Special feature: tutorial

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Cutting-edge mass spectrometry characterization of originator, biosimilar and biobetter antibodies

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The approval process for antibody biosimilars relies primarily on comprehensive analytical data to establish comparability and high similarity with the originator. Mass spectrometry (MS) in combination with liquid chromatography (LC) and electrophoretic methods are the corner stone for comparability and biosimilarity evaluation. In this special feature we report head-to-head comparison of trastuzumab and cetuximab with corresponding biosimilar and biobetter candidates based on cutting-edge mass spectrometry techniques such as native MS and ion-mobility MS at different levels (top, middle and bottom). In addition, we discuss the advantages and the limitations of sample preparation and enzymatic digestion, middle-up and -down strategies and the use of hydrogen/deuterium exchange followed by MS (HDX-MS). Last but not least, emerging separation methods combined to MS such as capillary zone electrophoresis-tandem MS (CESI-MS/MS), electron transfer dissociation (ETD), top down-sequencing (TDS) and high-resolution MS (HR-MS) that complete the panel of state-of-the-art MS-based options for comparability and biosimilarity evaluation are presented. Copyright © 2015 John Wiley & Sons, Ltd.

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Keywords: antibody; biosimilar; biobetter; trastuzumab; cetuximab; glycosylation; mass spectrometry; critical quality attribute

Introduction

Biosimilar antibodies are copy versions of already approved biologics that are or will be out of patent soon.[1] In contrast to generic versions of small molecules (150 to 600 Da range), it is not possible to produce exact molecular copies of such large and complex tetrameric glycoproteins (150 000 Da). By nature, biosimilars are produced from different cell clones and different manufacturing processes as the originators. As a consequence differences in glycosylation and other microvariations are observed and may impact (or not) on quality, safety and potency of the drug.[1,2] Only very small differences between biosimilar and reference mAbs, with reassurance that these are not of clinical relevance, may be accepted by health authorities. In 2007, copies of rituximab (Dr Reddy's Reditux®) and abciximab (Isu Abxis', Clotinab®) were first approved in India and in South Korea, respectively, paving the ways for regulatory guidelines for biosimilar antibodies in other parts of the world including highly regulated regions such as Europe, the USA and Japan.[3]

The European Medicine Agency (EMA) has pioneered the regulatory framework for approval of biosimilar products since 2005,[4] resulting to date in marketing authorizations for 16 recombinant drugs including somatropins, erythropoetins, filgastrims and more recently the first two mAbs in the European Union (EU).[4,5] Both mAbs were biosimilar versions of infliximab (Celltrion's Remsima® and Hospira's Inflectra®) that should be followed by biosimilar versions of other blockbuster mAbs such as rituximab, trastuzumab, cetuximab, adalimumab, bevacizumab as well as etanercept, an Fc-fusion protein. Following several workshops starting in 2009, the EMA has released a guideline for 'similar biological medicinal products'. The document discusses relevant animal-model, nonclinical and clinical studies that are recommended to establish the similarity and the safety of a biosimilar compared with a reference mAb or Fc-fusion product approved in the EU.[4,6] Importantly, a revised version was recently released and will come into effect in December 2014 (www.ema.europa.eu).

The approval process for biosimilars relies primarily on comprehensive analytical data to establish comparability and high similarity with the originator biologic. On a practical side and from a regulatory standpoint, antibodies that are not highly similar must undergo greater preclinical and clinical evaluation. Conversely, abbreviated clinical programs may be suitable for biosimilars mAbs showing molecular profiles and characteristics within the range of originator versions (quality target product profile, QTPP). Manufacturing process changes are tightly regulated by the International Committee of Harmonization (ICH) guidelines. Changes of quality attributes are only acceptable if they do not alter safety and efficacy (Critical Quality Attributes). The biosimilar release specifications should be close to the current originator specifications. In the leading generic companies, high degree of

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similarity is achieved based on extensive characterization and on iterative process development. In such laboratories, only when a biosimilar antibody is shown to be 'highly similar' at the analytical level, pre-clinical and clinical studies are engaged. When used very early in the R&D process, the state-of-the-art MS methods described below contribute to the successful development of biosimilar antibodies.

Biosimilar antibodies and Fc-fusion proteins in clinical trials in Europe

MAbs and related products represent the fastest growing segment of the pharmaceutical industry,[7] resulting to date in approval of more than 50 drugs in multiple indications.[8] Patent expiry in Europe and in the USA of major first-generation mAbs has led to the development of numerous copies.[9] Many head-to-head clinical trials are ongoing to evaluate their biosimilarity with originator versions. By the end of 2011, the EMA was asked for 35 scientific advice procedures for biosimilar mAbs and Fc-fusion proteins[3] resulting recently in the first marketing-authorization for biosimilars of infliximab by the end of 2013.[5]

Extensive structural and functional comparison of biosimilar and reference products is the foundation of biosimilar development. The comparison exercise must be scientifically tailored using state-of-the-art analytical tools and sensitive tests to detect small product-related differences between the biosimilar and the reference product. Structural differences determine the amount of biological, non-clinical and clinical studies that are needed for development of biosimilar mAbs and Fc-fusion proteins. The primary amino acid sequence for example is expected to be the same for the biosimilar and the reference product in Europe and in the USA. Surprisingly, as recently reported in several papers, a number of biosimilar candidates of trastuzumab,[10] rituximab[11] and etanercept[12] do not fulfill these criteria. Moreover, a number of antibody sequences available in public data bases contain errors that can be identified and corrected by MS as illustrated for rituximab[13] and cetuximab.[14] When done early in the R&D process, the correct sequence selection results in saving significant time and money. In addition to the correct primary sequence, small differences in the micro-heterogeneity pattern of mAbs may be acceptable if appropriately justified with regard to their potential impact on safety and efficacy.

As illustrated in this tutorial, intact mAb, middle-up and bottom-up mass measurements in combination with a panel of electrophoretic and chromatographic methods must be used for the evaluation of the small differences between the biosimilar and the originator mAbs.[15] Among the emerging MS methodologies, native MS, ion mobility coupled to MS (IM-MS) and hydrogen/deuterium exchange followed by MS (HDX-MS) will play increasing roles in comparability studies of biosimilars candidates and originators as highlighted here for trastuzumab and a biosimilar candidate (trastuzumab-B) and for cetuximab and a biobetter candidate (cetuximab-B), respectively.

Trastuzumab

Trastuzumab (Herceptin®) is a humanized IgG1 antibody highly potent for treating HER2-overexpressing breast cancer. Multiple mechanisms of action are thought to contribute to the tumor-inhibitory effect of this mAb. Among them, trastuzumab can mediate the effector functions of immune cells through the constant region (Fc) by binding to Fc gamma receptor III (FcγRIII) and triggering antibody-dependent cell-mediated cytotoxicity (ADCC). Based on the published amino acid sequence of both light and heavy chain of trastuzumab,[16] the calculated mass is $C_{6560}H_{10132}O_{2090}N_{1728}S_{44}$ = 148 057 Da. The calculation includes 16 disulfide bridges (−32 Da), two main glycoforms (G0F; +1445 Da) and near 99% cleavage of two heavy chains C-terminal lysines (−128 Da × 2). Partial cyclization of one or two N-terminal glutamic acid (−18 Da) may also occur as well as methionine oxidations (+16 Da). Three Asn deamidation/Asp isomerization (+1 Da, acidic charge variant) hot spots have also been described in the CDRs and shown to impact negatively to HER2 antigen binding when degraded (Fig. 1). Damen *et al.* reported the use of such type of analysis for four different production batches of trastuzumab compared in terms of global glycosylation profiles as well as in terms of variability at each glycosylation site.[17] The results showed that each batch of trastuzumab shared the same types of glycoforms but that the relative abundance of each glycoform was relatively variable. This gives a first idea of inherent batch variability of recombinant antibodies and the level of glycosylation heterogeneity that may be acceptable by regulatory authorities for approved products.

