose glycans decreases *in vivo* half-life in both animal models and in humans [106-112].

The most abundant three glycoforms in the Fc part are biantennary complex carbohydrates with either zero, one or two terminal Gal [102, 113]. A comparable galactosylation pattern is present in human endogenous IgG [24, 101]. Slight conformational variations depend on the galactosylation level [94-96, 114, 115], but have no influence on the protein propensity to aggregation under thermal stress [105, 116]. Galactosylation of the Fc glycans does not affect the clearance of the mAbs *in vivo* [108, 111, 112]. However, glycans at Fab region with a high level of Gal, which are not capped by sialic acids, may lead to a reduction of half-life due to binding to the asialoglycoprotein receptor [117]. Recently conducted studies demonstrated, that galactosylation of the Fc part has a confined impact on ADCC activity, but some effect on CDC [118, 119]. Regarding safety, it is essential to mention that mAb N-glycans may additionally bear α1,3-linked Gal, forming the highly immunogenic αGal-epitope [120, 121].

α1,6-linked Fuc attached to the core GlcNAc in complex glycans is the most abundant pattern in human IgG [101]. Similar structures are found in recombinant mAbs together with

a low amount of afucosylated glycans. Alteration of the fucosylation quantity induces subtle structural variations around the glycosylation site only [96, 104, 122, 123], and has a slight impact on binding of antibodies to FcγRI, FcγRII, C1q, and FcRn [124, 125]. However, minimized core-fucosylation leads to enhancement of antibody binding to FcγRIIIa [124-127] and strongly increases ADCC activity [124-126, 128]. This positive impact of reduced core-fucosylation on the ADCC activity leads to higher efficacy in animal disease models [126, 127] and improvement of mAb´s mechanism of action in tumor therapy in human subjects [129, 130]. Since core-fucosylation is crucial for ADCC, monitoring its degree is essential for effector functions, especially when ADCC is involved in the therapeutic effect or for targeting cell surface antigens by means of antibodies [14].

Neu5Ac is the most abundant sialic acid in human endogenous IgG [24, 113]. It is bound to terminal Gal trough an α2,3- or α2,6-linkage. In CHO cells, the most common cell system for the production of recombinant glycoproteins, Neu5Ac is found only in α2,3- linkages [131]. Biotherapeutics manufactured in murine NS0 and mouse myeloma cell lines contain the potentially antigenic *N*-glycolylneuraminic acid (Neu5Gc) [24, 113]. Fc-glycans of mAbs present low amounts of sialic acids (about 5%) [102, 113, 132]. However, the rare N-linked glycans at the Fab region may contain relatively high levels of sialic acid [132], ranging between 11% and 15% in human endogenous IgG [101, 113, 133]. Current studies pointed out that the existence of low degree of Fc-sialylation only cause a slight conformational variation [103, 134-138] in comparison to their asialylated counterparts. Moreover, the aggregation propensity under thermal stress is not influenced by the presence of Fc-sialylation [105]. This latter has no impact on antigen binding [139-141] and no impact on FcγR1 binding, ADCC and CDC [108, 139, 142].

However, modifications leading to an elevated level of sialylation have been shown to be effective in increasing the half-life of other biotherapeutics such as erythropoietin [143]. The longest half-life is observed on highly branched, highly sialylated glycoforms. Glycosylation supports *in-vivo* targeting and trafficking of a newly synthesized protein to its final destination. For example, the removal of EPOs N-linked glycosylation sites leads to a product that will be secreted very poorly from a producer cell. Human follicle-stimulating hormone (hFSH) predominantly bears sialic acid as the terminal moiety in glycans, which affects the hormones's survival in the circulation [144]. Exposure of terminal Gal residues affects the mechanism of hepatocyte receptors towards asialo-galactose-terminated glycans, leading to a

dramatical increase of glycoprotein clearance [144]. Finally, sulfated glycans present in Luteinizing Hormone and hFSH accelerate clearance of the glycoprotein [144].

1.6 Molecular Structure Analysis

The ICH-guideline Q6B (specifications: test procedures and acceptance criteria for biotechnological/biological products) summarizes a set of specifications, and suggests a scientifically sound technical approach for complete physicochemical characterization of protein molecular structures [27]. This guidance presents the appropriate analytical method for each molecular structure characteristic [2].

