Qualitative and Quantitative Characterization of Therapeutic Antibodies by Native Mass Spectrometry

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Qualitative and Quantitative Characterization of Therapeutic Antibodies by Native Mass Spectrometry

Karakterisering van therapeutische antilichamen met behulp van massaspectrometrie onder natieve condities
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof. dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op woensdag 3 september 2014 des ochtends te 10.30 uur

door

Sara Rosati

geboren op 15 maart 1985 te Termoli, Italië

Promotor	Prof. dr. A.J.R. Heck
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Chapter I

A Short Introduction into Native Mass Spectrometry and Antibodies

Partially based on:

Performing Native Mass Spectrometry Analysis on Therapeutic Antibodies

N. J. Thompson, S. Rosati, A.J.R. Heck

Methods 65, 11-17 (2014)

My thesis collects the work of four years, wherein I enjoyed looking for what is invisible to the naked eye. For the work described in my thesis, mass spectrometers somehow became my eyes, and, if you hold on and keep on reading, you will see what good eyes we managed to develop in these years to see the things we wanted to study!

If you are really willing and have time to read the whole thesis, (and I would be glad if someone really does) be warned: you will read the term "native mass spectrometry" or "Native MS" hundreds of times! Since I am particularly kind (after all I am a native MS person, gentleness is my specialty!) I'm going to introduce my PhD work by spending a few words on this peculiar terminology, extremely common for my colleagues and me, but maybe a little arduous for most people, even experts in chemistry, pharmacy and biology.

The word "native" was coined for the first time as an adjective to describe "mass spectrometry" by my promotor Albert Heck in 2004[1,2] and has since then become widely accepted in the field, with even workshops and conferences organized around this technique. The unique feature that led to the choice of such adjective is the capability of native mass spectrometry to mass analyze protein and protein complexes in the gas-phase, whereby non-covalent interactions can be retained. Therefore, proteins can be analyzed retaining properties of the tertiary and quaternary structures of the samples as it occurs in more conventional and broader known techniques of size-exclusion chromatography and native (non-reducing) gels.

Are intrinsic properties really conserved during native MS analysis? Is a question my colleagues and myself get questioned many times. Most people may argue that proteins transferred into the gas-phase, completely desolvated, carrying a number of charges, are very unlikely to retain their "real" physiological structure, conformation and function. Still, cumulating from many reports from our group and others it has now been established that proteins during this transition can keep a certain level of conformational memory[3]. Notwithstanding, we are aware that a certain level of disruption will occur. However, if this disruption does occur this is evidently of such low extent that most non-covalent interactions remain intact. That is very fortunate for me as it allowed me to investigate interesting properties of proteins, and in particular of therapeutic antibodies, described throughout this thesis.

1. Native Mass Spectrometry

Mass spectrometry (MS) is a technique with a broad range of applications, in part due to the universal nature of its capability to separate and identify multiple components in mixtures by differences in mass. Although MS has been around for more than a century, it was initially limited to the analysis of small molecules due to the inability to transfer large biomolecules into the gas phase efficiently, without breaking them apart. However, with the introduction of so-called "soft" or "gentle" ionization techniques, *i.e.*, matrix-assisted laser desorption ionization (MALDI)[4] and electrospray ionization (ESI)[5], MS became capable of studying much larger biomolecules. This has been regarded as such a breakthrough for science that it has been awarded the Nobel Prize in 2002. The application area of ESI was subsequently even further extended via the incorporation of volatile buffers at neutral pH as solvents, *e.g.*, aqueous ammonium acetate. This combination allowed the retention of non-covalent interactions and thus protein complexes in the gas phase, a technique as explained above termed native MS[1,2,6]. Native MS has further evolved to utilize nanoflow ESI for even more gentle ionization conditions at much higher sensitivity and coupled to quadrupole-time-of-flight (Q-TOF) analyzers, with a theoretically infinite mass range. The dream of Nobel Laureate John Fenn that electrospray could make "elephants fly" has not been achieved yet, although molecular elephants such as whole intact ribosomes and viruses[7,8] can now be ionized and thus studied by native mass spectrometry. Therefore, it is worthwhile to describe the technology used in a bit more detail (Figure 1).

Nano-electrospray ionization (nano-ESI)

Nowadays, ESI is one of the most widespread methods of ionization used in MS, used for amongst other small molecules, lipids, sugars, polymers and peptides and proteins. In conventional ESI a voltage difference ranging from 3 to 6 kV is applied across a capillary containing the sample and the counter-electrode placed at atmospheric pressure. The flow through the capillary is typically in the µl/min range. The resulting electric field induces the formation of a Taylor-cone consisting of small charged liquid droplets at the tip of the capillary. The evaporation of the solvent causes the shrinking of the droplets around the analyte. For large molecules, such as intact proteins, it is believed that ions enter the gas-phase via the so-called charge residue model, in which Coulombic repulsion between charges induces a number of fission events, drastically reducing the droplet size, until the bare, completely desolvated, charged analyte is transferred into the gas-phase[10-12].

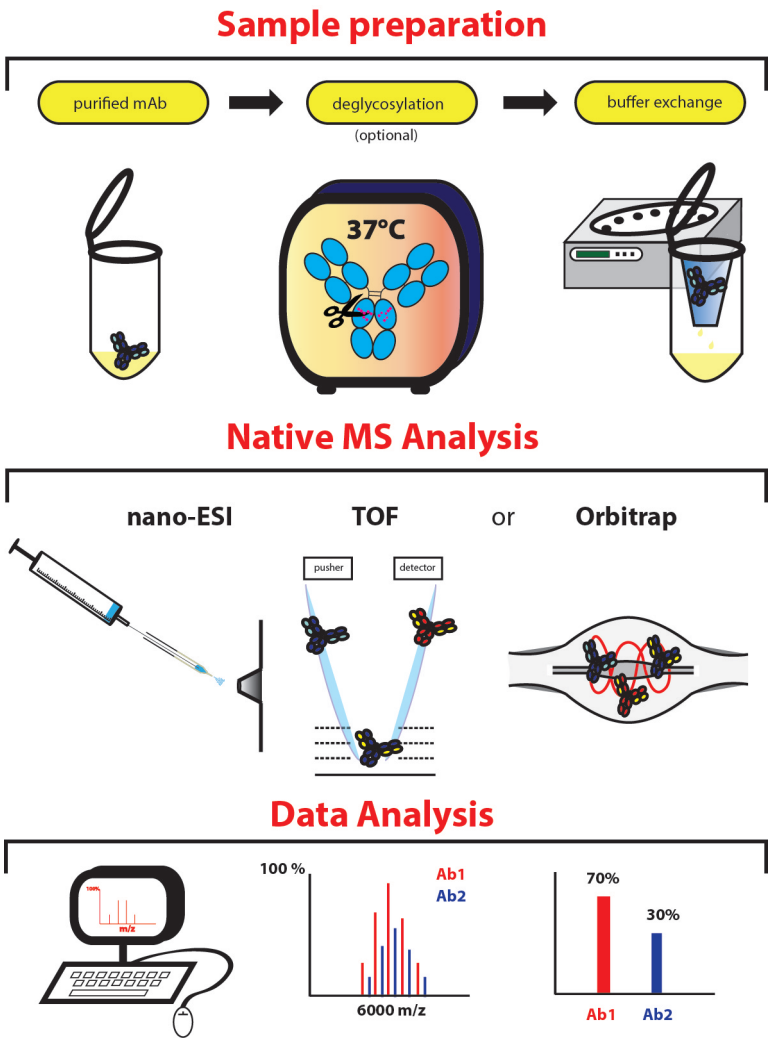

Figure 1. Experimental workflow of native MS analysis of mAbs pictured in three main steps: sample preparation, native MS analysis, and data analysis. Starting from the purified sample, a deglycosylation step can be performed followed by buffer exchange. Instrumental analysis is performed on TOF or Orbitrap instruments, directly infusing the sample using a nano-ESI source. Finally, several software programs can be used to analyze the raw data thus obtaining both qualitative and quantitative information. Reprinted from[9].

Two unique features have made of ESI "the" ionization method for native MS: i) the mildness of the ionization process, and ii) the formation of multiply charged ions. While the first feature is necessary to preserve non-covalent interactions, the second allows the detection of large biomolecules at (relatively) low m/z.

Yet, these two very important features are not enough to make a native protein or

protein complex “fly” in the gas phase. Nano-ESI was initially described by Wilm and Mann in the 1990s[13] and technological enhancements reduced droplet size (150 nm for nano-ESI versus 1.5 µm for ESI) via a smaller capillary column and orifice (diameter of 1-10 µm for the nano-ESI versus 130 µm for the standard ESI). As only a minor volume of liquid solution needs to be removed during the ionization process, less volatile buffers are now tolerable, such as the aqueous buffers used in native MS. Moreover, a capillary voltage of 1 to 1.5 kV is normally sufficient to ensure an efficient ionization. Another advantage of nanospray is the reduction of the sample volume, requiring typically just a few microliters containing a few picomole of sample.

Although commercially available, nano-ESI capillaries can be prepared in-house, by using a capillary puller. A borosilicate glass capillary is heated by a filament, and is pulled apart to generate two capillaries ending with extremely thin (and fragile) tips. Subsequently, a sputter coater is used to coat the capillaries with a thin layer of gold to ensure conductivity.

At this point of the introduction, it is worth reminding the reader that, in native MS, in some contrast with applications in proteomics and metabolomics, the introduction of the sample into the mass spectrometer takes place typically via direct infusion. Therefore, mass spectrometers used for native MS are typically not coupled with any separation devices at the front end. The reason for the latter has its prime origin in the non-native conditions that are used in a majority of the separation techniques such as organic solvents and acidifiers in liquid chromatography (LC). Thus, 1 to 5 µl of sample, with an analyte concentration between 1 and 10 µM, is loaded directly in the capillary. When using nano-ESI, the spray is assured by the electrical field between the capillary and the counter-electrode, therefore, no back-pressure is required.