Cetuximab

Cetuximab (Erbitux®) is a chimeric mouse-human IgG1 monoclonal antibody targeting Epidermal Growth Factor receptor (EGFR) and approved worldwide as a treatment for colorectal cancer and squamous cell carcinoma of the head and neck. Like other IgGs, cetuximab has a conserved N-glycosylation site in the CH2 domain but also a second consensus site within the framework 3 of the variable portion of the heavy chain (-Asn^{88}AspThrAla-). The detailed structures of these oligosaccharides have been investigated based on MALDI-Q-TOF MS and MS/MS. These orthogonal MS-based methods revealed the presence of 21 distinct oligosaccharide structures differing in degree of sialylation with *N*-glycolyl neuraminic acid (NGNA) and additional galactoses (zero-, mono-, di- and α-1,3-galactose).[18,19] More recently, 37 N-glycans were identified by LC-MS/MS on cetuximab and the 10 most abundant confirmed by NMR.[20] On the clinical side, a high prevalence of hypersensitivity reactions to cetuximab has been reported in some areas of the USA. Among 76 cetuximab-treated subjects, 25 had a hypersensitivity reaction to the drug.[21] The immune response was shown to be specific for oligosaccharides containing the galactose-α-1,3-galactose glycotype. This motive is present on the Fab portion of the cetuximab heavy chain when the molecule is produced in the murine SP2/0 cell line used for commercial manufacturing, but not in the Chinese Hamster Ovary (CHO) cells used as control.[21] These results suggest that such a cetuximab 'bio-better' version could be obtained by production in CHO rather than in SP2/0 cells or by glyco-engineering.[22] Nevertheless, the resulting mAb is not a highly similar version in regards to the very different glycoprofiles but can be expected to show the same efficacy and a better safety profile. Recently, data of clinical phase I have been reported for such a 'biobetter' version of cetuximab produced in CHO cells.[23]

Experimental

IdeS digestion

To 30 μg of mAb in 50 mM Na_2HPO_4 and 150 mM NaCl 30 UI of IdeS enzyme was added at pH 6.6, following the instructions of the enzyme kit (FabRICATORKit, Genovis, Lund, SW). The mixture was incubated at 37 °C for 60 min. Before reduction, the digested

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Figure 1. Amino acid sequences of studied mAbs. Trastuzumab and trastuzumab-B light chains (A), trastuzumab heavy chain (B), trastuzumab-B heavy chain (C), Cetuximab and cetuximab light (D) and heavy chain (E).

sample was diluted three times in 8 M guanidine HCl. TCEP is added to reach a final concentration of 50 mM. After 60-min incubation at 57 °C, the reaction is quenched by adding 1% trifluoroacetic acid (TFA).

LC-MS

LC-MS analysis was performed using an Alliance 2695 (Waters, Manchester, UK) coupled to a micrOTOF-Q (Bruker, Bremen, GE). A volume equivalent to 7 μg of sample preparation was injected on an XBridge BEH300, C4, 3.5 μm, 2.1 × 150 mm column (Waters, Manchester, UK) set at 60 °C. The gradient was generated at a flow rate of 250 μl/min using 0.1% TFA for mobile phase A and acetonitrile containing 0.08% TFA for mobile phase B. B was raised from 20 to 50% in 30 min followed by a 2 min washing step at 90% B and a 25-min reequilibration period. The micrOTOF-Q was operated in positive mode with a capillary voltage of −4500 V. Acquisitions were performed in the mass range of 500–5000 *m/z* with a 1-s scan time. Calibration was performed using the singly charged ions produced by an ES-TOF tuning mix (Agilent, Waldron, GE). Data analysis was performed by Compass Data Analysis 4.2 (Bruker, Bremen, GE).

Trypsin digestion for glycopeptide analysis

Twenty micrograms of mAbs was lyophilized and solubilized in 90-μl 50 mM NH_4HCO_3, 0.1% RapiGest™ (Waters, Manchester, UK), pH 7.8. Disulfide reduction was performed by incubating the mAb solution with 5 mM DTT for 30 min at 60 °C. The enzyme is prepared by suspending 20 μg at 0.2mg/mL of trypsin in 100-μl H_2O. Digestion was performed by adding 1 μl of trypsin, which corresponds to a 1:50 enzyme:substrate ratio. Samples were incubated at 37 °C for 5 h. The reaction was stopped by adding 1 μl of TFA. RapiGest™ was eliminated by heating at 37 °C for 30 min and by centrifugation at 10 000 *g* for 5 min.

Pepsin digestion for peptide mapping

Twenty micrograms of mAb was lyophilized and solubilized in 85-μl 50 mM NH_4HCO_3, 0.1% RapiGest™. Disulfide reduction was performed by incubating the mAb solution with 5 mM DTT for 30 min at 60 °C. To the mAb solution 1 N HCl was added to obtain a pH of 1–2. The pepsin is prepared in H_2O pH 5.5 to obtain a concentration of 0.2 μg/μl. Digestion was performed by adding 1 μl of pepsin solution, which corresponds to a 1:50 enzyme:substrate ratio. Samples were incubated at 37 °C for 5 h. The reaction was stopped by heating at 95 °C for 10 min. RapiGest™ was eliminated by centrifugation at 10 000 *g* for 5 min.

nanoLC-MS and nanoLC-MS/MS

Chromatography conditions on nanoAcquity chromatography pump

The analysis was performed using a nanoACQUITY Ultra-Performance-LC (UPLC; (Waters, Manchester, UK). The samples were trapped on a nanoACQUITY UPLC precolumn (C18, 180 μm × 20 mm, 5 μm particle size), and the peptides were separated on a nanoACQUITY UPLC column, (C18, 75 μm × 250 mm, 1.7-μm particle size). The solvent system consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). Trapping was performed during 3 min at 5 μl/min with 99% of solvent A and 1% of solvent B. Elution was performed at a flow rate of 300 nl/min, using 8–35% gradient (solvent B) over 20 min at 50 °C.

MS and MS/MS conditions on SYNAPT HDMS G1 mass spectrometer

The MS and MS/MS analyses were performed using a SYNAPT™ High Definition Mass Spectrometry (Waters, Manchester, UK), a hybrid quadrupole orthogonal acceleration time-of-flight tandem mass spectrometer equipped with a Z-spray ion source and a lock

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mass system. The capillary voltage was set at 2.8 kV and the cone voltage at 35 V. Mass calibration of the analyzer was achieved using fragments of Glu-fibrino-peptide B on the *m/z* [50;2000] range. Online correction of this calibration was performed with Glu-fibrino-peptide B as the lock-mass. The ion $(M+2H)^{2+}$ at *m/z* 785.8426 was used to calibrate MS data, and the fragment ion $(M+H)^{+}$ at *m/z* 684.3469 was used to calibrate MS/MS data during the analysis.

For MS experiments, the system was operated with a cone voltage at 35 V. Acquisitions were performed on the mass range 150–2000 *m/z* with a 1-s scan time.

For tandem MS experiments, the system was operated with automatic switching between MS and MS/MS modes (MS 0.5 s/scan on *m/z* [250;1700] range and MS/MS 0.7 s/scan on *m/z* [50;2000] range). The three most abundant peptides (intensity threshold >50 counts/s), preferably doubly and triply charged ions, were selected on each MS spectrum for further isolation and CID fragmentation with 2 energies using collision energy profile. Fragmentation was performed using argon as the collision gas. The complete system was fully controlled by MassLynx 4.1 ((Waters, Manchester, UK).

Data analysis and peptide identification

Raw data collected during nanoLC-MS/MS analyses were processed and converted with ProteinLynx Browser 2.3 (Waters, Manchester, UK) into .pkl peak list format. Normal background subtraction type was used for both MS and MS/MS with 5% threshold and polynomial correction of order 5. Savitzky–Golay smoothing was performed on MS/MS spectra (2 iterations and window of 3 channels). Medium and fast deisotoping was performed for MS and MS/MS spectra, respectively. The MS/MS data were analyzed using the MASCOT 2.3.02 algorithm (Matrix Science) to search against the sequence of the light and heavy chains of the mAb. Spectra were searched with a mass tolerance of 15 ppm for MS and 0.05 Da for MS/MS data. No enzyme and variable modifications were specified: carbamidomethylation of cysteine residues, oxidation of methionine residues, pyro-glutamylation of N-terminus and deamidation of asparagine and glutamine residues. Peptide identifications were validated with a minimal Mascot ion score of 35.

Native mass spectrometry

Prior to native mass spectrometry experiments, mAbs were desalted against 150 mM ammonium acetate solution buffered at pH 7.4 using six cycles of concentration/dilution on micro-concentrators (Vivaspin, 10-kD cutoff, Sartorius, Göttingen, GE). Protein concentration was determined by UV absorbance using a NanoDrop spectrophotometer (Thermo Fisher Scientific, France). mAbs deglycosylation was achieved by incubating (37 °C—30 min) one unit of IgGZERO™ (Genovis, Lund, Sweden) per microgram of mAb prior to buffer exchange desalting step.

Mass spectrometry experiments were performed on a hybrid electrospray quadrupole time-of-flight mass spectrometer (Synapt G2 HDMS, Waters, Manchester, UK) coupled to an automated chip-based nanoelectrospray device (TriversaNanomate, Advion Biosciences, Ithaca, NY) operating in the positive ion mode.