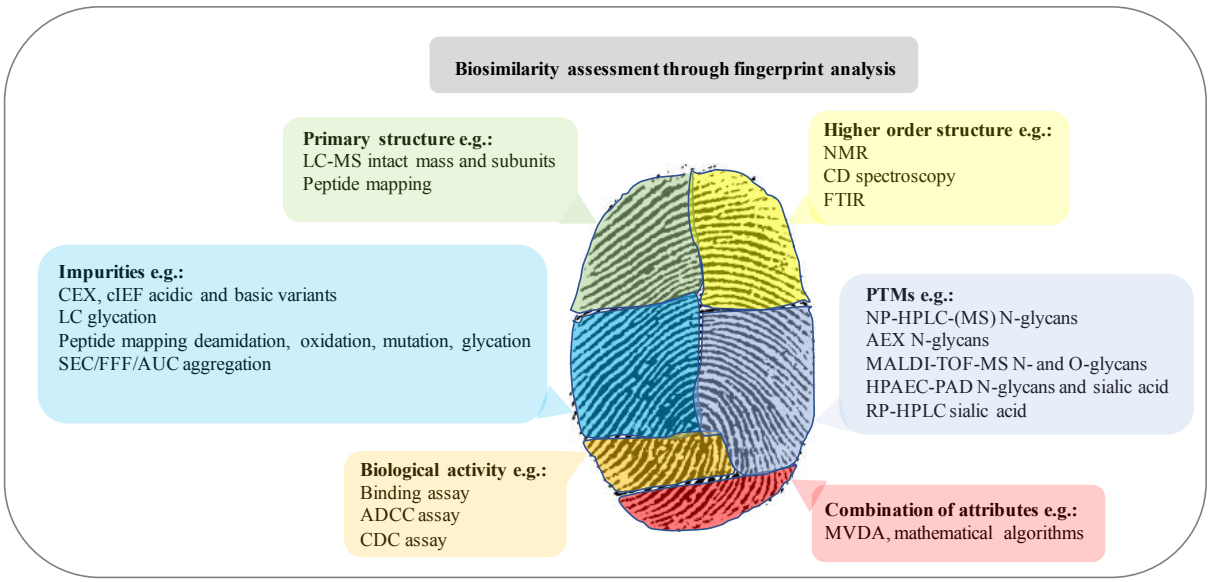
ICH Q6B is emphasizing five times that the manufacturers are encouraged to establish new analytical technologies and the existing methodologies must be continually modified and developed. Biosimilar applicants are not only committed to exhibit the biosimilarity of their biopharmaceutical product, but they should demonstrate a deep understanding of the molecular structure of the biosimilar product. The importance of state-of-the-art physicochemical molecular structural analysis is illustrated in a biosimilarity study as requirement of the regulatory authority [2]. Though the revolutionary development in the analytical methodology, one approach may not be able to investigate all relevant structural variations in therapeutic proteins. For this reason, the application of several, complementary and orthogonal analytical techniques are required to gain more information about the biopharmaceutical's structure. The major changes of molecular structures that may occur need to be investigated. These are primary sequence, PTMs and HOS [2]. The review in this thesis (see paragraph 3.1) therefore demonstrates the most applicable techniques to characterize a biopharmaceutical.

2 Scope of Work

This work aims at to assess the biosimilarity of three different reference biopharmaceuticals approved in Europe and US with the trade names Mabthera® (Roche), NovoSeven® (Novo Nordisk) and Enbrel® (Pfizer) and their respective follow-on products produced for the Asian market called Zytux™ , AryoSeven™ and Altebrel™ (AryoGen Pharmed). This is referred to as an external comparison. It is also a focus to analyse possible batch-to-batch variations of at least three batches of each biopharmaceutical that may occur during the biotechnological production process of reference and follow-on biopharmaceutical. This is referred to as an internal comparison. Furthermore, the internal and external comparability study will be performed based on ICH guidelines. Since regulatory authorities request extensive comparison of CQA that may impact pharmacological response, it is an aim to apply ICH Q5E and Q6B guidelines to assess physicochemical characteristics such as intact mass and subunit analysis, PTMs and HOS. A combination of state-of-the-art analytics will be applied to assess CQA concerning physical, chemical and biological characteristics mentioned in ICH Q5E. Furthermore, ICH Q6B guideline proposes suitable techniques to characterize physicochemical properties, immunochemical properties, biological activity, purity and impurities of the biopharmaceutical. Hence, these will be applied to the biopharmaceuticals mentioned in this work. Comparison of the data will be performed to investigate the biosimilarity as well as batch-to-batch variations over an extended time period. Moreover, different mass spectrometry-based analytical techniques typically used for the state-of-the-art analytics of the biopharmaceuticals will be established. Intact mass and subunit analysis, PTMs and HOS will be investigated using a UHPLC-QTOF-MS^E, UHPLC-IMS, nLC-ESI-MS/MS and MALDI-TOF/TOF-MS systems. Since glycosylation is highly sensitive to product quality differences arising from the biological production process, the N- and O-glycosylation will be investigated on protein (intact mass and subunits), glycopeptide and released glycan level. This includes a thorough investigation of glycosylation as an important CQA to confirm the preset specifications as a range by manufacturer in a GMP regulated framework.

3 Publications

3.1 Physicochemical Characterization of Biopharmaceuticals



This chapter was published in the following journal:

Parr MK, Montacir O, Montacir H, *J Pharm Biomed Anal*. 130 (**2016**), 366-389.

The interest of biopharmaceuticals for therapy is increasing due to their high target selectivity. When early approved blockbuster biopharmaceuticals run out of patent protection, proper analytical characterization and comparison of reference product and biosimilar is needed.