As mentioned above, ESI generates multiple-charged ions. Mostly protonated ions are formed when using ESI in the positive ion mode, used for peptides and proteins, and deprotonated when using ESI in the negative ion mode, typically used for RNA and DNA analysis. However, not all the molecules will attain the same number of charges. As a consequence, the resulting ESI-MS-spectrum of an intact protein is characterized by a number of peaks corresponding to a distribution of different charge states. When native MS is performed, a substantial lower number of charge-state peaks are observed compared to MS performed under denaturing conditions. This is due to the retained folded conformation, making many chargeable sites on the proteins less accessible for protonation. An example of a nano-ESI MS spectrum of an antibody sprayed under denaturing and native

conditions is shown in Figure 2. In general, when the sample is analyzed under native conditions, five to seven charge states are observed around m/z 6000. The mass spectrum appears very different when denatured conditions are used. In the latter case, because of the unfolded conformation, a higher number of residues become accessible for protonation resulting in a significant increase of number of charges that causes a shift of the signal toward a lower m/z range.

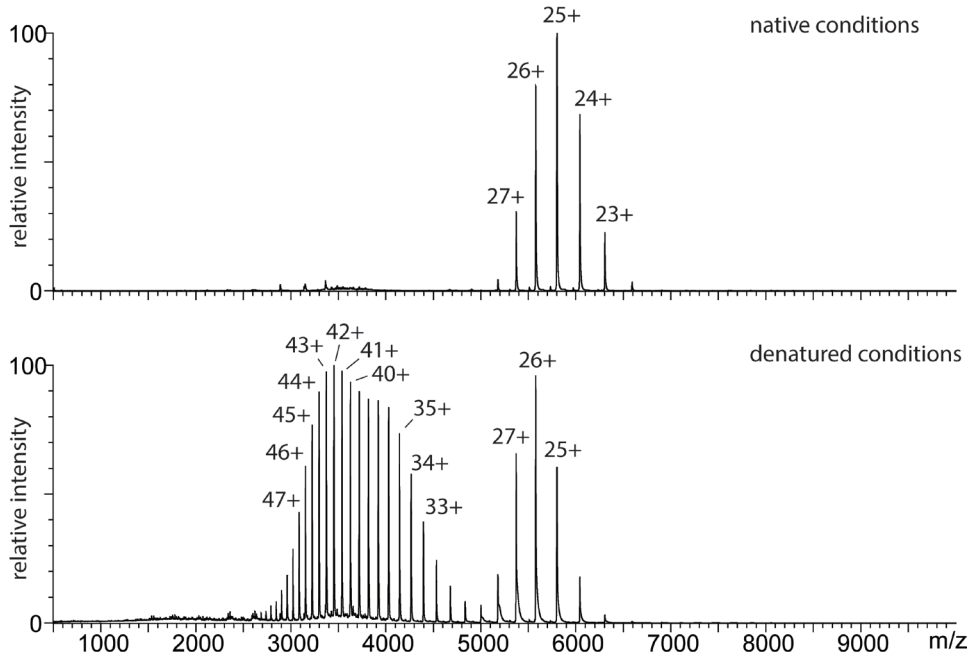

Figure 2. MS spectra of an intact mAb. An intact mAb has been analyzed by nano-ESI MS under native (top panel) and denatured (bottom panel) conditions. Under native conditions the folded conformation of the protein shields a number of residues from protonation, therefore fewer charge state are detected. On the contrary, under denaturing conditions more residues become accessible for protonation resulting in a higher number of charge states and a shift toward a lower m/z range.

Time-of-flight (TOF) analyzer-based mass spectrometers

Because of its theoretically unlimited mass range, the time-of-flight (TOF) mass analyzer is the most commonly used mass analyzer in native MS (Figure 3). In a TOF analyzer, the ratio mass-to-charge (m/z) is determined by measuring the time the ions take to reach the detector travelling through a field-free flight tube, with a defined length, kept under high vacuum (10^{-7} mbar). A packet of ions is pushed toward the flight tube by applying a difference of potential in the first acceleration

region where all ions gain the same kinetic energy. Once the ions enter the field-free region, the velocity is inversely proportional to the m/z, thus the ions are separated and characterized by different arrival times.

With regard to the analysis of mAbs, an instrument, consisting of just a nano-ESI-source, a few hexapoles for the transmission of the ions, and a TOF analyzer, such as the in our field popular LCT (Waters, Manchester, UK), can be a perfect choice for routine analysis[14-16]. With a resolution of 1500 at 6000 Th, such an instrument is capable of separating a mixture of two or more antibodies differing by 150 Da in mass (at 50% resolution). When the antibody sequence is known, a mass accuracy within 5 Da not only allows protein identification with high confidence, but can also be useful for the identification of typically occurring modifications, such as C-terminal lysine clipping (128 Da). It is worth mentioning that as the natural isotopic peak width of an intact 150 kDa antibody is estimated to be 25 Da[17] therefore, unfortunately, small mass modifications, such as deamidation (+1 Da), are not easily detectable by native MS.

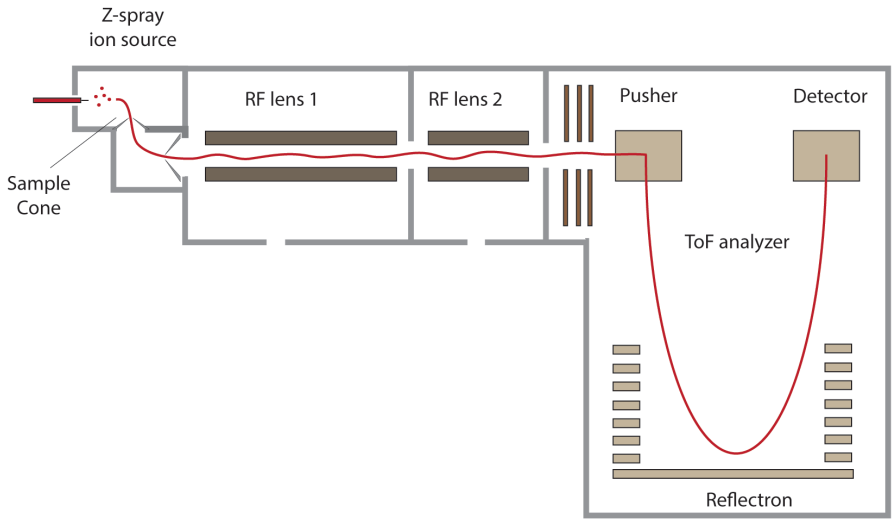

Figure 3. Schematic of a time-of-flight based instrument: the LCT (Waters).The LCT mass spectrometer comprises of a Z-spray ion source where intact mAbs are ionized in their native-like folded conformation using nESI. The ions enter the instrument through the sample cone and are transmitted and focused by two hexapoles (RF lens 1 and RF lens 2). The pusher sends packages of ions in the TOF for their m/z measurements. Adapted from[21].

With the purpose of improving transmission of high m/z ions and preventing the dissociation of non-covalent complexes, this type of ESI-TOF instrument generally works with a relatively high pressure in the first vacuum-stage, just behind the

sample orifice. It has been demonstrated that decreasing the pressure difference between the atmosphere pressure and the first vacuum stage, promotes collisional cooling of the ions and improves transmission of large ions through the ion guides[18-20]. Generally, a valve located in proximity of the first vacuum chamber, allows the reduction of the efficiency of the rotary pump, thus resulting in higher pressures in the first stage of vacuum. For the analysis of mAbs, a pressure between 6 and 7 mbar is optimal.

Another instrument often used in native MS is the Q-TOF (Waters, Manchester, UK or AB Sciex, Toronto, Canada). In such an instrument, a mass-selecting quadrupole (Q) analyzer and a TOF analyzer are coupled with a collision cell in between. This set-up allows tandem-MS (MS/MS) experiments, in which a precursor ion is selected in the quadrupole, dissociated in the collision cell via collision-induced dissociation (CID), and the product ions are analyzed in the TOF. The Q-TOF is the instrument of choice for stoichiometry and stability studies of protein complexes. As normally the mAb structure is already well known and needs not to be analyzed, tandem-MS experiments on mAbs might not be of the highest interest. However, this Q-ToF instruments become of interest when dealing with engineered antibodies or when studying mAbs bound to other proteins, such as antigens, drugs, receptors, etc.[15]. Though one can use a Q-TOF in a scanning mode, thus without selection in the quadrupole and dissociation in the collision cell, the simpler LCT instrument may be the instrument of choice for routine analysis of mAbs. This is not only because of its simplicity and lower cost, but also because the Q-TOF requires additional modifications to allow transmission of large ions, the most important modification being a low frequency quadrupole that allows transmission and selection of ions above 4000 m/z[22-24].

By implementing on the Q-TOF instrument an ion-mobility (IM) cell conformational studies of mAbs and other proteins and protein complexes can be performed. IM is a technique that allows the separation of ions based on their mass, charge and gas-phase collision cross-section[25]. The separation occurs in a gas-filled cell located between the quadrupole and the TOF. Ions with different sizes, crossing the cell under the influence of an electric field, will experience different interactions with the inert-gas molecules, thus influencing their velocity. IM-MS can thus be regarded as the gas-phase analogues of size-exclusion chromatography, which also separates molecules on mass, size, and shape. Currently, the only commercially available instrument for IM-MS that can handle high mass ions as generated by native MS is the Synapt™ from Waters[3].