Denatured MS analysis was performed using classical interface tuning parameters of the mass spectrometer (Vc = 40 V, Pi = 2.1 mbar) and external calibration with the multiply charged ions produced by 2-μM horse heart myoglobin solution diluted in water/acetonitrile/formic acid (50v/50v/1v) with same tuning.

For native MS experiments, external calibration was performed using singly charged ions produced by a 2 mg/ml solution of cesium iodide in 2-propanol/water (1v/1v), and samples were infused at 5 μM (Vc = 180v, Pi = 6 mbar). Native MS data interpretations and deconvolutions were performed using MassLynx 4.1 (Waters, Manchester, UK).

Ion mobility mass spectrometry

IM-MS experiments were performed using a hybrid quadrupole/ion mobility separator/time-of-flight instrument (Synapt G2 HDMS, Waters, Manchester, UK). Separation parameters such as wave height (WH) and velocity (WV) were optimized to reach the best separation resolution: Vc = 80 V, Pi = 6 mbar, Trap Bias = 37 V, Trap CE = 2 V, Transfer CE = 15 V, P_{He} = 130 ml/min, P_{N2} = 45 ml/min, IMS WV = 923 m/s, IMS WH = 40 V. IM-MS measurements were performed in triplicate, and data interpretation was performed using MassLynx 4.1 and Driftscope 2.2 version (Waters, Manchester, UK.).

Results and discussion

Trastuzumab-B and cetuximab-B

Trastuzumab-B and cetuximab-B described here are model biosimilar candidates. They have been designed and generated to illustrate the potency of state-of-the-art analytical and structural methods for biosimilarity assessment in a didactic way.

(i) Trastuzumab-B, produced in a similar cell line (CHO) than the originator, displays the same amino acid sequence in the light chains (Fig. 1A) except for one amino acid (R217K) out of 450 in the heavy chain and corresponding to a different allotype ($G1m3/R^{214}$ instead of $G1m17/K^{217}$)[24] (Fig. 1B, C).

(ii) Cetuximab-B displays the same amino acid sequence as cetuximab (Fig. 1D,E) but was produced in a CHO cell line instead of SP2/0 to illustrate the impact of the production system on both N-glycosylation sites[25,26] and the challenges that such a 'biobetter' candidate will have to face to get approval.

Trastuzumab/trastuzumab-B, cetuximab/cetuximab-B head to head comparison by MS (top, middle and bottom levels)

Intact antibodies can be analyzed by denaturing (Fig. 2), native MS (Fig. 4) and ion mobility MS (Fig. 5) to gain structural insights directly at the top level ('150 kDa') or after profile simplification by enzymatic treatments[27] like N-deglycosylation (IgGZERO®, Fig. 3), carboxypeptidase B digestion[28] or glutaminyl–peptide cyclotransferase treatment.[29] At the middle level, smaller mAb fragments are generated either by reduction (light/heavy chains 25/50 kDa), papain digestion (Fab/Fc fragments of 50 kDa) or IdeS digestion (Fabricator®) followed by DTT reduction (Fc/2, LC and Fd fragments of 25 kDa) (Figs. 6 and 7). At the bottom level, mAbs are cut into peptides and glycopeptides produced by mAbs enzymatic digestions (e.g. trypsin, Lys-C, Asp-N, pepsin; 0.5 to 4 kDa) and further analyzed by LC-MS/MS (Figs. 8 and 9) or CESI-MS/MS.[30] In addition HDX-MS can be used to investigate structural changes in mAbs[31] as well as aggregates.[32]

Intact mAb analysis by denaturing MS (top level)

Intact analysis of IgGs and their large domains has been reported by either MALDI (matrix-assisted laser desorption/ionization) or ESI (electrospray ionization) coupled to almost all types of mass analyzers including time-of-flight (TOF), quadrupole (Q), ion trap (IT) and orbitrap. A mass shift of the mAb ion distribution can serve

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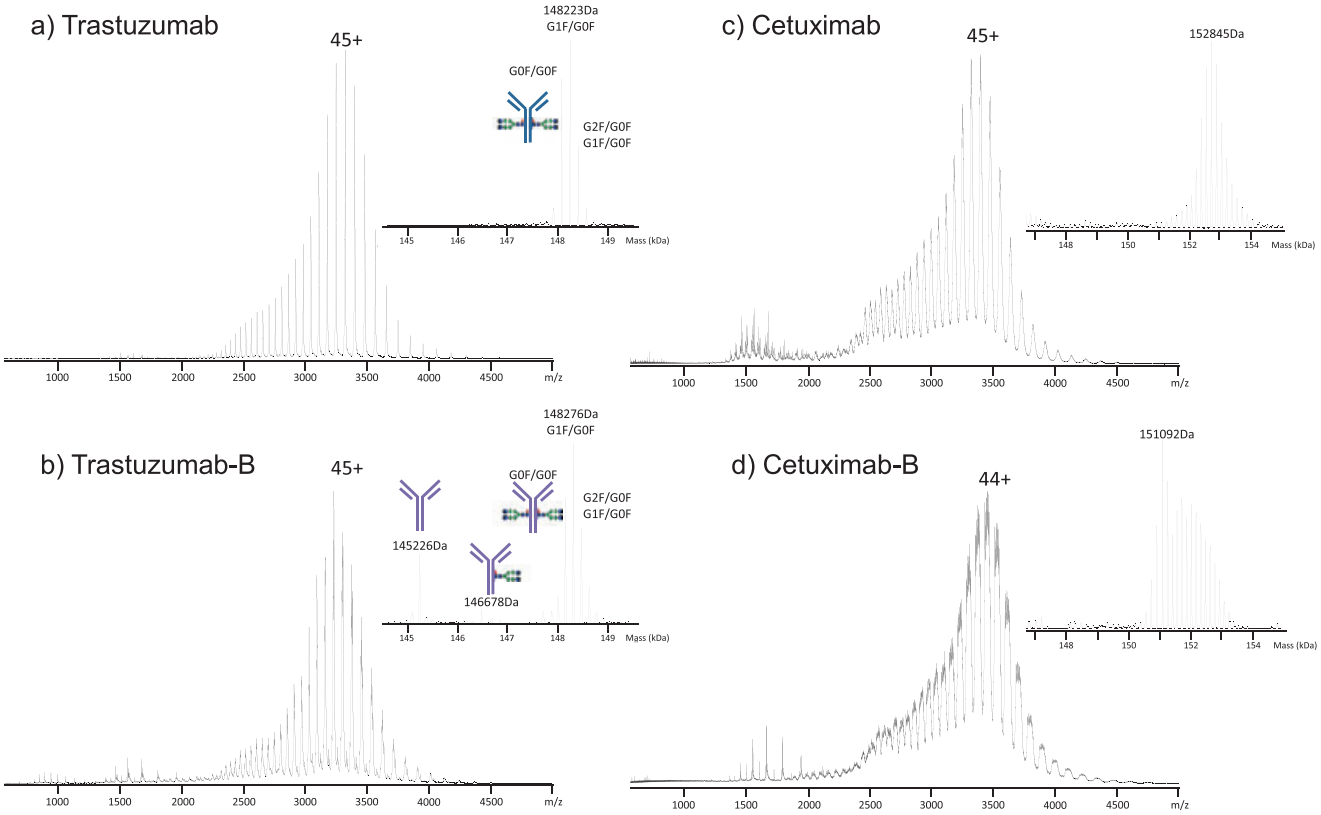


Figure 2. Denaturing MS analysis of intact trastuzumab (A), trastuzumab-B (B), cetuximab (C) and cetuximab-B (D). Deconvoluted mass spectra and corresponding masses of intact mAb species are given as inserts.