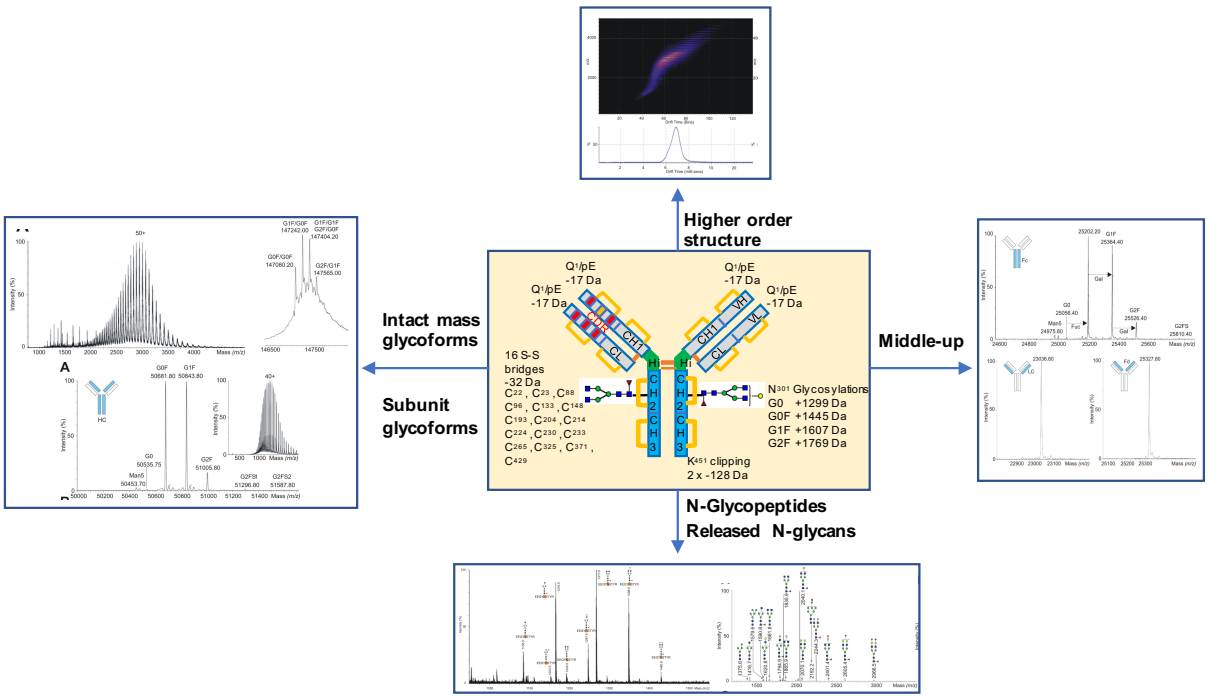
This review gives an overview on analytical methods for characterization of biopharmaceuticals. Classical methods such as gel electrophoresis and liquid chromatography are summarized and complemented with state-of-the-art mass spectrometric approaches.

The preparation of the manuscript was performed in the scope of this dissertation under supervision of Prof. Dr. Maria Kristina Parr. Coauthors provided content-related support.

The original review is available online at:

https://doi.org/10.1016/j.jpba.2016.05.028

3.2 Comparability Study of Rituximab Originator and Follow-on Biopharmaceutical



This chapter was published in the following journal:

Montacir O, Montacir H, Eravci M, Springer A, Hinderlich S, Saadati A, Parr MK, *J Pharm Biomed Anal*. 140, (**2017**), 239-251.

The goal of this study was to perform a head-to-head comparability of Mabthera® and its biosimilar Zytux™, which needs implementation of regulatory guidelines ICH Q6B and Q5E. Hence, middle-up approach to assess specific differences in LC, Fc and Fd region as well as bottom-up approach to screen for deamidation, pyroglutamic acid formation and oxidation is essential. IgG N^{301}-glycosylation site needs to be investigated by comparison of intact mass and subunit (heavy chain) glycoforms, glycopeptides and released N-glycans. Finally, binding assay and CDC will be used to verify the biological impact of possible physicochemical differences.

Rituximab intact mass analysis of reference molecule (Mabthera®) and biosimilar (Zytux™) using middle-up approach and subunit (LC and HC) measurement enabled to specifically detect the presence of Lys-variants within different glycoforms. Analysis of glycosylation on protein level (intact mass and subunits), glycopeptide level and released glycans showed qualitatively similar glycosylation profiles but different sialic acid contents in reference product, which may impact half-life *in vivo*. Furthermore, the glycan analysis of three mAb

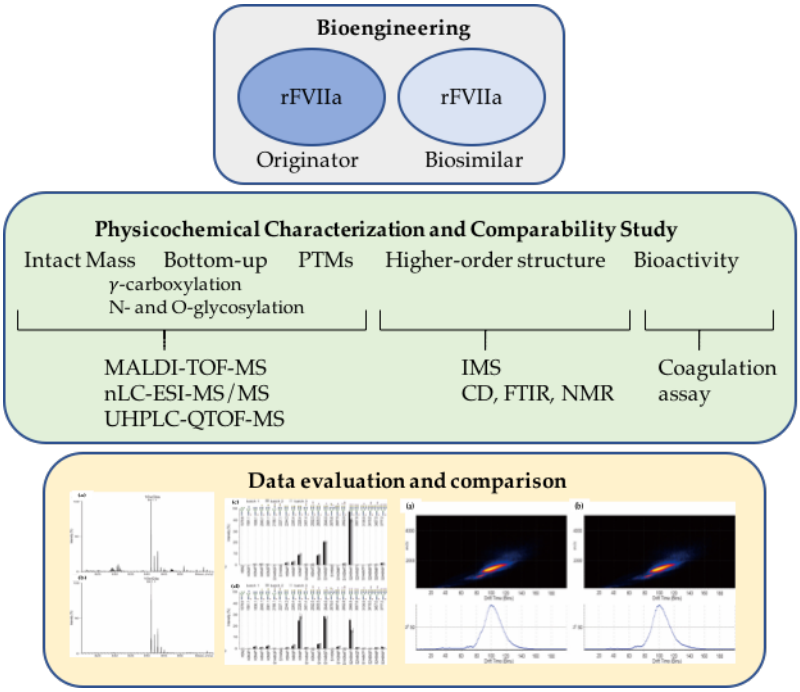
batches revealed manufacturing process changes in case of the follow-on mAb. However, a comprehensive investigation of functional properties using binding and *in vitro* cell-based (CDC) bioassays revealed that the biosimilar has the same bioactivity as the reference molecule.