Orbitrap™ analyzer-based mass spectrometers

Since its introduction on the market in 2005, the Orbitrap™ mass analyzer (Thermo Scientific, Bremen, Germany) has been primarily used for the analysis of small molecules and peptides[26]. Only recently, we demonstrated that this type of mass analyzer can also be adapted for the analysis of large complexes including mAbs using native MS[27,28], as described in detail in Chapter IV of this thesis.

The Orbitrap™ is a particular type of mass analyzer, consisting of two electrodes: an external barrel-shaped electrode and an internal spindle-shaped one. An electrostatic voltage, applied to the central electrode, generates a field that forces the ions to oscillate along the central electrode with a frequency determined by the m/z ratio. A Fourier Transform converts the current generated by the oscillating ions into single frequencies and intensities yielding the mass spectrum.

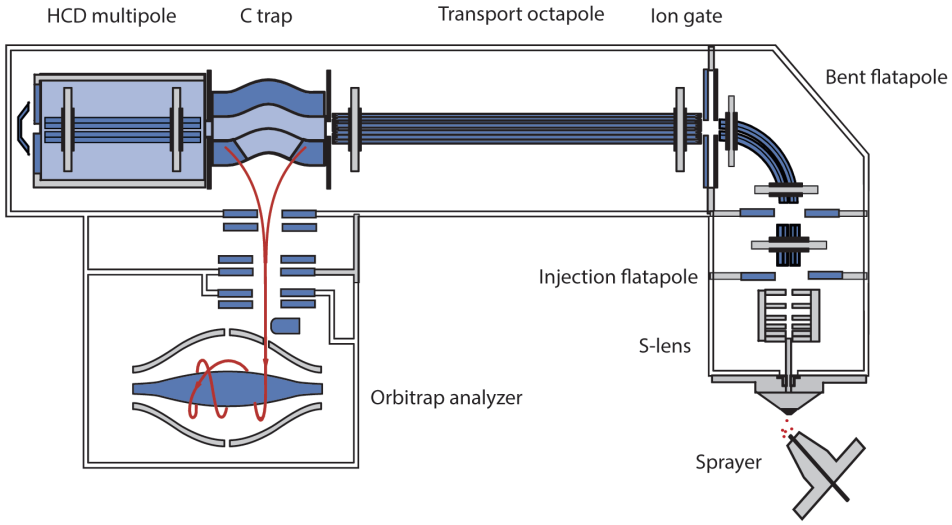

Figure 4. Exactive Plus EMR (ThermoFisher Scientific). The Exactive Plus mass spectrometer consists of a nESI source wherein ions are generated. The ions are focused and transmitted through a bent flatapole, transport octapole and C-trap until they enter the HCD cell. In the HCD cell, the ions are trapped and eventually sent to the Orbitrap for accurate mass analysis. Adapted from[21].

Initially, the Exactive Plus™ (Thermo Fisher Scientific, Bremen, Germany) Orbitrap™ instrument (Figure 4) has been modified for usage in native MS mode. The instrument is equipped with an higher energy collision induced dissociation (HCD) cell in which the ions are stored for improved transmission and desolvation. Native MS experiments are possible on such a commercial instrument only after hav-

ing applied a number of modifications. The majority of these are software-related modifications that allow the user to manually tune voltage offsets to improve the transmission of large ions. The HCD cell, normally filled with nitrogen, can be filled with a heavier gas, such as argon or xenon, when working with big molecular complexes. For the analysis of mAbs, both nitrogen and xenon yield intense, desolvated ions. Following our initial work, this modified instrument has been commercialized and is now available as the Exactive™ Plus EMR Orbitrap™. The main advantage of this type of instrument is the improved mass resolving power. Currently, it is believed that this is to a large degree due to more efficient desolvation of the ions in the Thermo ion source. In the field of native mass spectrometric analysis of mAbs, this becomes the instrument of choice when dealing with complex antibody mixtures or whenever it is essential to discriminate between two species very close in mass[29]. Indeed, another application of this instrument is in the analysis of glycosylation profiles of antibodies as different isoforms can be resolved very nicely[21,30]. Examples of such analysis of glycosylation profiles by this new mass analyzer can be found in chapter V and VI of this thesis.

2. Therapeutic Monoclonal Antibodies

In the last decade therapeutic monoclonal antibodies (mAbs) have become some of the most successful classes of protein therapeutics. Although their history is relatively short compared to small molecule drugs, the first therapeutic mAb was already approved in 1986. From then on their success has made them the fastest growing class of protein therapeutics with 34 mAbs approved in USA and Europe in the last two decades[31,32]. The key of this success relies in their high target specificity, that made many people think of the “magic bullets” theory postulated by the noble prize Paul Ehrlich already at the beginning of the 20th century[33]. In addition, mAbs are very versatile molecules: different mAbs can be generated against as many different targets and thus used in many therapeutic disease areas.

Structural features of antibodies

Natural occurring antibodies, also known as immunoglobulines (Igs) are glycoproteins that are produced by plasma cells in response to immunogens. Based on their amino acid sequence and other structural features they are often classified in 5 different categories: IgA, IgG, IgD, IgM and IgE (Figure 5). Among these, IgG, the most abundant Ig class in human serum (75% of all Igs), is the class that has been used as therapeutic agent. IgGs are further divided into 4 subclasses: IgG1, IgG2, IgG3 and IgG4 based on differences in their amino acid sequences and number and arrangement of disulfide linkages they contain. With the exception of

IgG3s, which have unfavorable pharmacokinetics, all other IgG subclasses have, at least, one representative among all marketed mAbs, with IgG1 being, by far, the most popular subclass (around 80%)[34].

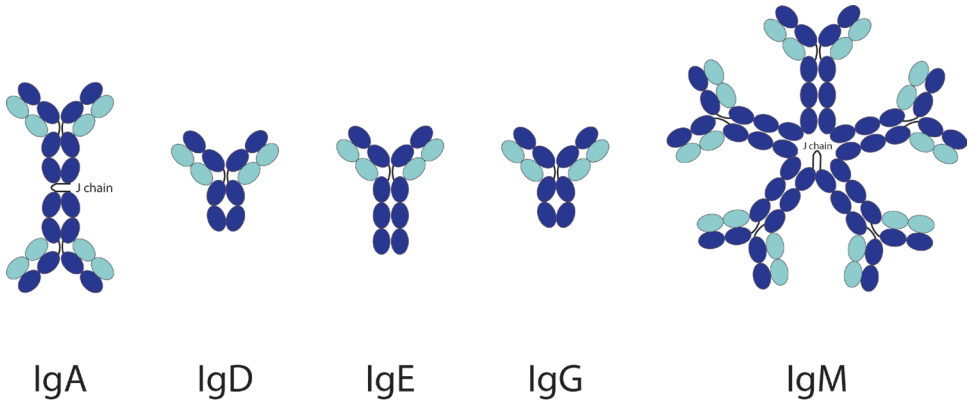

Figure 5. Schematic structure of human antibody classes. Human immunoglobulins are classified in 5 classes. Main differences are found in the polymerization of IgAs (mostly dimers) and IgMs (mostly pentamers and hexamers) aided by the presence of the J chain. IgEs and IgMs are characterized by the presence of an extra domain in the heavy chain. IgDs and IgGs are both monomeric and conserve the same number of domains, however, they are characterized by significant structural differences.

In illustrative schemes IgGs are typically displayed by a typical Y-shape, which arises from the association of four polypeptide chains: two identical light chains and two identical heavy chains (Figure 6). Each light chain is covalently bound to one heavy chain through an inter-chain disulfide bond, forming what in this thesis is referred as "half-antibody" with a molecular weight of about 75 kDa. The association of two half-antibodies gives rise to the antibody monomer (around 150 kDa), which is the most common form of the antibody. This association occurs through the interaction of the two heavy chains via strong, and multiple non-covalent interactions between the CH3-CH3 domains and a variable number of disulfide bonds occurring in the hinge region, "above" the CH2 domains. While wild type IgGs are prevalently monomers, some other Ig classes have the ability to polymerize into higher order structures. IgAs often form dimers, *i.e.* two antibody monomers are associated via the J-chain, while IgM are mostly pentamers and hexamers, whereby five or six antibody monomers interact via the Fc domains giving rise to a ring-shape structure (Figure 5).

Looking at the intact IgG molecule, various different regions can be distinguished: two Fab (antigen binding fragment) arms, each one consisting of one light chain and the VH and CH1 domains of its associated heavy chain, and one Fc region

(crystallizable fragment) consisting of the CH2 and CH3 domains of the two heavy chains. While most of the protein sequence is highly conserved, each of the two Fab arms contains a highly variable CDR region (complementarity-determining regions) located at the N-terminus. These regions are responsible for antigen recognition, which they can bind with extremely high specificity and affinity, *i.e.* below nanomole.

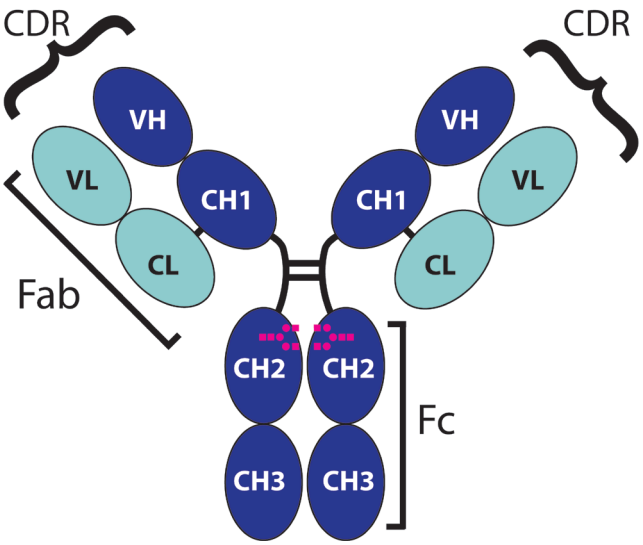

Figure 6. Schematic structure of typical IgG antibodies. Two heavy chains (dark blue) and two light chains (light blue) assemble to give rise to the antibody monomer. The distinctive Y-shape, typical of all antibodies, highlights two Fab arms and a Fc region linked via the hinge region. N-glycosylation (here depicted in pink) occurs in the CH2 domain at the conserved Asn297 site.