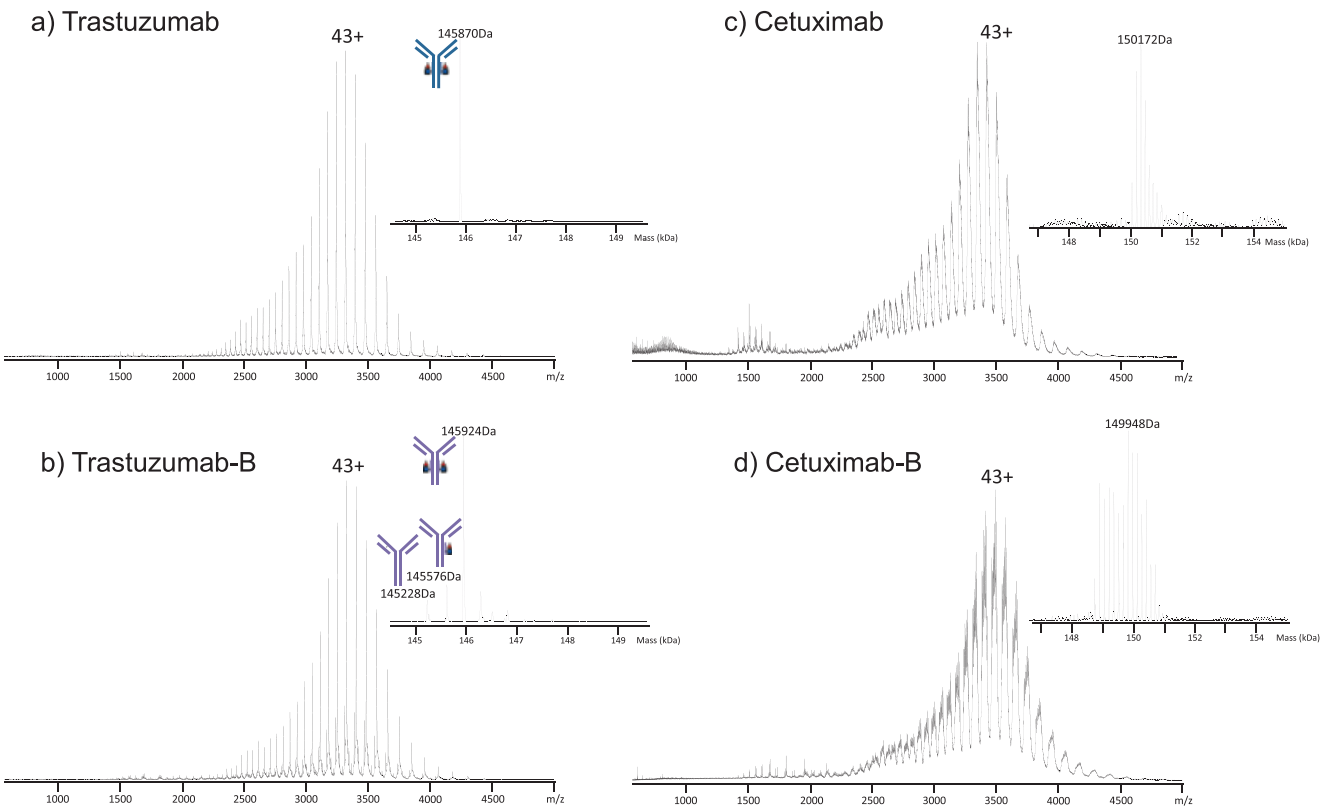


Figure 3. Denaturing MS analysis of trastuzumab (A), trastuzumab-B (B), cetuximab (C) and cetuximab-B (D) after IgGZERO™ treatment. Deconvoluted mass spectra and corresponding masses of intact mAb species are given as inserts.

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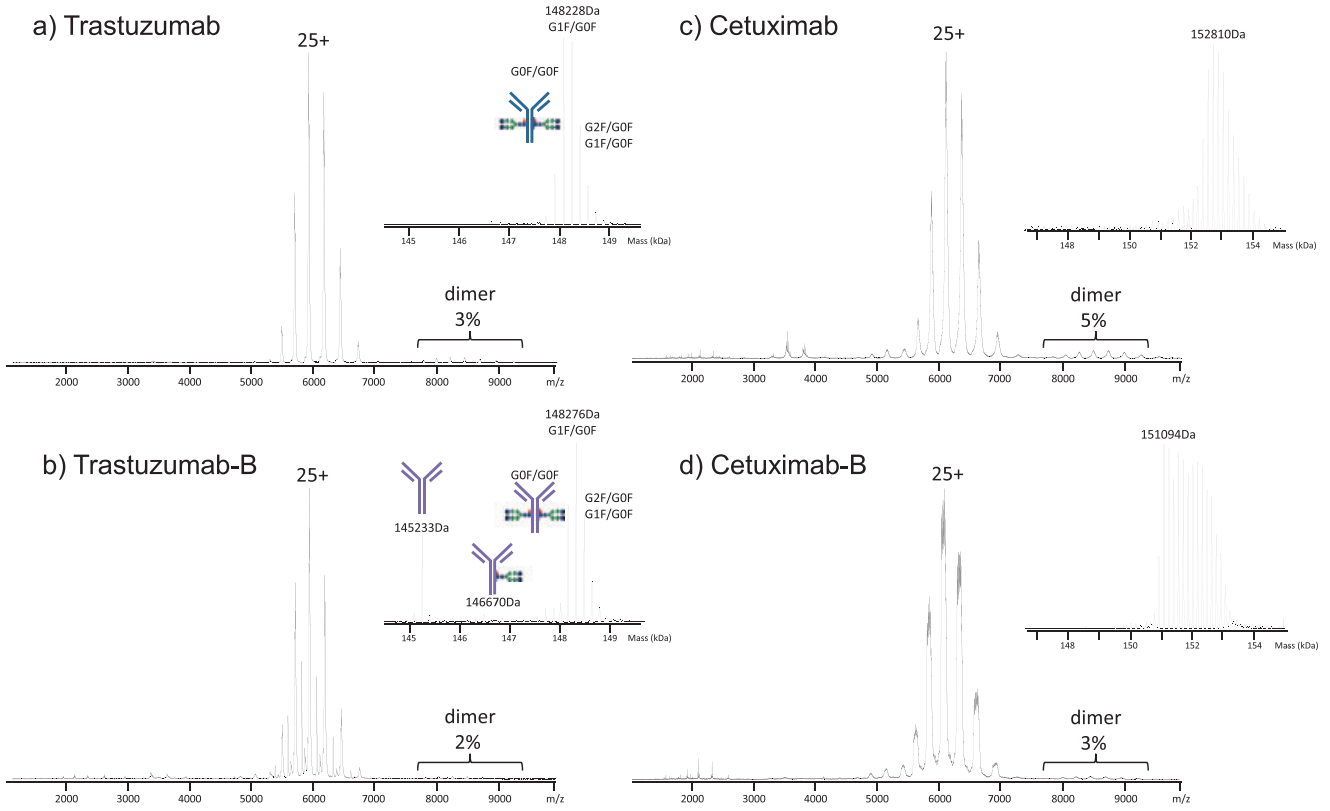


Figure 4. Native MS analysis of trastuzumab (A), trastuzumab-B (B), cetuximab (C) and cetuximab-B (D). Deconvoluted mass spectra and corresponding masses of monomeric mAbs are given as inserts. Dimer ratios were deduced from charge states intensities.

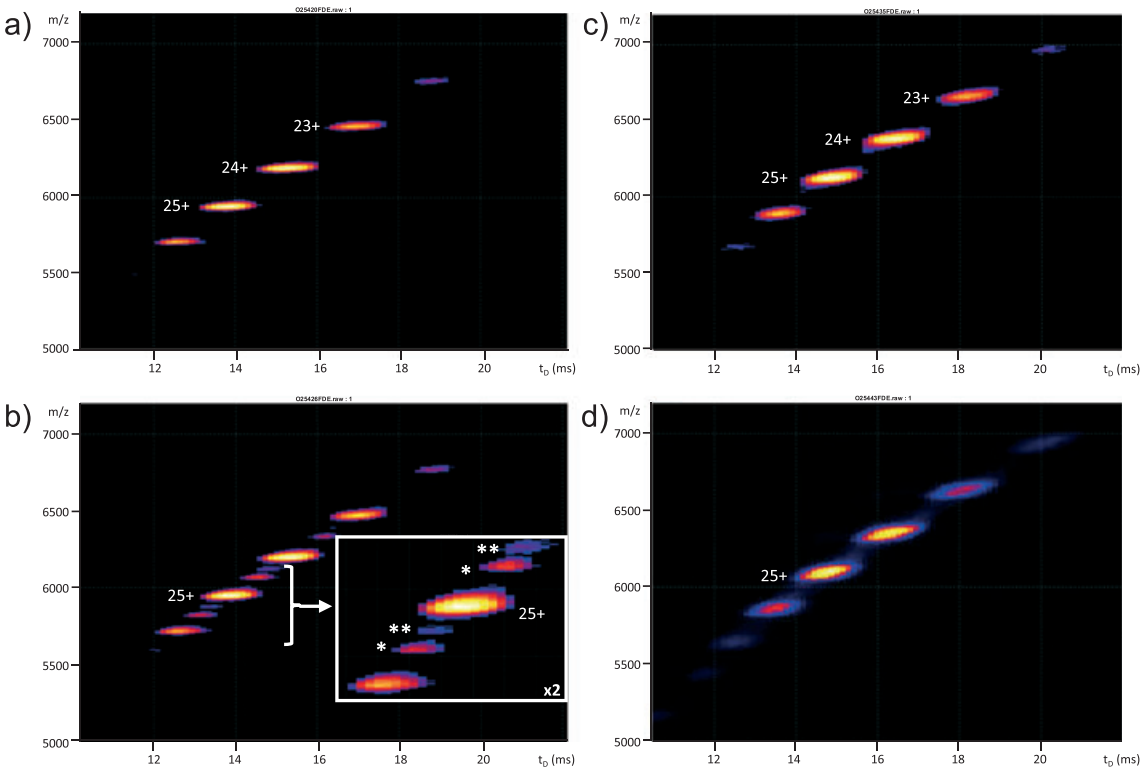


Figure 5. Intact native IM-MS analysis of trastuzumab, cetuximab and their biosimilar/biobetter versions. Driftscope plots of trastuzumab (a), trastuzumab-B (b), cetuximab (c) and cetuximab-B (d). *trastuzumab-B bearing 0 GlcNAcFuc; **trastuzumab-B bearing 1 GlcNAcFuc.