Conception and execution of all experiments and the preparation of the manuscript were performed in the scope of this dissertation under supervision of Prof. Dr. Maria Kristina Parr. Coauthors provided preliminary findings, practical assistance or content-related support.

The original research article is available online at:

https://doi.org/10.1016/j.jpba.2017.03.029

3.3 Bioengineering of rFVIIa Biopharmaceutical and Structure Characterization for Biosimilarity Assessment



This chapter was published in the following journal:

Montacir O, Montacir H, Eravci M, Springer A, Hinderlich S, Mahboudi F, Saadati A, Parr MK, *Bioengineering (Basel)* 5(1), (**2018**), 1-16.

Bioengineering of Eptacog alfa rFVIIa follow-on biopharmaceutical AryoSeven™ will be thoroughly investigated by assessment of physicochemical properties including intact mass, PTMs and higher-order structure in this study. The focus of this work was to analyse PTMs such as N- and O-glycosylation as well as γ-carboxylation based on intact glycoprotein, glycopeptide level and released glycans using high-resolution UHPLC-QTOF-MS^{E} and MALDI-TOF-MS/MS.

Bioengineering was successfully performed for the Eptacog alfa follow-on biopharmaceutical AryoSeven™. In-depth characterization using mass spectrometry showed comparable intact masses for reference product (NovoSeven®) and biosimilar (AryoSeven™). However, small mass shifts observed for the biosimilar are most likely from variances in PTMs. γ-Carboxylated peptides were found to be comparable in NovoSeven® and AryoSeven™ since the fully γ-carboxylated peptide containing 10-times Gla exits in both biopharmaceuticals.

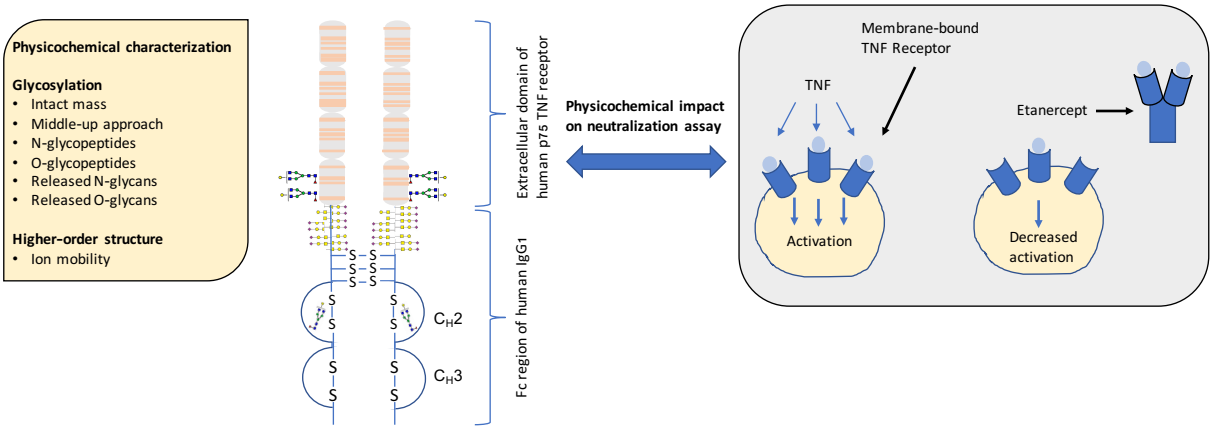
O- and N-glycopeptide mass spectra of follow-on biodrug (AryoSeven™) and reference product (NovoSeven®) were comparable. The analytical strategy enabled to confirm each O- and N-glycosylation sites (Ser52, Ser60, Asn145 and Asn322). GalNAc-bearing N-glycan structures™, which are often correlated with an overall lower sialylation, were found to be much more abundant in AryoSeven. The follow-on biopharmaceutical production obviously promotes mainly the non-sialylated structures ending with Gal and GalNAc (galacto- and agalacto-type), respectively. This may result in accelerated clearance from blood due to high affinity binding to asialoglycoprotein receptor. The bioactivity assay of the biosimilar is almost identical to the reference product NovoSeven®. Thus, the lower amount of sialylated structures found in AryoSeven, may be considered of low relevance.

Conception and execution of all experiments and the preparation of the manuscript were performed in the scope of this dissertation under supervision of Prof. Dr. Maria Kristina Parr. Coauthors provided preliminary findings, practical assistance or content-related support.