As already mentioned, numerous inter-chain disulfide bonds contribute to dictate the three-dimensional structure of IgGs. A disulfide bond between the light and the heavy chain is well conserved among all IgG subclasses. On the contrary, the number of disulfide bonds in the hinge region varies: 2 for IgG1s and IgG4s, 4 for IgG2 and 11 for IgG3[35] (Figure 7). It has often been reported that disulfide bonds undergo a "scrambling" phenomenon where a disulfide bond is broken to make a new disulfide bond with a different cysteine, a phenomenon that can severely affect the antibody three-dimensional structure[36,37]. This phenomenon is especially observed in IgG4s, where the inter-chain disulfide bonds in the hinge region are broken to form intra-chain bonds[38], but also when samples are heated, freeze-thawed or stressed by other means. In addition to the inter-chain disulfide bonds, 12 well conserved intra-chain disulfide bonds are well spread throughout the whole structure, one for each protein domain.

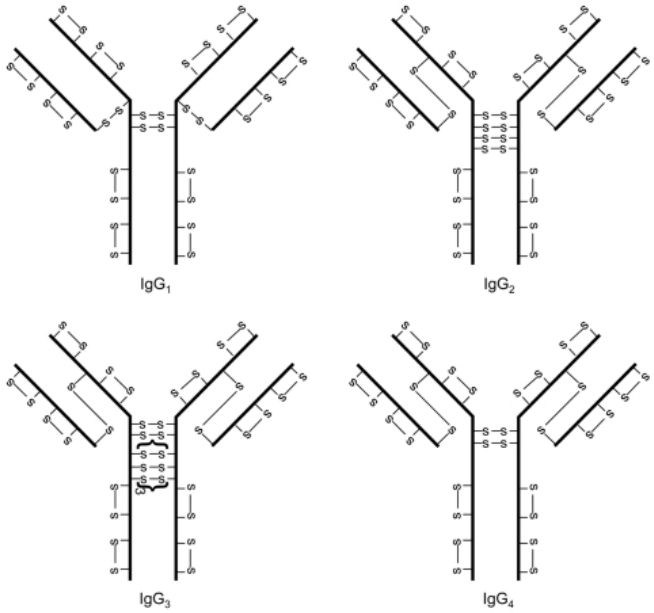

Figure 7. Disulfide bond structures of IgG molecules. IgG subclasses are distinguished by their differences in the disulfide bonds that occur throughout the whole antibody molecule. This figure schematically represents the disulfide bonds occurring in the IgG subclasses. Reprinted from[35].

Although disulfide bond rearrangements can contribute to a certain level of protein heterogeneity in mAbs, especially when they are stressed as in storage and/or formulation, the main source of structural heterogeneity arises from other post-translational modifications (PTMs). Glycosylation, N-terminal glutamine cyclization, C-terminal lysine processing, deamidation and oxidation are the modifications that are most commonly detected on mAbs. In the context of therapeutic mAbs, glycosylation is certainly the most studied modification for several reasons: it is highly dependent on the host system used for the expression of the mAb, it plays an important role in mAb effector functions, plasmatic clearance and immunogenicity, it occurs stoichiometrically and it represents 2-3% of the total mass. mAb glycosylation significantly increases the molecular heterogeneity, turning a single protein backbone often in tens of different co-occurring isoforms, potentially having all different functionality and efficacy.

Therapeutic IgG mAbs bear the well-conserved Fc glycosylation motif Asn-X-Ser/Thr in each heavy chain where Asn297 is linked to a complex type glycan. More rarely, such as for Cetuximab (Erbitux®), an additional N-glycosylation site is present in the Fab region[39]. The typical wild-type Fc glycosylation of therapeutic mAbs consists of a heptasaccharide diantennary core, often referred to as G0 that can

be extended by the addition of galactose (Gal) and sialic acid (Sia or Neu5Ac and Neu5Gc) residues (Figure 8). Moreover, the first N-acetylglucosamine (GlcNAc) of the core can be fucosylated and additional GlcNAc residues can be linked to the β1-4-Man (bisetting GlcNAc).

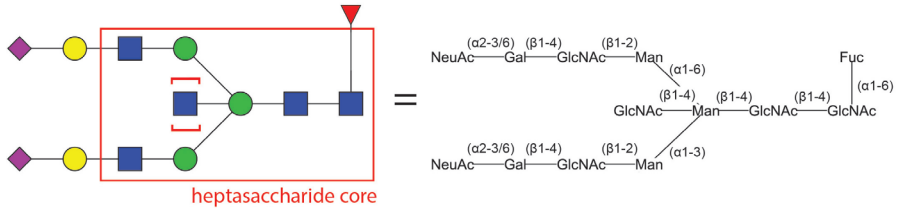

Figure 8. Schematic of typical Fc glycan structures. Fc glycosylation is characterized by a conserved heptasaccharide core (boxed) consisting of four GlcNAc residues (blue squares) and three mannose residues (green circles). The glycan chains can be then elongated with the addition of extra residues such as galactoses (yellow circles) or sialic acids (pink diamonds). A bisetting GlcNAc (in the brackets) can also be found linked to the β1-4-Man.

To improve mAbs pharmaceutical properties, protein engineering has become a common practice. It has been demonstrated that even a single mutation in the protein sequence can heavily effect the glycosylation profile of mAbs giving rise to very extended or truncated glycan structures. An example of analysis of complex glycosylation profiles in engineered mAbs is given in chapter V, where extensive tri- and tetra-antennary structures were observed in engineered IgG4 antibodies differing by single amino acid mutations.

Therapeutic indication

To date, therapeutic mAbs have been developed against several different targets and they are currently used in the clinic for a wide range of diseases. Although the first mAbs were developed for the treatment of transplant reactions, nowadays, a vast majority of mAbs are used in the treatment of different cancer types. The anti-CD20 mAb Rituximab (MabThera®, Rituxan®), the first therapeutic mAb approved for cancer therapy, entered the clinic in 1997 for the treatment of non-Hodgkin's lymphoma. Subsequently, more mAbs followed, such as the anti-HER2 Trastuzumab (Herceptin®) for the treatment of breast cancer and the anti-VEGF Bevacizumab (Avastin®) for the treatment of colorectal cancer.

Another therapeutic area, wherein pharmaceutical and biotechnological companies currently focus their interest, is the development of mAbs for immunological diseases. Crohn disease, rheumatoid arthritis and psoriasis can now be

treated with antibody therapy. Examples are: the anti-TNF antibodies Infliximab (Remicade®) and Adalimumab (Humira®) used for treatment of Crohn disease and rheumatoid arthritis diseases, respectively.

Furthermore, the efficacy of therapeutic antibodies has been successfully explored also in other therapeutic areas. Palivizumab (Synagis®) developed against the RSV virus is an example of mAb employed for the treatment of infectious diseases. Other mAbs are at the moment already in Clinical Phase 3 either for other infectious diseases or diseases such as hypercholesterolemia and Alzheimer[40,41].

Pharmacodynamic properties

Of the whole antibody molecule, the regions mostly involved in pharmacodynamics are the two CDR regions and the Fc region. As mentioned above, CDRs are responsible for the antigen recognition. Normally, therapeutic mAbs act by blocking their targets. However, depending on the nature of the target, the mAb-antigen complex can proceed via different mechanism of actions. For example, some mAbs are designed to bind cytokines with the result of neutralizing them as in the case of anti-TNF mAbs. Obstructing the overexpression of TNF, as it happen in some immune diseases, mAbs are able to reduce inflammation. Other therapeutic mAbs are designed to block membrane receptors such as anti-HER2. Some tumor cell types overexpress the membrane protein HER (epidermal growth factor receptor) favoring the HER dimerization that leads to a pro-tumor signal transduction. The binding of a mAb to HER inhibits its dimerization, decreasing cell growth[42].

The Fc region plays an important role in antibody effector functions. It binds to FcR (Fc-receptors), expressed on the surface of immune cells, inducing antibody-depended cytotoxicity (ADCC), thus helping the immune system to recognize and eliminate aberrant cells. Moreover, the binding to the FcRn (neonatal Fc-receptor) is involved in the antibody clearance and therefore influences the half-life of mAbs; upon binding the FcRn, antibodies are protected against proteolytic degradation[43].

The antibody-FcR binding affinity is influenced by Fc glycosylation. Afucosylated mAbs have been shown to result in enhanced ADCC activity[44]. Hence, not surprisingly, both Fc glycosylation and protein backbone have been object of engineering in order to improve the binding affinity to Fc-receptors to augment ADCC activity or to modulate antibody half-life.

Another mechanism of action of antibodies is by complement activation. It was already known that mAbs have CDC (complement-dependent cytotoxicity) activi-

ty[45] though at a variable degree depending of different antibodies. Recently, it has been shown that the Fc region plays an important role in this mechanism. mAbs have been successfully engineered to promote antibody hexamerization that occurs through Fc interactions giving rise to a ring-shape hexamer. This complex can bind C1q with enhanced affinity triggering the complement cascade that, in turn, leads to lysis of the target cells[46]. In this work, native MS contributed to the firm and unambiguous establishment of the hexamer antibody stoichiometry (Figure 9).