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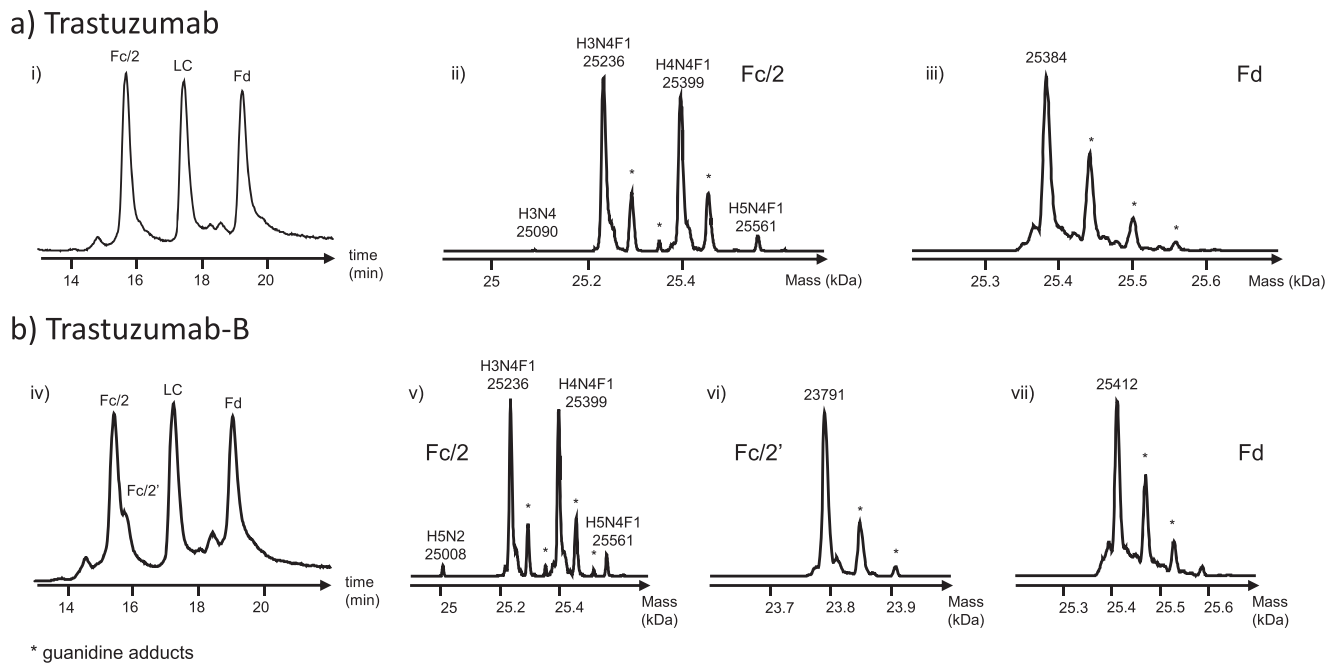


Figure 6. Middle-up LC-MS analysis of trastuzumab (a) *versus* trastuzumab-B (b). Total ion chromatograms of IdeS treated and DTT reduced trastuzumab (i) and trastuzumab-B (iv). Deconvoluted mass spectra of corresponding Fc/2 (ii, v and vi) and Fd (iii, vii) fragments. For clarity, deconvoluted mass spectra of trastuzumab and trastuzumab-B light chains are not shown (see Table 1).

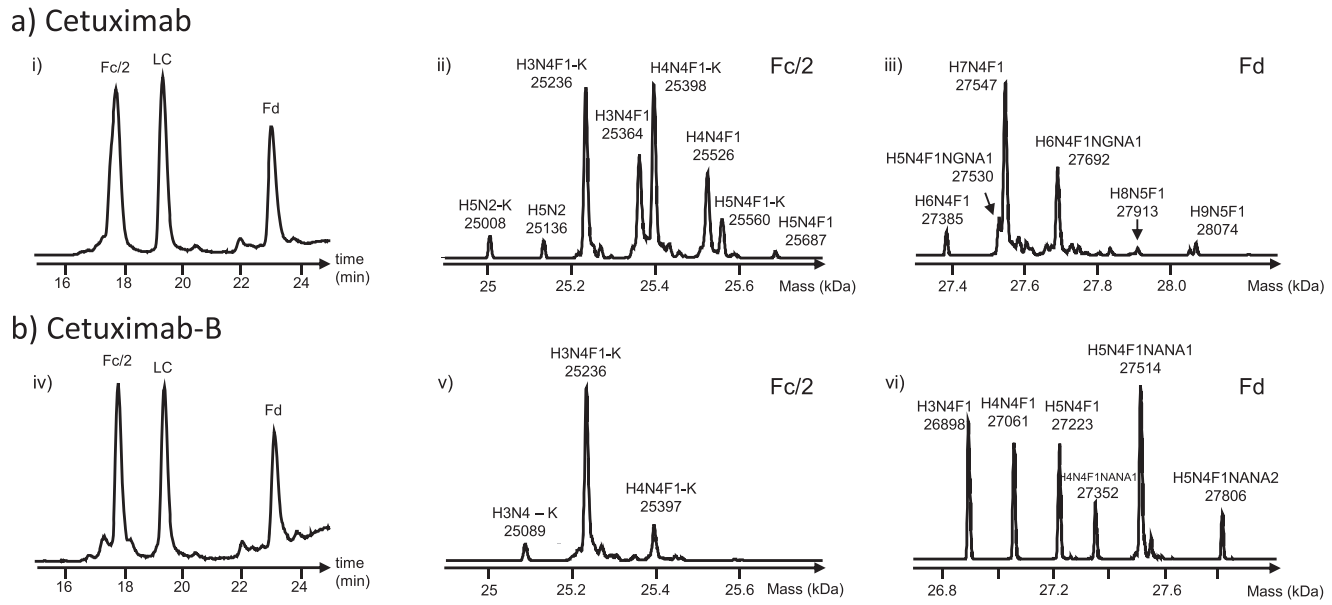


Figure 7. Middle-up LC-MS analysis of cetuximab (a) *versus* cetuximab-B (b). Total ion chromatograms of IdeS treated and DTT reduced cetuximab (i) and cetuximab-B (iv). Deconvoluted mass spectra of corresponding Fc/2 (ii, v) and Fd (iii, vi) fragments. For clarity, deconvoluted mass spectra of trastuzumab and trastuzumab-B light chains are not shown (see Table 2).

to detect and monitor the presence of post-translational modifications (PTMs) even if the site of modification cannot be determined. LC-MS is also a method of choice to measure intact mAb masses and to distinguish batch-to-batch heterogeneities. Intact IgG mass analysis performed either by LC-MS or by direct ESI-MS infusion is relatively fast and does not require lengthy sample preparation and induces fewer modifications compared to peptide mapping (e.g. no trypsin-induced deamidation). Based on the deconvoluted masses, peaks observed were found to be very close to the calculated masses and assigned to trastuzumab main glycoforms: G0/G0F (147,913 Da), G0F/G0F (148,063 Da), G0F/G1F (148,223 Da), G0F/G2F or G1F/G1F (148,384 Da) (Fig. 2A, Table S1). In the case of trastuzumab-B there is a mass discrepancy of +53 Da for example for the G0F/G1F isoform (Fig. 2B). This indicates that the K217R difference in both heavy chains can be suspected at the intact antibody level but must be confirmed at the middle and bottom levels. Surprisingly, a significant amount of an additional species that may correspond to a non N-glycosylated variant on both heavy