The original research article is available online at:

https://doi.org/10.3390/bioengineering5010007

3.4 Physicochemical Characterization, Glycosylation Pattern and Biosimilarity Assessment of the Fusion Protein Etanercept



This chapter was published in the following journal:

Montacir O, Montacir H, Springer A, Hinderlich S, Mahboudi F, Saadati A, Parr MK, Protein J. 37(2), (**2018**), 164-179.

Physocochemical characterization of Etanercept reference product (Enbrel®) and biosimilar (Altebrel™) comprises studies about intact mass and N- and O-glycosylation using different complement analytical approaches due to the complex molecular structure of Etanercept. Since Etanercept glycans are highly sialylated, different purification and enrichment steps are necessary to isolate the glycopeptides accordingly. The impact of structural differences between the biopharmaceuticals will be assessed via the TNF-sensitive neutralization assay.
Intact mass analysis of Etanercept reference product (Enbrel®) and biosimilar (Altebrel™) was performed using two different mass spectrometric approaches and revealed an approximate mass of about 126 kDa for desialylated reference product and follow-on product. Calculation from subunit analysis in this study results in an approximate mass of 64.1 kDa for the monomer (38.3 kDa for TNFR/2 (tumor necrosis factor receptor) and 25.8 kDa for Fc/2). Hence a total intact mass of 128 kDa for the homodimer Etanercept is calculated. UHPLC-QTOF-MS^E glycopeptide analysis of reference product (Enbrel®) and biosimilar (Altebrel™) confirmed all N-glycosylation sites and their similarity. Using a mixture of three different enzymes (Lys-C, trypsin and Asp-N), the complexity of glycopeptides was reduced by minimizing their size and thereby allowed to identify the N-glycopeptides corresponding to the three different N-glycosylation sites (Asn149, Asn171 and Asn317) of Etanercept reference and follow-on product. Using an enrichment step that

selectively removes non-glycosylated peptides, it was possible to enhance the ionization efficiency and identify most of the N-glycopeptides of Etanercept. N-Glycopeptides assigned to Asn317 and hence the Fc-part of Etanercept were shown to bear the Fc-specific glycans such as Man5, G0, G0F and G1F, as already found in Fc domain analysis. N-Glycopeptides corresponding to Asn149 and Asn171 and hence the TNFR domain of Etanercept, were shown to possess a higher heterogeneity and are mainly sialylated structures. In this study, O-glycopeptide structures were confirmed to be similar for the reference and follow-on biopharmaceutical Etanercept. Four different O-glycopeptides at T8, T184, S199, T200 and T245 were detected without prior cleavage of terminal sialic acids, but using a mixture of different proteases followed by an O-glycopeptide specific HILIC-based enrichment step. The data revealed that three peptides have one occupied O-glycosylated site to which core 1-type O-glycans (N1, H1N1, S1N1, S1H1N1 and S2H1N1) are attached. Glycosylation profiling in this study revealed that the most abundant structures on Etanercept reference as well as follow-on biopharmaceutical are biantennary and bigalactosylated structures bearing one Fuc and/or sialic acid residue. About half of the total N-glycan pool of reference and follow-on biopharmaceutical were found to be sialylated and O-glycosylation mapping shows that most of the O-glycans contain sialic acid as well. Furthermore, a neutralization assay, investigating the impact of altered PTMs on potency, indicated that the differences found in PTMs within all batches are still in the acceptable range for biosimilarity.

Conception and execution of all experiments and the preparation of the manuscript were performed in the scope of this dissertation under supervision of Prof. Dr. Maria Kristina Parr. Coauthors provided preliminary findings, practical assistance or content-related support.

The original research article is available online at:

https://doi.org/10.1007/s10930-018-9757-y

4 Personal Contribution

The following table shows the assignment of personal contribution to the papers of this dissertation.

Table 3: Assignment of personal contribution to publications for this dissertation shown in %.

Publication	Conception	Execution	Data evaluation	Preparation of manuscript
1 (review)	40	-	-	40
2 (original research)	90	90	90	90
3 (original research)	90	90	90	90
4 (original research)	90	90	90	90

5 Conclusion and Future Perspectives

This work represents biosimilarity assessment studies performed between three branded biopharmaceuticals (Mabthera®, NovoSeven® and Enbrel®) and their respective follow-on products commercialized in the Asian market (Zytux™ , AryoSeven™ and Altebrel™). Three batches of reference product and biosimilar were investigated for possible variations in bioprocess manufacturing, using various mass spectrometric approaches such as UHPLC-QTOF-MS^E, UHPLC-IMS, nLC-ESI-MS/MS and MALDI-TOF/TOF-MS. The data was correlated to a set of bioassay experiments to evaluate the impact on the bioactivity.

The physicochemical characterization of biopharmaceuticals is a challenging task for industry as well as for regulatory agencies, which requires state-of-art analytical methods to meet the requirements of Q6B and Q5E ICH-guidelines. Comparability studies between reference products and non-originators demand in-depth investigation on CQA and batch-to-batch variations occurring during manufacturing. However, an analytical workflow was proposed to analyze physicochemical properties.