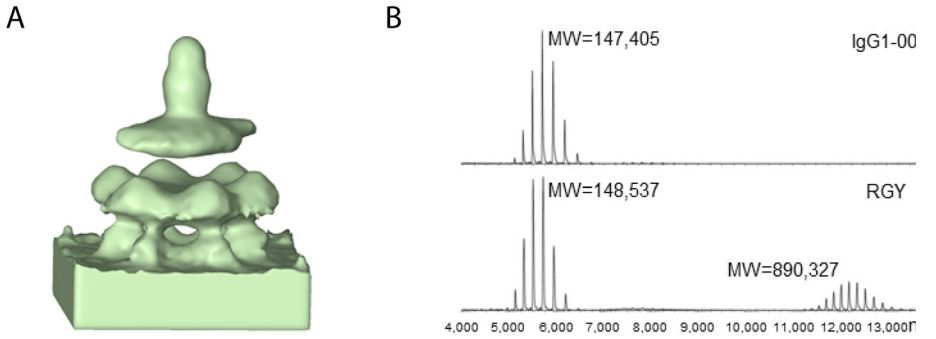

Figure 9. Hexameric mAbs. A) Cryo-electron tomography (cryo-ET) of the antibody-C1 complex. 107 particles representing antibody-C1 complexes were averaged resulting in an electron density map at low (<6 nm) resolution. B) A mAb engineered to enhance antibody hexamerization has been analyzed by native MS (bottom panel). As a control its corresponding wild-type antibody has been analyzed under the identical conditions (top panel). Both spectra show the signal of the monomeric species (MW 148537 Da), however, only the engineered mAb shows a significant amount of antibody hexamer (MW 890327 Da). Adapted from[46].

From murine to human mAbs: the issue of immunogenicity

Muromonab-CD3 (Orthoclone OKT3), the first therapeutic mAb approved in 1986, was developed as a fully murine mAb. However, very soon it became apparent that murine derived mAbs were often themselves the cause of immunogenic responses when used in humans. To overcome this issue, engineering strategies were developed to produce chimeric and humanized mAbs, which contain a lower content of mouse-derived sequences and showed reduced immunogenicity. Only since the 1990s new methods became available allowing the production of entirely human mAbs from transgenic mouse or phage display methods[47,48]. As a consequence, the majority of mAb used clinically are now of human origin[49].

Unfortunately, even human mAbs can still show some level of immunogenicity suggesting that factors, other than protein backbone sequence, may be involved.

One of these factors is certainly mAb glycosylation. Often therapeutic mAbs are expressed in non-human host cells such as chinese hamster ovary (CHO), mouse NSO or mouse Sp2/0 cells. These host cells will normally use their own glycosylation machinery to glycosylate the "human" antibody protein backbone. Therefore, altered glycosylation patterns, such as Gal-α1,3-Gal epitope and N-glycolylneuraminic acid (Neu5Gc), can trigger immunogenic responses. However, such issues can be overcome by selecting particular cell lines that minimize the introduction of abnormal glycoforms or even by changing the glycosylation machinery of the host, making it more human-like.

mAb formats

With the aim of improving pharmacologic properties, mAbs have been optimized even further, using strategies that resulted into a variety of antibody-based products. Natural occurring mAbs are mono-specific, as they have two identical CDR regions, which theoretically should be able to bind two identical antigens. However, efforts have been made to engineer mAb molecules that resulted in the association of two different Fab fragments in order to give rise to bispecific antibodies, *i.e.* an antibody containing of two different CDR regions[50-52]. The clever idea behind the development of bispecific antibodies is to exploit the dual binding capability to either simultaneously block two different antigens (*e.g.* two cytokines to reduce inflammation) or to bring in close proximity a tumor cell and a immune cell by binding a tumor antigen to one Fab arm and a specific antigen of the immune cell with the other Fab arm. Although the idea behind this antibody format is extremely clever, the outcomes from clinical trials are, so far, somewhat disappointing, with the main problem being the critical distance between the two antigens[53]. To date, only one bispecific antibody, catumaxomab (Removab®), has reached the clinic.

Another new mAb format that is very promising is the antibody-drug conjugate (ADC) format[54,55]. The strategy behind this format is to obtain higher efficacy properties by conjugating a toxic "small" drug to the antibody backbone via a chemical linker. Such a format could for instance be used to first internalize the mAb inside the cell where, subsequently, the cytotoxic drug is released. However, the drug conjugation process entails a number of challenges during the development process. Firstly, the linker needs to be stable in the extracellular environment and the drug only needs to be released inside the target cell to reduce side-effects, caused by circulating free drug. Secondly, the conjugation reaction often leads to a mixture of molecular structures carrying different drug payloads at different positions in the mAb. This, significantly increases the heterogeneity of the sample

and thus, putting a strain on their structural analysis. An example of analysis of ADCs mAbs is shown in chapter V of this thesis.

Another mAb format, which has two representatives in the clinic, ranibizumab (Lucentis®) and certolizumab pegol (Cimzia®), is the antibody fragment format. In these cases, the pharmaceutical product consists of only one fragment, the Fab fragment, rather than the whole antibody molecule. Fab fragments bind their antigens monovalently. Moreover, the lack of the Fc fragment causes that they lack CDC and ADCC activity and display an enhanced clearance. Therefore, strategies to modulate the clearance speed have been used, such as pegylation in the case of certolizumab pegol (Cimzia®)[56].

Lastly, an emerging mAb format is to use a mixture of different mAbs, rather than a single one, in order to recognize multiple targets involved in the same disease at the same time resulting in an improved efficacy[57,58].

mAb Biosimilars

As for small-molecule drugs, ‘‘copy versions’’ of biopharmaceuticals can be and are commercialized after expiry of the patent of the original products. However, the size and the complexity of mAbs, partly caused by their ‘‘non-synthetic’’ production in biological cellular platforms, makes it very hard to produce exact copies of a biopharmaceutical reference product. Hence, small differences compared to the reference product are at present accepted by the regulatory authorities (*e.g.*, FDA and EMA), who have termed these types of generic drugs ‘‘biosimilars’’[59].

How similar does a biosimilar mAb have to be in order to enter the market and how may this be evaluated? Generally, qualitative and quantitative differences in PTMs are still accepted by the regulatory authorities, but primary sequence variations are not allowed[60]. Yet, as even minimal structural variations can affect efficacy and safety, preclinical and clinical trials, along with biological and physicochemical characterization, are generally required. The extent of molecular similarity may influence the level of preclinical and clinical trials requested by the regulatory authorities.

It may be clear that mAbs themselves are, from a structural point-of-view, already complex heterogeneous molecules. With all these new exciting formats emerging this structural heterogeneity will only increase. Therefore, there is an urgent need to also enhance the analytical capabilities to characterize such molecules, which is the main aim of the work described in my thesis.

3. Thesis Outline

In this thesis I will continue in chapter II reviewing how MS can and has been applied in the analysis of therapeutic mAbs trying to cover some of the main challenges often encountered in this field. In the following chapters, the focus will be mostly narrowed to native MS. In chapter III, a new analytical method, based on native MS, for the quantitative and qualitative characterization of mixtures of therapeutic mAbs using time-of-flight (TOF) instruments is demonstrated. In chapter IV, the new instrument developed by us for native MS, the orbitrap Exactive PlusTM (ThermoFisher Scientific) is introduced, and its exciting features described especially for the analysis of mAbs. The astonishing performance of this instrument led to new applications for native MS that are objects of study in the last chapters of my thesis. Chapter V and chapter VI describe new methodologies to study mAbs glycosylation at the intact protein level by native MS. A summary and future perspective finally completes my thesis.

References

1 van den Heuvel, R. H. & Heck, A. J. Native protein mass spectrometry: from intact oligomers to functional machineries. Curr Opin Chem Biol 8, 519-526 (2004).

2 Heck, A. J. Native mass spectrometry: a bridge between interactomics and structural biology. Nat Methods 5, 927-933 (2008).

3 Bush, M. F. et al. Collision cross sections of proteins and their complexes: a calibration framework and database for gas-phase structural biology. Anal Chem 82, 9557-9565 (2010).

4 Karas, M., Bachmann, D., Bahr, U. & Hillenkamp, F. Matrix-Assisted Ultraviolet-Laser Desorption of Nonvolatile Compounds. Int J Mass Spectrom 78, 53-68 (1987).

5 Fenn, J. B., Mann, M., Meng, C. K., Wong, S. F. & Whitehouse, C. M. Electrospray ionization for mass spectrometry of large biomolecules. Science 246, 64-71 (1989).

6 Loo, J. A. Electrospray ionization mass spectrometry: a technology for studying noncovalent macromolecular complexes. International Journal of Mass Spectrometry 200, 175-186 (2000).

7 Sharon, M. & Robinson, C. V. The role of mass Spectrometry in structure elucidation of dynamic protein complexes. Annu Rev Biochem 76, 167-193 (2007).

8 Uetrecht, C. & Heck, A. J. Modern biomolecular mass spectrometry and its role in studying virus structure, dynamics, and assembly. Angew Chem Int Ed Engl 50, 8248-8262 (2011).

9 Thompson, N. J., Rosati, S. & Heck, A. J. Performing native mass spectrometry analysis on therapeutic antibodies. Methods (2013).

10 Fenn, J. B., Mann, M., Meng, C. K., Wong, S. F. & Whitehouse, C. M. Electrospray Ionization for Mass-Spectrometry of Large Biomolecules. Science 246, 64-71 (1989).

11 Kebarle, P. & Verkerk, U. H. Electrospray: from ions in solution to ions in the gas phase, what we know now. Mass Spectrom Rev 28, 898-917 (2009).

12 de la Mora, J. F. Electrospray ionization of large multiply charged species proceeds via Dole's charged residue mechanism. Anal Chim Acta 406, 93-104 (2000).