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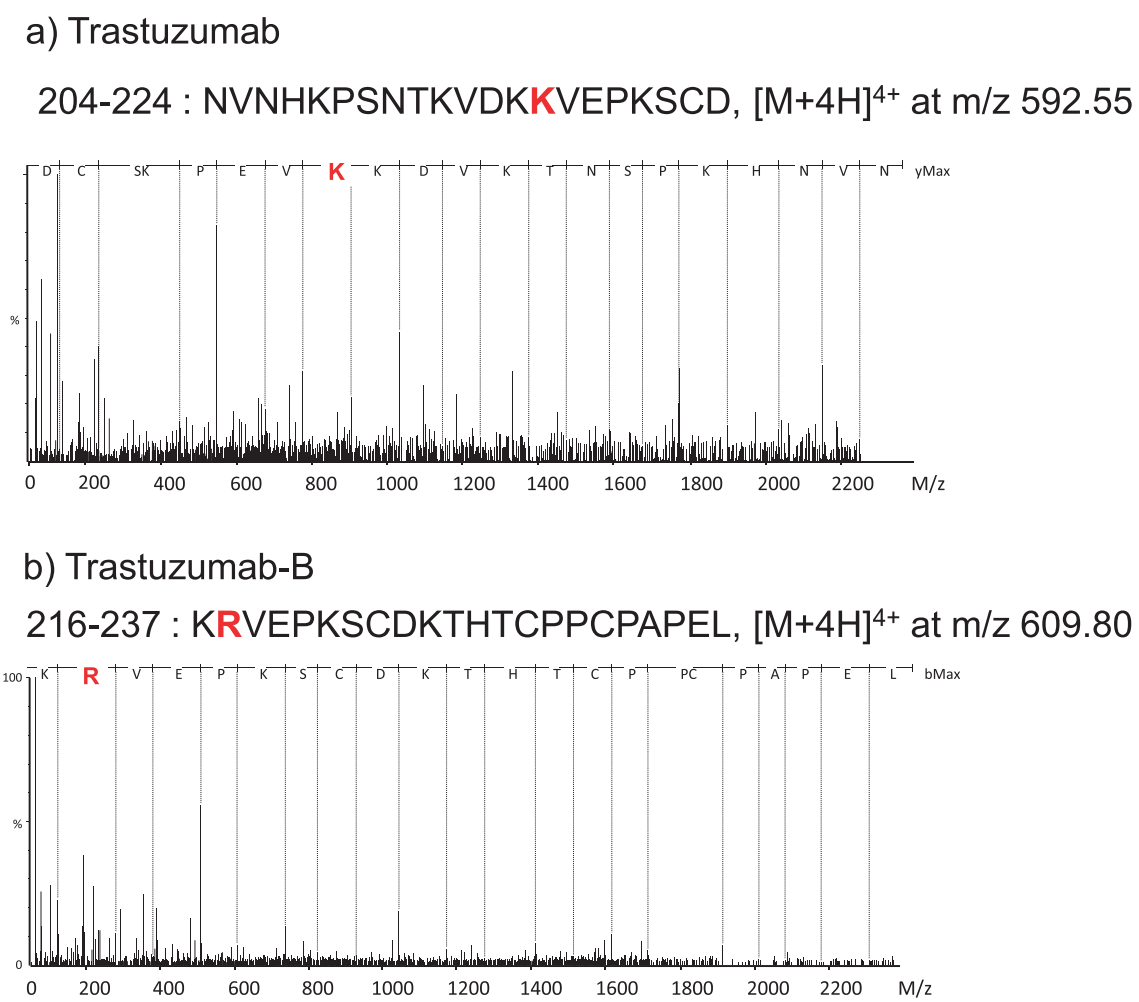


Figure 8. Bottom-up analysis of trastuzumab *versus* trastuzumab-B. Tandem mass spectra of: (a) trastuzumab 204-224 NVNHKPSNTKVDKKVEPKSCD peptide and (b) trastuzumab-B 216–237 KRVEPKSCDKTHTCPPCPAPEL peptide. Differential amino acids are in bold red.

chains is also visualized (−2890 Da, 2xG0F) in trastuzumab-B (15% by CE-SDS-UV, data not shown). Both structural features are critical and indicate that trastuzumab-B would not be a suitable biosimilar candidate in highly regulated countries. The following additional experiments must be used to confirm these working hypotheses. In the case of cetuximab-B, the amino-acid sequence is the same as for cetuximab, but the MS profiles are much more complicated for the intact IgGs (Fig. 2C,D). Middle and bottom analyses confirmed that the heterogeneity is linked to the additional glycosylation site in the Fd domain along with larger amounts of remaining C-terminal Lys on one or two heavy chains.[33]

Antibodies EndoS Fc deglycosylation prior to denaturing MS (top level)

IgGZERO™ (EndoS, www.genovis.com) is an endoglycosidase specific for N-glycans on the Fc domain of IgGs which are cleaved after the first Glc-NAc. The reaction is fast (30 min *vs* 10 h for PNGAses F) and mild under physiological conditions with no need for detergents. After IgGZero™ treatment, the most intense ion series correspond to expected trastuzumab-B with 2 GlcNAcFuc (1 on each HC). More surprisingly two additional species were detected by denatured MS corresponding to heavy chains bearing either 0 or 1 GlcNAcFuc, a strong proof of the presence of non glycosylated heavy chains in relatively large amounts (Fig. 3A,B). Due to the presence of 2 N-glycosylation sites for cetuximab and cetuximab-B heavy chains, only partial deglycosylation is observed (in the Fc, not in the Fab), and denatured mass spectra interpretations remain still complicated (Fig. 3C,D).

Intact mAb analysis by native MS (top level)

Native MS based on nanoESI-MS technology can be used to precisely and unambiguously characterize mAb/antigen systems especially in terms of binding stoichiometries.[34] This technique has also been successfully applied to highlight the presence of higher order structure including dimers, trimers and tetramers,[35] sometimes even in combination with size exclusion chromatography.[36] Native MS was also reported to study wild type IgG4 structural dynamics.[37] Native mass spectra of trastuzumab, trastuzumab-B, cetuximab and cetuximab-B are displayed in Fig. 4A–D, respectively. Native mass spectra lead to easier data interpretation compared to denatured mass spectra (compare Fig. 2 and Fig. 4) due to a lower number of charge states and fewer charges carried by protein ions. For all mAbs, glycosylation patterns correspond to those observed in denaturing conditions. Regarding higher order structures, minor populations of dimers are detected for both trastuzumab and cetuximab, and to a lower extent in their corresponding biosimilar versions. Importantly, native MS analysis allowed

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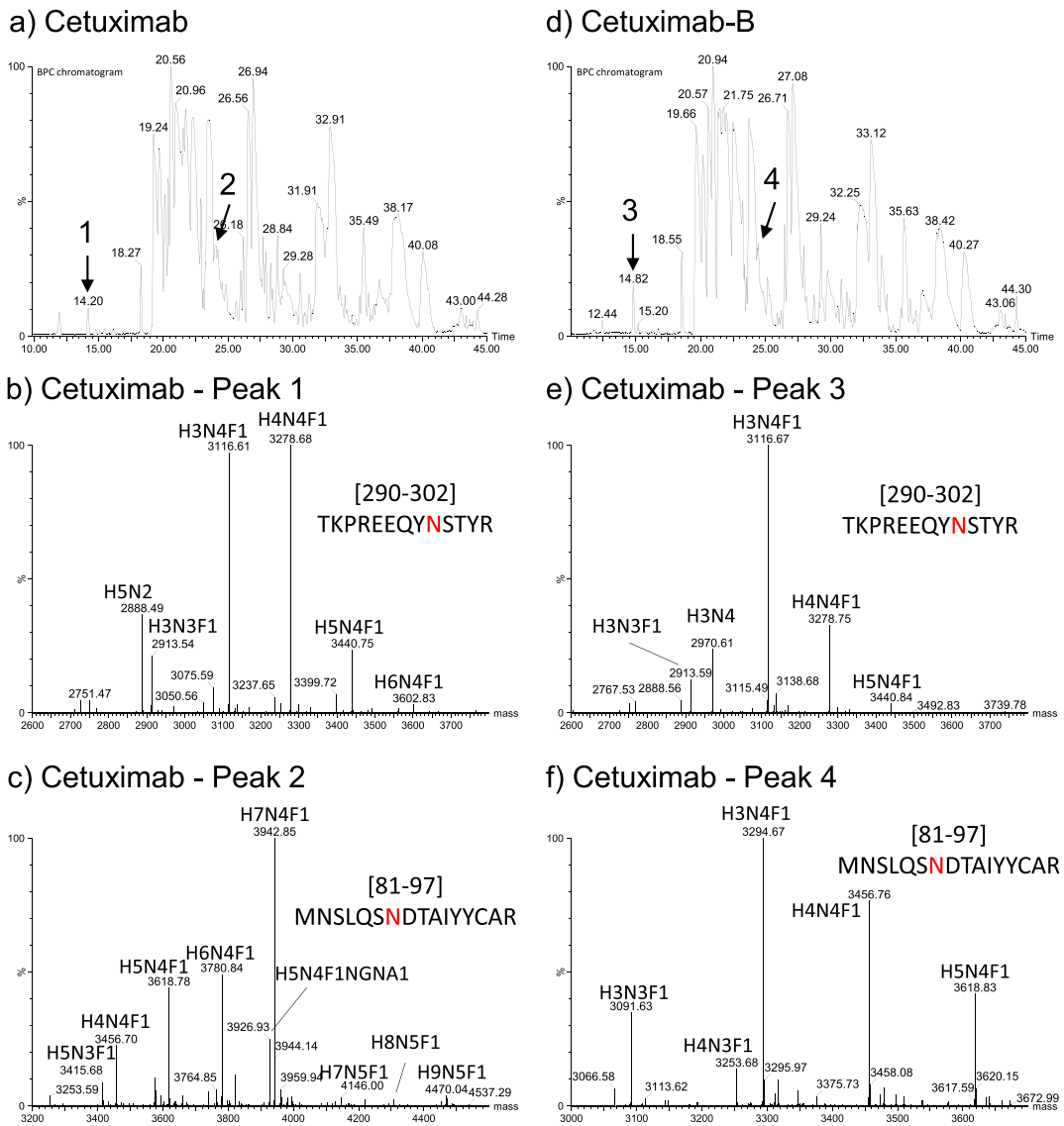