In parallel to the growing interest in the production of biopharmaceuticals, quality assurance is gaining more and more interest. Robust methods for daily quality control are needed that should be easy to use and available at affordable costs. Gel electrophoresis and HPLC-UV separation using orthogonal stationary phases will remain the main pillars in this case. A combination of different orthogonal separation techniques allows for sufficient characterization of heterogeneity (isoforms) in batch-to-batch studies of biopharmaceuticals.

However, in product development and approval, approaches for in-depth characterization to investigate all CQA are needed. Mass spectrometry (MS)-based methods play crucial roles due to their ability to provide precise, confident and information-rich data regarding intact mass, primary sequence, PTMs and HOS. The implementation of regulatory guidance ICH Q6B and Q5E requires state-of-the-art MS-based methods such as MALDI-TOF/TOF-MS and UHPLC-QTOF-MS^E in combination with IMS to provide a comprehensive comparability data set. Indeed, MS is offering comprehensive results and facilitating the interaction with regulatory departments. In addition, structural variances detected by these analytical methods determine the amount of biological, non-clinical and clinical studies that are needed for development of biosimilar mAbs, Fc-fusion proteins and other biopharmaceuticals. Furthermore, these data may help to select appropriate cell lines, to develop robust production, and to support process control. In addition, evaluation of MS data regarding the degree of similarity between a biosimilar and a reference product should be done at the

beginning of the biosimilar production as the correct primary structure may save a lot of time and money.

MS-based methods will gain further importance to comprehensively characterize the therapeutic protein in comparability studies of reference product and biosimilar. In addition, the development of more sensitive and accurate MS-techniques may facilitate the biosimilarity assessment. However, in addition to MS-based techniques other analytical approaches should be used for full physicochemical characterization. The abbreviated procedure for drug approval of biosimilars require comprehensive data to demonstrate structural similarity. Knowledge on CQA and their variances during production of biopharmaceuticals is needed to evaluate the analytical results. Biological assays should be applied in parallel to the physicochemical characterization to assess the potential impact of analytical differences on clinical effects in safety and efficacy.

To conclude, it was possible to establish different MS-based approaches that may detect also small differences in the physicochemical profile of different biopharmaceuticals. These state-of-the-art approaches are of great importance for internal and external comparability studies and enable to detect possible variations regarding the intact mass and PTMs of reference product and follow-on drug. Finally, the impact of these variations on the bioactivity were correlated to a set of bioassays and it was possible to show that they were minor, since they had no influence on biopharmaceutical's potency.

6 Abstract

Most of the recently approved biopharmaceuticals represent drugs that are produced by biotechnological processes involving recombinant DNA. Protein-based biopharmaceuticals are emerging on the pharmaceutical market due to their high target selectivity in specific diseases. In parallel, a growing interest by other companies to produce similar or highly similar follow-on biopharmaceuticals exits, once the patent of blockbuster biotherapeutics is about to expire. In correlation to their complex structure, an analytical challenge is facing the approval of these biosimilars. Since the biological product's critical quality attributes (CQAs) could be influenced by manufacturing process conditions, health authorities (e.g. FDA and EMA) have issued several guidelines to define CQA during manufacturing process changes. Investigation of possible batch-to-batch variations that may occur during the biotechnological production process of reference and follow-on biopharmaceutical is referred to as an internal comparison. Comparing two biopharmaceutical products from different manufacturers to demonstrate biosimilarity is extremely challenging. Here, we refer to this type of comparability as an 'external' comparison. Since biosimilars differ from generic small molecules, the demonstration of biosimilarity requires extensive analytical tools.

In the current study, physicochemical characterization using state-of-the-art analytics was performed to analyze intact and subunit masses, post-translational modifications (PTMs), higher order structure (HOS) and potency of the reference biotherapeutics Mabthera® (Roche), Enbrel® (Pfizer) and NovoSeven® (Novo Nordisk), and their respective biosimilars Zytux™, AryoSeven™ and Altebrel™ (AryoGen Pharmed) in accordance to CQA of biopharmaceuticals. A special focus was given to N-glycosylation due to its potential to monitor the batch-to-batch consistency and alteration during the production bioprocess.

Head-to-head comparability of three batches Mabthera® and Zytux™ comprising intact mass analysis, middle-up approach as well as subunit analysis revealed similar glycoforms but additional lysine (Lys) variants in the biosimilar. The N-glycosylation site was confirmed for both, the reference product and biosimilar. PTMs and HOS were confirmed to be comparable. However, comparison of the N-glycosylation profiles obtained from three batches of the biosimilar and the reference product showed quantitative variations, although the N-glycans were qualitatively similar. Furthermore, functional properties were compared to investigate the impact of glycosylation alteration and PTMs on potency within the biosimilar batches and between reference and follow-on biodrug. The data affirm that the analytically detected differences are still in the acceptable range for biosimilarity.

Regarding Eptacog alfa biopharmaceuticals, physicochemical properties were analyzed based on mass spectrometry, including intact mass, PTMs and higher-order structure. N-glycopeptide analysis with UHPLC-QTOF-MS^E confirmed all N-glycosylation sites as well as two different O-glycopeptide sites. Serine (Ser)60 was found to be O-fucosylated and Ser52 had O-glucose or O-glucose-$(xylose)_{1,2}$ motifs as glycan variants. Both biotherapeutics (NovoSeven®, AryoSeven™) exhibit a batch-to-batch variability in their N-glycan profiles. Traveling wave ion-mobility spectrometry (TWIMS) and NMR (nuclear magnetic resonance) spectroscopy affirm close similarity of the higher-order structure of both biopharmaceuticals. Potency of the biodrugs was analyzed by a coagulation assay demonstrating comparable bioactivity.