13 Wilm, M. & Mann, M. Analytical properties of the nanoelectrospray ion source. Anal Chem 68, 1-8 (1996).

14 Rosati, S. et al. Qualitative and Semiquantitative Analysis of Composite Mixtures of Antibodies by Native Mass Spectrometry. Anal Chem 84, 7227-7232 (2012).

15 Rose, R. J. et al. Quantitative Analysis of the Interaction Strength and Dynamics of Human IgG4 Half Molecules by Native Mass Spectrometry. Structure 19, 1274-1282 (2011).

16 Atmanene, C. et al. Extending Mass Spectrometry Contribution to Therapeutic Monoclonal Antibody Lead Optimization: Characterization of Immune Complexes Using Noncovalent ESI-MS. Anal Chem 81, 6364-6373 (2009).

17 Zhang, Z. Q., Pan, H. & Chen, X. Y. Mass Spectrometry for Structural Characterization of Therapeutic Antibodies. Mass Spectrom Rev 28, 147-176 (2009).

18 Tahallah, N., Pinkse, M., Maier, C. S. & Heck, A. J. R. The effect of the source pressure on the abundance of ions of noncovalent protein assemblies in an electrospray ionization orthogonal time-of-flight instrument. Rapid Commun Mass Sp 15, 596-601 (2001).

19 Schmidt, A., Bahr, U. & Karas, M. Influence of pressure in the first pumping stage on analyte desolvation and fragmentation in nano-ESI MS. Anal Chem 73, 6040-6046 (2001).

20 Chernushevich, I. V. & Thomson, B. A. Collisional cooling of large ions in electrospray mass spectrometry. Anal Chem 76, 1754-1760 (2004).

21 Rosati, S., Yang, Y., Barendregt, A. & Heck, A. J. Detailed mass analysis of structural heterogeneity in monoclonal antibodies using native mass spectrometry. Nat Protoc 9, 967-976 (2014).

22 van den Heuvel, R. H. H. et al. Improving the performance of a quadrupole time-of-flight instrument for macromolecular mass spectrometry. Anal Chem 78, 7473-7483 (2006).

23 Sobott, F., Hernandez, H., McCammon, M. G., Tito, M. A. & Robinson, C. V. A tandem mass spectrometer for improved transmission and analysis of large macromolecular assemblies. Anal Chem 74, 1402-1407 (2002).

24 Lorenzen, K., Versluis, C., van Duijn, E., van den Heuvel, R. H. H. & Heck, A. J. R. Optimizing macromolecular tandem mass spectrometry of large non-covalent complexes using heavy collision gases. International Journal of Mass Spectrometry 268, 198-206 (2007).

25 Uetrecht, C., Rose, R. J., van Duijn, E., Lorenzen, K. & Heck, A. J. R. Ion

mobility mass spectrometry of proteins and protein assemblies. Chem Soc Rev 39, 1633-1655 (2010).

26 Thevis, M., Makarov, A. A., Horning, S. & Schanzer, W. Mass spectrometry of stanozolol and its analogues using electrospray ionization and collision-induced dissociation with quadrupole-linear ion trap and linear ion trap-orbitrap hybrid mass analyzers. Rapid Commun Mass Sp 19, 3369-3378 (2005).

27 Rose, R. J., Damoc, E., Denisov, E., Makarov, A. & Heck, A. J. R. High-sensitivity Orbitrap mass analysis of intact macromolecular assemblies. Nat Methods 9, 1084-1086 (2012).

28 Rosati, S. et al. Exploring an orbitrap analyzer for the characterization of intact antibodies by native mass spectrometry. Angew Chem Int Ed Engl 51, 12992-12996 (2012).

29 Thompson, N. J., Hendriks, L. J., de Kruif, J., Throsby, M. & Heck, A. J. Complex mixtures of antibodies generated from a single production qualitatively and quantitatively evaluated by native Orbitrap mass spectrometry. MAbs 6, 197-203 (2014).

30 Rosati, S. et al. In-depth qualitative and quantitative analysis of composite glycosylation profiles and other micro-heterogeneity on intact monoclonal antibodies by high-resolution native mass spectrometry using a modified Orbitrap. MAbs 5 (2013).

31 Reichert, J. M. Metrics for antibody therapeutics development. MAbs 2, 695-700 (2010).

32 Society, T. A. <http://www.antibodysociety.org/news/approved_mabs.php> (

33 Strebhardt, K. & Ullrich, A. Paul Ehrlich's magic bullet concept: 100 years of progress. Nat Rev Cancer 8, 473-480 (2008).

34 Reichert, J. M. Marketed therapeutic antibodies compendium. MAbs 4, 413-415 (2012).

35 Liu, H. & May, K. Disulfide bond structures of IgG molecules: structural variations, chemical modifications and possible impacts to stability and biological function. MAbs 4, 17-23 (2012).

36 Bagal, D., Valliere-Douglass, J. F., Balland, A. & Schnier, P. D. Resolving disulfide structural isoforms of IgG2 monoclonal antibodies by ion mobility mass spectrometry. Anal Chem 82, 6751-6755 (2010).

37 Dillon, T. M. et al. Structural and functional characterization of disulfide isoforms of the human IgG2 subclass. J Biol Chem 283, 16206-16215 (2008).

38 Schuurman, J., Perdok, G. J., Gorter, A. D. & Aalberse, R. C. The inter-heavy chain disulfide bonds of IgG4 are in equilibrium with intra-chain disulfide bonds. Mol Immunol 38, 1-8 (2001).

39 Ayoub, D. et al. Correct primary structure assessment and extensive glyco-profiling of cetuximab by a combination of intact, middle-up, middle-down and bottom-up ESI and MALDI mass spectrometry techniques. MAbs 5, 699-710 (2013).

40 Reichert, J. M. Antibodies to watch in 2014. MAbs 6 (2013).

41 Reichert, J. M. Antibodies to watch in 2013: Mid-year update. MAbs 5, 513-517 (2013).

42 Hudis, C. A. Trastuzumab--mechanism of action and use in clinical practice. N Engl J Med 357, 39-51 (2007).

43 Golay, J. & Introna, M. Mechanism of action of therapeutic monoclonal antibodies: promises and pitfalls of in vitro and in vivo assays. Arch Biochem Biophys 526, 146-153 (2012).

44 Nakagawa, T., Natsume, A., Satoh, M. & Niwa, R. Nonfucosylated anti-CD20 antibody potentially induces apoptosis in lymphoma cells through enhanced interaction with FcgammaRIIIb on neutrophils. Leuk Res 34, 666-671 (2010).

45 Hughesjones, N. C. & Gardner, B. Reaction between the Isolation Globular Subunits of the Complement Component Clq and Igg-Complexes. Mol Immunol 16, 697-701 (1979).

46 Diebolder, C. A. et al. Complement is activated by IgG hexamers assembled at the cell surface. Science 343, 1260-1263 (2014).

47 McCafferty, J., Griffiths, A. D., Winter, G. & Chiswell, D. J. Phage antibodies: filamentous phage displaying antibody variable domains. Nature 348, 552-554 (1990).

48 Lonberg, N. Human antibodies from transgenic animals. Nat Biotechnol 23, 1117-1125 (2005).

49 Reichert, J. M. Monoclonal antibodies as innovative therapeutics. Curr Pharm Biotechnol 9, 423-430 (2008).

50 Xie, Z., Guo, N., Yu, M., Hu, M. & Shen, B. A new format of bispecific antibody: highly efficient heterodimerization, expression and tumor cell lysis. J Immunol Methods 296, 95-101 (2005).

51 Chelius, D. et al. Structural and functional characterization of the trifunctional antibody catumaxomab. MAbs 2, 309-319 (2010).

52 Gunasekaran, K. et al. Enhancing antibody Fc heterodimer formation through electrostatic steering effects: applications to bispecific molecules and monovalent IgG. J Biol Chem 285, 19637-19646 (2010).

53 Riethmuller, G. Symmetry breaking: bispecific antibodies, the beginnings, and 50 years on. Cancer Immun 12, 12 (2012).

54 Beck, A. et al. The next generation of antibody-drug conjugates comes of age. Discov Med 10, 329-339 (2010).

55 Katz, J., Janik, J. E. & Younes, A. Brentuximab Vedotin (SGN-35). Clin Cancer Res 17, 6428-6436 (2011).

56 Jevsevar, S., Kusterle, M. & Kenig, M. PEGylation of antibody fragments for half-life extension. Methods Mol Biol 901, 233-246 (2012).

57 Logtenberg, T. Antibody cocktails: next-generation biopharmaceuticals with improved potency. Trends Biotechnol 25, 390-394 (2007).

58 de Kruif, J. et al. Generation of stable cell clones expressing mixtures of human antibodies. Biotechnol Bioeng 106, 741-750 (2010).

59 Weise, M. et al. Biosimilars-why terminology matters. Nat Biotechnol 29, 690-693 (2011).

60 Reichert, J. M., Beck, A. & Iyer, H. European Medicines Agency workshop on biosimilar monoclonal antibodies: July 2, 2009, London, UK. MAbs 1, 394-416 (2009).