Figure 9. Bottom-up glycopeptide analysis of cetuximab and cetuximab-B. Base peak chromatograms of cetuximab (a) and cetuximab-B (d) obtained after trypsin digestion and nanoLC-MS/MS analysis performed on a Q-TOF instrument. Deconvoluted mass spectra of glycopeptides [290–302] (b and e) and [290–302] (c and f) showing different glycosylations for cetuximab and cetuximab-B.

unambiguously detecting and quantifying a significant fraction of non N-glycosylated variant on one heavy chain (18% as deduced from native MS, close to the 15% resulting from CE-SDS-UV). IgGZERO™ is also compatible with native MS (see Fig. S1).

Intact mAb characterization by ion mobility-MS (IM-MS) (top level)

Ion mobility spectrometry is a charge and shape-selective separation technique. Combined to native MS, ion mobility provides a simple and direct shape/size measurement of mAbs. A first application of IM-MS is to detect disulfide bridge pairing heterogeneity on intact IgG antibodies as well as on mAb/Ag complexes. For example, two to three gas-phase conformers have been observed by IM-MS for IgG2 antibodies known to exhibit disulfide-linked isomers.[38] These gas-phase conformers were also maintained on deglycosylated IgG2s. Analysis of redox refolded IgG2 as well as an IgG2 cysteine to serine single point mutant clearly demonstrated that the observed gas-phase conformers were related to disulfide variants. Structural differences between IgG1 and IgG4 have also been recently reported based on IM-MS.[39] Conformational changes induced during dynamic processes, like IgG4 fab-arm exchange, can also be monitored by native IM-MS.[37] For comparability studies, IM-MS is an interesting methodology for the characterization of intact antibodies and may be useful to routinely fingerprint higher order structures. IM-MS measurements can be extended to investigate conformational changes induced upon antigen binding to individual mAb isoforms in order to gain more structural insights. As an illustration IM-MS spectra of trastuzumab, trastuzumab-B, cetuximab and cetuximab-B recorded under native conditions are displayed in Fig. 5A–D, respectively. Except tratstuzumab-B, all other mAbs display homogeneous IM-MS driftscope plots. For trastuzumab-B, heterogeneities related to trastuzumab-B bearing 0 or 1glycan per Fc are detected, suggesting that IM-MS can be an interesting visualization tool for comparability studies. IM-MS can also be performed in denaturing conditions.[2,13]

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IdeS digestion of IgGs followed by LC-MS analysis (middle level)

Middle approaches, which refer to mAb analysis after its cleavage into large fragments (25–100 kDa), appear as valuable alternatives to intact mass analysis. Smaller mAb fragment sizes allow easier and more straightforward LC-MS analysis, potentially with higher mass accuracies. The bacterial cysteine protease immunoglobulin-degrading enzyme of *Streptococcus pyogenes* (IdeS, Fabricator®, www.genovis.com) is now widely used in mAb characterization and generates three 25-kDa fragments. This approach has the advantage of being rapid (less than 2 h for the entire analysis including digestion and LC-MS analysis), informative and low material consuming.

Theoretically protein downsizing and sequencing can be achieved by gas phase fragmentation in the mass spectrometer, referred to as top down MS. However, top down sequencing to reach 100% sequence coverage is still difficult to obtain especially on large biomolecules like mAbs (150 kDa). Tsybin *et al.* used top down MS to get sequence confirmation of terminal regions as well as variable mAb domains, for the identification and localization of major modifications or for the characterization of major glycoforms.[40]

The middle-down approach could be a good alternative to top-down MS. In a recent study, Fornelli *et al.* analyzed by reversed phase liquid chromatography coupled online with an electron transfer dissociation (ETD) orbitrap mass spectrometer several IdeS digested IgGs.[41] Upon optimization of ETD and product ion transfer parameters, up to 50% sequence coverage for selected IgG fragments was reached in a single LC run, while up to 70% was reached when data obtained by distinct LC-MS runs were averaged. This middle-down approach allowed also the identification of oxidized methionine residues.

More classically, middle-up approaches, consisting of intact mass analysis of 25-kDa mAb fragments generated by IdeS treatments and further reduction, can be set up on current instrumentations available in most labs. Here we have performed middle-up analysis of trastuzumab, cetuximab and their related biosimilar/biobetter versions on a Q-TOF instrument (Figs. 6 and 7). As expected after IdeS treatment followed by reduction, chromatographic profile obtained for originators and biosimilars was very similar with three main peaks corresponding to Fc/2, LC and Fd fragments, respectively. As expected, two Fc/2 fragments are observed for trastuzumab-B (Fig. 6, Table 1): one corresponding to reduced Fc/2 fragment bearing glycosylations and the other fully deglycosylated, in agreement with trastuzumab-B intact mass analysis (Fig. 6B, Table 1). Similarly discrepancies in masses measured for Fd fragments confirmed trastuzumab-B K217R modification compared to originator trastuzumab (+28 Da as expected). Middle-up analysis is even more informative for highly glycosylated mAbs as cetuximab (Fig. 7, Table 2). Differences in glycosylation patterns of cetuximab and its biosimilar version can immediately be visualized on Fc/2 and Fd fragments.

Peptide and glycopeptide mapping by LC-MS/MS (bottom level)

The bottom-up approach refers to the analysis of protein digest mixtures after enzymatic digestion. Following denaturation, reduction and alkylation of Cys residues, antibodies or recombinant proteins are commonly digested with trypsin or endoproteases such as Lys-C, Asp-N or Glu-C. LC-MS/MS of proteolytic peptides is the method of choice for primary sequence confirmation and for PTM localization, as for example aspartic acid isomerization and asparagine deamidation in therapeutic antibodies during their production and resulting in charge variants.[42] Peptide mapping is the key MS method to identify the various structures resolved by high-resolution chromatographic methods such as cationic chromatography exchange.[43,44] In a previous study, the trastuzumab structure was compared to a biosimilar candidate version.[10] LC-MS intact mass measurement revealed that the biosimilar mAb had a mass difference of −64 Da compared to the innovator one. The mass difference was located on heavy chains (~32 Da each). nanoLC-MS/MS analyses allowed attributing the difference to a sequence variant of tryptic peptide HT35. Heavy chain sequences with two different amino acids on HT35 (Asp^{359} and Leu^{361} instead of Glu^{359} and Met^{361}) produced a 32-Da lower mass for the biosimilar compared to the innovator trastuzumab. No other sequence differences were detected between the two mAbs. The identified sequence variants demonstrate that the biosimilar candidate was derived from a different allotype compared to the innovator mAb.[45][24] Trastuzumab, cetuximab and their biosimilar/biobetter versions were analyzed by bottom-up analysis after trypsin digestion and nanoLC-MS/MS analysis on a Q-TOF instrument. Bottom-up analysis allowed confirmation of K214R mutation in trastuzumab (Fig. 8). In case of cetuximab and cetuximab-B, deconvoluted mass spectra interpretation of selected glycopeptides allows to depict glycosylation differences at the peptide level between both samples (Fig. 9).