Physicochemical characterization using state-of-the-art analytics was performed to reference product Enbrel® and its biosimilar Altebrel™. Intact mass and subunit analysis revealed a size of about 126 kDa for both biopharmaceuticals. Similar glycoprotein species for the complete monomer and the fragment crystallizable (Fc) region domain of reference and follow-on product were observed, however, small differences in Lys variants and oxidation were found. N-Glycopeptide analysis with UHPLC-QTOF-MS^E confirmed the N-glycosylation sites (Asn149, Asn171 and Asn317) as well as Fc-specific glycosylation on Asn317, and tumor necrosis factor receptor (TNFR)-specific highly sialylated glycans on Asn149 and Asn171 for both investigated products. Small quantitative variations in the N-glycan profile were detected, although the N-glycans were qualitatively similar. Four different O-glycopeptides bearing core 1-type glycans were detected. For both, N- and O-glycopeptide analysis, determination was achieved without prior cleavage of the sialic acid residues for the first time. In addition, ion-mobility spectrometry confirmed close similarity of higher-order structure of both biopharmaceuticals. Furthermore, a neutralization assay, investigating the impact of altered PTMs on potency, indicated that the differences within all batches are still in the acceptable range for biosimilarity.

7 Zusammenfassung

Die meisten der kürzlich zugelassenen Biopharmazeutika repräsentieren Therapeutika, die durch biotechnologische Prozesse unter Verwendung rekombinanter DNA produziert werden. Proteinbasierende Biopharmazeutika sind wegen ihrer hohen Targetselektivität in spezifischen Erkrankungen immer häufiger auf dem pharmazeutischen Markt vertreten. Zudem existiert ein immer größer werdendes Interesse anderer Firmen ähnliche oder sehr ähnliche Nachfolger-Biologika zu produzieren, sobald das Patent der teuren Originator-Biotherapeutika dabei ist abzulaufen. Aufgrund ihrer komplexen Struktur steht der Zulassung der Biosimilare eine analytische Herausforderung gegenüber. Da die kritischen Qualitätsattribute eines biologischen Produktes durch die Schritte des Herstellungsprozesses beeinflusst werden können, haben die Gesundheitsorganisationen (z. B. FDA und EMA) verschiedene Richtlinien erlassen, um die kritischen Qualitätsattribute bei Veränderungen des Herstellungsprozesses zu definieren. Allerdings ist der Nachweis der Biosimilarität beim Vergleich zweier biologischer Produkte von verschiedenen Herstellern sehr herausfordernd. In der vorliegenden Arbeit wird diese Art des Vergleichs als “externer Vergleich” bezeichnet. Da Biosimilare sich von generischen Molekülen unterscheiden, setzt die Darstellung der Biosimilarität den Einsatz umfassender analytischer Methoden voraus.

In der vorliegenden Arbeit wurde eine physikochemische Charakterisierung unter Verwendung von *State-of-the-Art*-Analytik durchgeführt, um die Masse des intakten Moleküls sowie Untereinheiten (Fc, LC und Fd) davon, post-translationalen Modifikationen, dreidimensionale Struktur und biologische Stärke der Original-Biotherapeutika Mabthera® (Roche), Enbrel® (Pfizer) und NovoSeven® (Novo Nordisk) sowie ihrer jeweiligen Biosimilare Zytux™, AryoSeven™ und Altebrel™ (AryoGen Pharmed) unter Berücksichtigung ihrer kritischen Qualitätsattribute, zu analysieren. Ein spezieller Fokus wurde dabei auf die N-Glykosylierung gelegt, da diese das Potential besitzt die Batch-zu-Batch-Konsistenz und Unterschiede während des biologischen Produktionsprozesses zu überwachen. Ein *Head-to-Head*-Vergleich der intakten Molekülmasse, *Middle-Up*-Analyse und Analytik der Untereinheiten dreier Batches Mabthera® und Zytux™ zeigte ähnliche Glykoformen aber zusätzliche Lysin (Lys)-Varianten im Biosimilar. Die N-Glykosylierungsstelle Asn301 wurde sowohl für das Referenzprodukt als auch dem Biosimilar bestätigt. Die post-translationalen Modifikationen und dreidimensionale Struktur des Referenzproduktes sowie Biosimilars wurden als vergleichbar nachgewiesen. Jedoch zeigte der Vergleich der N-Glykosylierungsprofile dreier Batches des Biosimilars und