Chapter II

Analysis of Monoclonal Antibodies by Mass Spectrometry

Partially based on:

The Impact of Mass Spectrometry on the Study of Intact Antibodies: From Post-Translational Modifications to Structural Analysis

N.J. Thompson, S. Rosati, R.J. Rose, A.J.R. Heck

Chem Commun (Camb) 49, 538-548 (2013)

and

Tackling the Increasing Complexity of Therapeutic Monoclonal Antibodies with Mass Spectrometry

S. Rosati, N.J. Thompson, A.J.R. Heck

Trac-Trend Anal Chem 48, 72-80 (2013)

The molecular composition of monoclonal antibodies (mAbs), even when produced by recombinant expression in well-defined hosts, can be very heterogeneous, due to a variety of modifications. IgG-based therapeutics possess a common global structure, yet there are several sources of heterogeneity that make detailed structural characterization of these compounds challenging. Variations in post-translational modifications (PTMs), such as glycosylation, disulfide bond formation and deamidation, as well as changes in primary sequence can result in a complex mixture within a single sample. Other modifications are engineered into the mAb structure, such as the covalent linking of cytotoxic drugs to the mAb in the case of antibody-drug conjugates (ADCs). Many of these modifications are quite small in mass compared to that of the intact antibody, others, such as disulfide bond scrambling, result in no change in mass but may induce a drastic change in structure. Whether mAb heterogeneity is engineered or a result of the production process, it is crucial that these mixtures are accurately characterized as differences can result in adverse effects of mAb-based therapeutics[1,2].

Mass spectrometry (MS) has become an essential tool in the biopharmaceutical industry, not only for the analysis of new antibody-based products, but also for the evaluation of biosimilars. This chapter focuses on MS as one of the most versatile technologies for the in-depth structural analysis of therapeutic mAbs, describing how MS is used to address challenges commonly encountered in the development process of therapeutic mAbs (Figure 1).

1. Sequence variants

Unintended amino acid substitutions, also known as sequence variants, are often detected in recombinant proteins produced in mammalian cell cultures[3]. These sequence variants can stem from multiple sources, including mutations at the DNA level, miss-incorporation of amino acids during protein assembly, or miss-cleavage during post-translational processing[4]. Relying on its strength in proteomics, MS has also been incorporated into mAb sequence variants analysis workflows. MS has the ability to detect and identify most types of sequence variants as these produce indicative mass shifts. Sequence variants, often consisting of a few point mutations throughout the protein structure, are commonly small in mass relative to the intact mAb mass. Although nowadays high-resolution mass spectrometric approaches allow the detection of sequence variants already at the intact protein level[5-8], bottom-up MS approaches, in which proteolytic fragments are analyzed, remain the most popular for this type of investigation[3,4,9].

Typically, the bottom-up MS-based methods incorporate one or more proteases

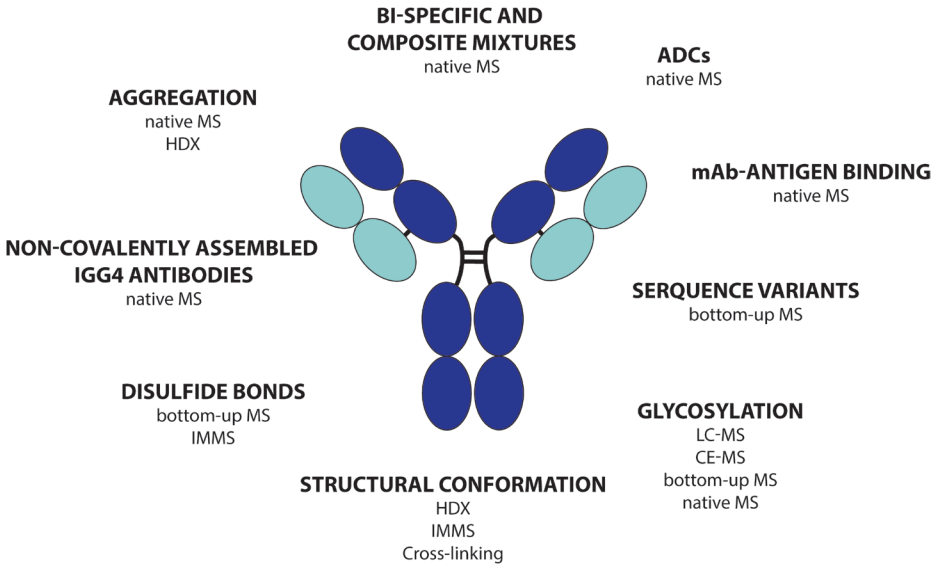

Figure 1. Numerous applications of MS for the analysis of mAbs. The great variety of different MS approaches allows the employ of this technique for many different applications. This figure summarizes how, by using MS, many different mAbs features can be studied.

used to produce peptides of the mAbs. These peptides are subsequently separated using liquid chromatography (LC) and analyzed using tandem MS (MS/MS). The MS/MS data are then searched against a database for identification. However, because peptides containing an amino acid substitution cannot be matched to the database, a strategy is needed in the data analysis process to identify sequence variants. In 2010, Yang *et al.* published a method that combined both UV and MS analysis after LC for the detection, identification, and quantification of low-level sequence variants[4]. This method utilizes the error tolerant search (ETS) mode, developed by Creasy and Cottrell[10] and currently available through Mascot, to facilitate the identification of sequence variants. The ETS method utilizes a substitution matrix making all possible substitutions. Subsequently, all unmatched MS/MS spectra are submitted to the ETS search. However, the ETS is only appropriate for those sequence variants that result from a single mutation as the software does not predict shifts due to multiple substitutions. Moreover, ETS produces several false positives, as caused by unwanted sodium adducts and S-carboxymethylation, making manual verification essential. Recently, Zeck *et al.* implemented the use of a biological reference instead of the ETS analysis using a theoretical reference[3]. In this method, LC-MS/MS runs of mAb digests are compared one-on-one using the SIEVE software in addition to the previous method of using the Mascot

ETS analysis. Differences detected by SIEVE, which analyzes the data set based on retention time and m/z window for peak matching, are plotted as a ratio and allows easy identification of those peptides that most likely contain a sequence variant. In this manner, those sequence variants that result from multiple amino acid substitutions can be detected, but confirmation of these substitutions still requires manual verification.

One of the main drawbacks of bottom-up approaches is the lengthy workflow that might introduce undesired modifications. To overcome this issue, in recent years top-down and middle-down approaches are being investigated as an alternative to bottom-up. In these approaches the protein of interest is either digested into large fragments by limited proteolysis (middle-down) or the digestion step is entirely skipped (top-down). Subsequently, protein fragments or the intact proteins are delivered to the the mass spectrometer where they are fragmented using fragmentation techniques such as electron-transfer induced dissociation (ETD), collision-induced dissociation (CID) or electron-capture induced dissociation (ECD)[11,12]. Although these methodologies are very promising and can significantly shorten the analysis time, they still suffer from low sequence coverage compared to bottom-up approaches. Therefore, further technical developments are required before they can be routinely implemented for the analysis of intact, proteins including therapeutic mAbs.

2. Glycosylation

Because of its intended applications, detailed characterization of the glycosylation profile of therapeutic mAbs is strictly required by regulatory authorities such as the FDA. If on the one hand glycosylation represents an extra challenge in the comprehensive structural analysis on therapeutic mAbs, on the other hand, IgGs bear just a single conserved N-glycosylation site (although more rarely an extra site can be found in the Fab region), which facilitates the analysis.

MS represents a versatile tool to investigate N-glycosylation allowing its analysis at three different levels: the released glycan level, the glycopeptide level and the intact protein level. N-glycans can be released enzymatically using peptide N-glycosydase F (PNGase F). Subsequently, they can be analyzed as intact glycans using for instance MALDI-TOF instruments, or, alternatively, electrospray ionization (ESI) mass spectrometers can be coupled to a separation technique whereby different separation methods can be used to separate the glycan mixture prior to MS analysis. Hydrophilic interaction chromatography (HILIC), reverse phase liquid chromatography (RP-LC), porous graphitized chromatography (PGC) and capillary

electrophoresis (CE) have been commonly used for this purpose[13]. In the case of the analysis of the released glycans, the identification relies on the accurate mass measurement of the intact glycan whereby monosaccharide composition and structural information can only be inferred based on known structures and known biosynthetic pathways. MS/MS using CID as well as ECD and ETD fragmentation techniques, can also be useful for further characterization of the glycosylation structure, as fragmentation spectra can provide insights on the sugar linkages. However, a comprehensive linkage analysis is merely possible upon derivatization of intact glycans, followed by acid hydrolysis and analysis of the monosaccharides by, for instance, GC-MS. Glycosylation analysis at the released glycan level is widely used and well-established, it ensures highly confident identifications an allows linkage studies. However, one main drawback of this approach is the identification of false positives originating from other contaminant glycoproteins.

As technical developments of mass spectrometric instrumentation is progressing, glycosylation analysis at the glycopeptide level and at the intact protein level is gaining momentum. The analysis at the glycopeptide level utilizes common bottom-up approaches, where the protein is digested into peptides using a protease such as trypsin. Subsequently, the peptides can be separated and analyzed using RP-HPLC, HILIC or PGC coupled to MS. Along with the accurate measurement of precursor ions, ion fragmentation performed using, for instance, a combination of ETD for the fragmentation of the peptide backbone, and CID for the fragmentation of the glycan, gives information on the glycan structure and sugar residues linkages[14-17].

Finally, glycoanalysis can be performed at the intact protein level. Most often this is done under denaturing condition, using (RP)-LC-MS on TOF instruments[18-20]. However, recently, the introduction of a Orbitrap-based instrument amenable for the analysis of protein and protein complexes under native conditions allowed the analysis of mAb glycosylation at the intact protein level under native conditions[6,7]. The advantage of this approach (described in more detail in chapter V and VI of this thesis) is the analysis speed: very little sample preparation is required and data acquisition normally takes only a few minutes. On the other hand, glycan identification relies solely on high accurate mass measurements, therefore glycan structure needs to be deduced from known biosynthetic pathways of the system used for protein expression and from a priori knowledge of the typical glycosylation profile of the sample under investigation. These particular features make this approach suitable for high-throughput routine analysis: it can be used, for instance, as a finger-print for batch-to-batch comparisons.