Peptide and glycopeptide mapping by CESI-MS/MS (bottom level)

Capillary zone electrophoresis coupled to mass spectrometry by a sheathless interface (CESI-MS/MS) has recently emerged as an orthogonal alternative to LC-MS/MS.[30] CESI-MS is capable of analyzing simultaneously very small (<300 Da) and very large peptides

Table 1. LC-MS middle up analysis of trastuzumab *versus* trastuzumab-B after Ides treatment

Trastuzumab				Trastuzumab-B			
Time (min)	Assignment	Theoretical masses (Da)	Experimental masses (Da)	Time (min)	Assignment	Theoretical masses (Da)	Experimental masses (Da)
15.7	Fc/2 + H3N4	25 089.9	25 090.9 ± 0.5	15.4	Fc/2 + H5N2	25 007.8	25 008.5 ± 0.5
	Fc/2 + H3N4F1	25 236.0	25 236.4 ± 0.5		Fc/2 + H3N4F1	25 236.0	25 236.4 ± 0.4
	Fc/2 + H4N4F1	25 398.2	25 398.7 ± 0.6		Fc/2 + H4N4F1	25 398.2	25 398.6 ± 0.6
	Fc/2 + H5N4F1	25 560.3	25 560.0 ± 0.7		Fc/2 + H5N4F1	25 560.3	25 561.0 ± 0.6
				15.7	Fc/2	23 790.7	23 791.4 ± 0.5
17.5	LC	23 442.9	23 443.7 ± 0.4	17.2	LC	23 442.9	23 443.3 ± 0.3
19.2	Fd	25 383.3	25 383.7 ± 0.5	19.0	Fd	25 411.3	25 411.8 ± 0.4

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Table 2. LC-MS middle-up analysis of cetuximab *versus* cetuximab-B after Ides treatment

Cetuximab				Cetuximab-B			
Time(min)	Assignment	Theoretical masses (Da)	Experimental masses (Da)	Time(min)	Assignment	Theoretical masses (Da)	Experimental masses (Da)
17.8	Fc/2 + H5N2-K	25 007.8	25 007.6 ± 0.3	17.8	Fc/2 + H3N4-K	25 089.9	25 089.9 ± 0.3
	Fc/2 + H5N2	25 136.0	25 135.9 ± 0.3		Fc/2 + H3N4F1-K	25 236.0	25 236.0 ± 0.3
	Fc/2 + H3N4F1-K	25 236.0	25 235.9 ± 0.3		Fc/2 + H4N4F1-K	25 398.2	25 397.9 ± 0.4
	Fc/2 + H3N4F1	25 364.2	25 364.1 ± 0.3				
	Fc/2 + H4N4F1-K	25 398.2	25 398.1 ± 0.3				
	Fc/2 + H4N4F1	25 526.4	25 525.8 ± 0.4				
	Fc/2 + H5N4F1-K	25 560.3	25 560.1 ± 0.4				
	Fc/2 + H5N4F1	25 688.5	25 687.4 ± 0.5				
19.3	LC	23 427.7	23 426.3 ± 0.2	19.4	LC	23 427.7	23 426.6 ± 0.3
23.0	Fd + H6N4F1	27 385.1	27 385.1 ± 0.4	23.1	Fd + H3N4F1	26 898.7	26 898.6 ± 0.3
	Fd + H5N4F1NGNA1	27 530.3	27 530.4 ± 0.7		Fd + H4N4F1	27 060.9	27 060.9 ± 0.4
	Fd + H7N4F1	27 547.3	27 547.0 ± 0.3		Fd + H5N4F1	27 223.0	27 222.8 ± 0.5
	Fd + H6N5F1	27 588.3	27 584.8 ± 0.7		Fd + H4N4F1NANA1	27 352.1	27 352.2 ± 0.5
	Fd + H6N4F1NGNA1	27 692.4	27 692.5 ± 0.4		Fd + H5N4F1NANA1	27 514.3	27 514.3 ± 0.3
	Fd + H7N5F1	27 750.5	27 750.6 ± 0.5		Fd + H5N4F1NANA2	27 805.5	27 806.3 ± 0.8
	Fd + H5N4F1NGNA2	27 837.5	27 838.8 ± 0.9				
	Fd + H8N5F1	27 912.6	27 912.9 ± 0.6				
	Fd + H7N5F1NGNA1	28 057.7	28 057.7 ± 0.6				
	Fd + H9N5F1	28 074.8	28 073.9 ± 0.6				
	Fd + H8N5F1NGNA1	28 219.9	28 219.6 ± 0.8				

(>8000 Da) under the same experimental conditions achieving 100% sequence coverage within one single run and one enzymatic digest. High ionization efficiencies provided by CESI and highly sensitive MS allowed in-depth analysis of glycosylations with relative abundance varying more than 3 orders of magnitude.[46] As a further evaluation, we have used CESI-MS/MS to compare trypsin maps of trastuzumab and trastuzumab-B and cetuximab and cetuximab-B.[47] Using only a single sample injection of 200 fmol, CESI-MS/MS data enabled 100% amino acids sequence characterization for the 4 mAbs allowing distinguishing precisely a difference of even one AA between trastuzumab and trastuzumab-B (R214K). Simultaneously N-glycoforms were characterized regarding their structures and position through fragmentation spectra and glycoforms semi quantitative profiling of 15 different glycoforms in trastuzumab. Other posttranslational modifications hotspots (Asn deamidation, IsoAsp and Met oxidation) were also characterized, while their relative occurrence levels were estimated and compared to the biosimilar candidates. More generally, capillary electrophoresis-mass spectrometry (CE-MS) approaches will play an increasingly role in the future for antibody characterization as illustrated for example by automated off-line CE-UV/MALDI-MS/MS with integrated delivery matrix system.[48]

HDX-MS (bottom level)

Among structural techniques able to investigate protein higher order structures and dynamics, HDX-MS has demonstrated suitability for conformational analysis of mAbs.[49] Conformational features for example of an intact glycosylated IgG1 relative to its deglycosylated version were reported, demonstrating how glycosylation affects IgG1 conformation.[50] HDX-MS can accurately provide useful new information about mAb conformation and solution dynamics, data that are more difficult to obtain with other analytical methods. H/D exchange coupled to pepsin proteolysis, HPLC chromatographic separation and MS analysis is the most common set up for HDX-MS experiments. Pepsic peptides are analyzed by LC-MS to assess the deuteration level of each peptide. Here again, ion mobility can be a valuable tool to introduce an additional level of separation for peptides with overlapping isotopic patterns, helping in correct peptide assignment and increased sequence coverage. HDX-MS combined to structural modeling has also been reported for detecting local conformational changes induced by common chemical modifications including methionine (Met) oxidation, aspartic acid (Asp) isomerization and asparagine (Asn) deamidation.[51] Physicochemical and functional comparability between rituximab and a biosimilar candidate (GP2013) were recently reported including HDX-MS.[52]

Conclusions and future directions

The approval process for biosimilars relies primarily on comprehensive analytical data to establish comparability and high similarity with the originator biologic. The development of biosimilar antibodies and Fc-fusion proteins is based on the scientific knowledge gained from the originator product. As a limitation, a manufacturer developing a biosimilar does not have access to all information that could allow an exhaustive comparison with the reference antibody. Biosimilars are manufactured and also controlled according to their own development. As highlighted here, multiple state-of-the-art mass spectrometry methods in combination with high-resolution separation methods such as liquid chromatography and capillary electrophoresis greatly help to show similarity between the reference product and the biosimilar candidates.

Top-level investigation of intact and reduced mAb by liquid chromatography coupled to an electrospray ionization mass spectrometry (LC-MS) provides fast and accurate profiles of entire mAbs at the top level and smaller fragments (Fc/2, LC and Fd) at the middle level. Fine structural characterization of mAbs is further performed by a classical 'bottom-up' approach after first digesting IgG and or

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light/heavy chain into peptides and glycopeptides, bearing in mind that enzymatic digestion may also increase the level of modifications, such as cyclization of N-terminus glutamine and deamidation depending on pH, temperature and duration.

Recently, a paradigm shift in regulatory thinking on the non-clinical development of biosimilars has emerged in Europe: biosimilar candidates may enter in the clinic without animal studies.[53] The mainstay for the establishment of biosimilarity is the pharmaceutical comparability based on extensive physicochemical and biological characterization. Pharmacodynamic comparability can be evaluated in *in vitro* assays, whereas pharmacokinetic comparability is evaluated in clinical studies. It is considered highly unlikely that new safety issues would arise when comparability has been demonstrated based on physicochemical and *in vitro* comparative studies. Obviously, MS will play increasing roles in comparability studies of biosimilars and originators and the limited use of *in vivo* studies in animals.

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