Referenzproduktes quantitative aber keine qualititativen Unterschiede. Zudem wurden die funktionellen Eigenschaften verglichen, um den Einfluss der Unterschiede in der Glykosylierung und der post-translationalen Modifikationen auf die Wirksamkeit der Biosimilar-Batches im Vergleich zum Referenzprodukt zu untersuchen. Die Daten bestätigen, dass die detektierten Unterschiede innerhalb des Toleranzbereiches für den Biosimilar liegen.
Die Eptagog alfa Biotherapeutika wurden physikochemisch mittels massenspektrometrischer Methoden auf intakte Masse, post-translationaler Modifikationen und dreidimensionaler Struktur analysiert. Die Analyse der N-Glykopeptide mittels UHPLC-QTOF-MS^E identifizierte alle N-Glykosylierungs- und zwei O-Glykosylierungsstellen. Es konnte gezeigt werden, dass Ser60 O-fucosyliert und Ser52 O-Glucose oder O-Glucose-$(Xylose)_{1,2}$ Motive als Glykan-Varianten trägt. Die N-Glykanprofile beider Biotherapeutika (NovoSeven®, AryoSeven™) besitzen Batch-zu-Batch Schwankungen. Ionenmobilitäts-Spektrometrie und NMR-Spektroskopie bestätigen die Ähnlichkeit der dreidimensionalen Struktur beider Biotherapeutika. Die Wirksamkeit beider Biopharmazeutika wurde mittels eines Koagulations-Tests analysiert und zeigte eine vergleichbare Bioaktivität.
Die Physikochemische Charakterisierung mittels *State-of-the-Art*-Analytik wurde für den Vergleich des Etanercept Referenzprodukt Enbrel® und seinem Biosimilar Altebrel™ verwendet. Die Analyse der intakten Masse und der Untereinheiten zeigte eine Gesamtmasse von etwa 126 kDa je Dimer für beide Biotherapeutika. Die Glykoproteinspezien der kompletten Monomere sowie der Fc-Region des Referenz-Produktes und Biosimilars waren, bis auf geringe Unterschiede in den Lys-Varianten sowie der Oxidation, ähnlich. Die N-Glykopeptid-Analyse mittels UHPLC-QTOF-MS^E bestätigte die N-Glykosylierungsstellen (Asn149, Asn171 und Asn317) sowie die Fc-spezifische Glykosylierung von Asn317. Zudem zeigte diese die Tumornekrosefaktor-Rezeptor (TNFR)-spezifschen hochsialyierten Glykane an Asn149 und Asn171 im Falle beider Biotherapeutika. Die N-Glykanprofile zeigten geringe quantitative aber keine qualitativen Unterschiede. Es konnten vier verschiedene O-Glykopeptide, die *core* 1-Typ-Glykane tragen, detektiert werden. Die N- und O-Glykopeptidanalyse wurde entgegen der aktuellen Literatur ohne vorherige Abspaltung der Sialinsäuren erfolgreich durchgeführt. Zudem bestätigte die Ionenmobilitäts-Spektrometrie eine große Ähnlichkeit der dreidimensionalen Struktur beider Biotherapeutika. Ein Neutralisationstest sollte den Einfluss der gefundenen Unterschiede in den post-translationalen Modifikationen auf die Wirksamkeit untersuchen. Dieser zeigte, dass die Unterschiede in allen Batches innerhalb des Toleranzbereiches des Biosimilars liegen.

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9 List of Peer-Reviewed Publications

Montacir O, Montacir H, Springer A, Hinderlich S, Mahboudi F, Saadati A, Parr MK, **Physicochemical Characterization, Glycosylation Pattern and Biosimilarity Assessment of the Fusion Protein Etanercept**, Protein J. 37(2), (**2018**), 164-179.
DOI: 10.1007/s10930-018-9757-y

Montacir O, Montacir H, Eravci M, Springer A, Hinderlich S, Mahboudi F, Saadati A, Parr MK, **Bioengineering of rFVIIa Biopharmaceutical and Structure Characterization for Biosimilarity Assessment,** *Bioengineering (Basel)* 5(1), (**2018**), 1-16.
DOI: 10.3390/bioengineering5010007

Montacir O, Montacir H, Eravci M, Springer A, Hinderlich S, Saadati A, Parr MK, **Comparability Study of Rituximab Originator and Follow-on Biopharmaceutical**, *J Pharm Biomed Anal*. 140, (**2017**), 239-251.
DOI: 10.1016/j.jpba.2017.03.029

Parr MK, **Montacir O**, Montacir H, **Physicochemical Characterization of Biopharmaceuticals**, *J Pharm Biomed Anal*. 130 (**2016**), 366-389.
DOI: 10.1016/j.jpba.2016.05.028

10 Abstracts/Posters

Montacir O, Eravci M, Springer A, Hinderlich S, Parr MK, Comparability study of Rituximab originator and follow-on biopharmaceutical, 3rd Biologics & Biosimilars Congress, March 2017, Berlin.

Montacir O, Eravci M, Springer A, Hinderlich S, Parr MK, Physicochemical Comparability between Originator and Biosimilar, American Society of Mass Spectrometry (ASMS), June 2016 San Antonio, TX, USA.

Montacir O, Eravci M, Springer A, Hinderlich S, Parr MK, Comparability study between Biosimilar and Originator, Global Engage 2nd Biologics & Biosimilars Congress, February 2016 Berlin.

11 Curriculum Vitae

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12 Declaration

I, Othman Montacir, do hereby declare that I have prepared the present cumulative dissertation independently and without unauthorized assistance. In preparing the dissertation, no other aids than those indicated in the text of this dissertation were used.

A doctoral procedure has never been applied for at another university or department.

Berlin, 30 September 2018

Othman Montacir