3. Disulfide bonds

A crucial aspect of the mAb structure is the correct localization of the existing disulfide bonds. It has become apparent that these bonds may "scramble": breaking and making new disulfide bonds. Such processes not only change the tertiary structure, but also can affect the efficacy of the therapeutic mAb. Therefore, the exact location of disulfide bonds must be characterized in high detail. For this purpose, bottom-up approaches can be used and, more recently, ETD fragmentation has been used in conjunction with CID to locate disulfide bonds in peptides from an enzymatically digested mAb. This combined approach results in complementary fragmentation patterns, where CID typically produces backbone fragmentations and ETD preferentially cleaves the disulfide bonds[21].

The effects of disulfide bond scrambling on the mAb structure can also be evaluated by ion mobility spectrometry (IMMS)[22]. For more details on this mass spectrometric approach the reader is referred to section 9 of this chapter.

4. Bi-specific mAbs and composite mixtures of mAbs

With conventional therapeutic mAbs penetrating the market of biopharmaceuticals, the industry is now looking forward to new improved products, in particular with regard to efficacy. Interesting classes of such molecules are the so-called bispecific mAbs, which are composed of specific parts of two different mAbs that consequently can bind to two different types of antigens and can be exploited to enhance pharmacodynamic properties. As bispecific IgG1s have not been found yet to occur naturally, molecular engineering is necessary for their production[23-25].

Even more complex than bispecific mAbs, composite mixtures of mAbs are also investigated as novel biopharmaceutical products. Despite not yet available as therapeutics, mixtures of mAbs seem to be promising alternatives to single mAbs therapeutics. A therapeutic product consisting of a mixture of antibodies can target multiple epitopes of the same antigens or different antigens involved in a particular disease, resulting in an improved efficacy[26].

Bispecific mAbs and composite mixtures of antibodies enlarge the molecular complexity of mAbs dramatically; putting even higher demands on the analytical tools to characterize them. Chapter III of this thesis describes a new method enabling the simultaneous qualitative and quantitative analysis of bispecifics and mixtures of mAbs using native MS[27]. Notably, these mixtures of antibodies had been produced by co-expressing multiple forms of monospecific antibodies in one cellular platform, an approach that also benefits from a significant reduction of costs[26,28].

Co-expression of antibodies resulted in a mixture of variable amounts of monospecific and bispecific antibodies. It was demonstrated that native MS represents a robust technique for antibody mixtures characterization.

More recently, the introduction of the new Obitrap instrument amenable for native MS analysis, significantly pushed the limits of sample complexity. Thompson *et al.* showed how the improved resolving power of the Orbitrap instrument allows the analysis of even more composite mixtures of mAbs. Mixtures of up to 15 mAbs could be confidently identified with an average mass accuracy of 7 ppm[8].

5. Antibody-drug conjugates (ADCs)

ADCs are a rapidly emerging class of therapeutic mAbs, in which the mAb backbone is covalently linked to small cytotoxic drugs through a linker molecule. This type of biopharmaceuticals exploits the high selectivity of antibodies to selectively deliver drugs to target (tumor) cells, thus potentially limiting side effects[29-31]. The drug is generally linked to either the cysteine or lysine residues in the mAbs. Unfortunately, this conjugation process can often not be fully controlled with regard to the number of drug molecules linked to the antibody and to their distribution and exact locations. Therefore, the final product will be a mixture of antibodies differing in load and location of the drug molecules, requiring thorough analysis to characterize them[32,33].

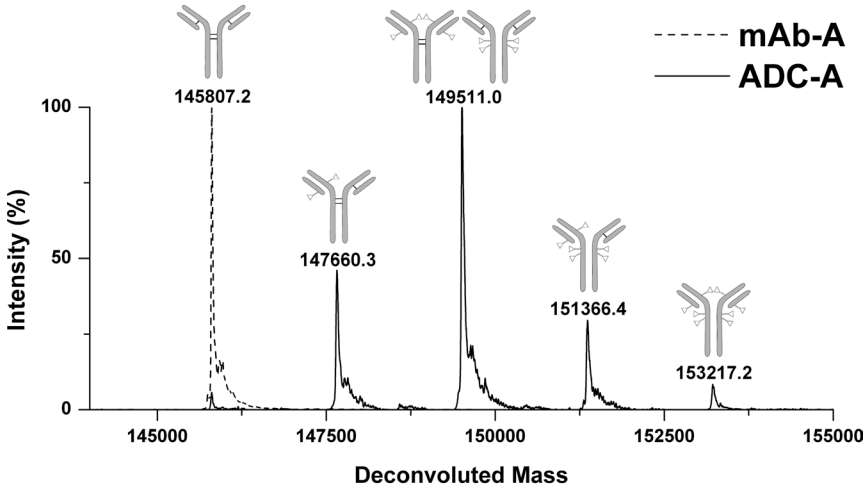

Figure 2. Deconvoluted native MS spectrum of a ADC product. The deconvoluted spectrum of the mcMMAF (maleimidocaproyl-monomethyl Auristatin F) ADC product shows multiple drug pay-loads present in different amounts. Superimposed (dashed trace) is the deconvoluted spectrum of the unconjugated antibody. Adapted from[34].

Because of differences in molecular mass caused by the different amount of drug loads, MS can be a valuable technique for the characterization of ADCs. To investigate the composition of the mixture, it is indispensable to perform the analysis at the intact protein level. In case of cysteine-linked ADCs, partial reduction of the disulfide bonds is required prior to conjugation. Consequently, these ADCs loose some of their inter-chain covalent bonds. As a result, under denaturing conditions of conventional LC-MS analysis, subunits could fall apart, thus losing any information about the composition of the intact functional antibody. In this case, native MS becomes the technique of choice because of its ability to preserve non-covalent interactions (Figure 2).

Because of the novelty of this new format of therapeutic mAbs, the field of MS analysis of ADCs is still in its infancy with only very few works published[6,33,34]. Therefore, major developments in this direction are to be expected.

6. Non-covalently assembled IgG4 antibodies

Another nice example of how native MS contributes to the analysis of biopharmaceuticals is described in the work done by Rose *et al.* who looked at hinge-deleted IgG4 constructs engineered to form half-molecules, or 'Unibodies'[35]. IgG4 antibodies exist both as covalently and non-covalently assembled molecules, *i.e.*, a fraction does not have inter-heavy chain disulfide bonds but rather intra-chain bridges. To study the non-covalent interactions involved between the heavy chains, a native MS study was carried out, providing a means to distinguish and measure simultaneously the abundance of the IgG4 half-molecules (mass of approximately 75 kDa) and intact IgG antibodies (mass of approximately 150 kDa).

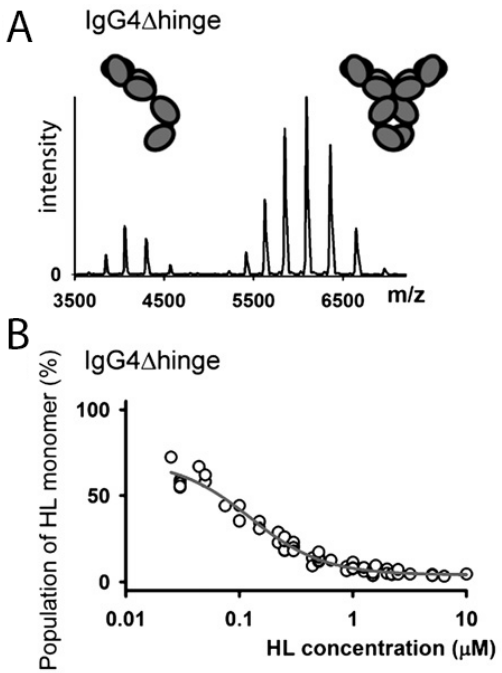

Figure 3. Relative distribution of half and intact IgG4 antibodies monitored by native MS. (A) The native MS spectrum of a hingeless IgG4 antibody shows the equilibrium between half and intact antibody. (B) Mass spectra were determined at different IgG4Δhinge concentrations. The proportion of IgG4Δhinge populating the half state is plotted as a function of overall protein concentration. Reprinted from[35].

The methodology was used to determine the solution phase K_D for wild-type hinge-deleted IgG4 (Figure 3).

Next, a series of single-point mutations made in the CH3 domain was evaluated revealing the sensitivity of this dimerization to the CH3-CH3 interaction, with the measured K_D varying from sub-nM to 100s of μM for individual mutants[35]. The strength of the non-covalent interaction within IgG4 was found to correlate well with the Fab-arm exchange phenomenon, *i.e.*, the mixing of IgG4 half-molecules to form bispecific antibodies[36-38].

Understanding this exchange is important due to its implications for the use of different IgG4 antibodies as therapeutics, as they may undergo Fab-arm exchange *in vivo* with endogenous antibodies[39]. Native MS was also used to measure the kinetics of formation of mixed antibodies for different mutants in real time; since data can be acquired in just a few minutes using this technique, such reactions can be monitored directly[35].

7. mAb-antigen binding

MS has also been explored by several groups as a technique to measure the binding of mAbs to their cognate antigens[40-43]. Since antibody-antigen complexes occur through non-covalent interaction, native MS can help determine the relative amount of unbound, singly-bound, and doubly-bound antigen. Most alternative techniques, *e.g.*, surface plasmon resonance (SPR), isothermal calorimetry (ITC), and SEC, only measure such populations as a weighted average. Already in 2001, Tito *et al.* measured the stoichiometry of antibody-antigen complexes using recombinant V antigen from *Yersinia pestis*. As expected, 1:2 antibody to antigen complexes were detected. This complex was established to be specific, since no binding was detected between the non-cognate antigen F1 and the antibody[40]. In another example, Atmanene *et al.* showed that two distinct mAbs bind the junctional adhesion molecule A (JAM-A) with ratios from 1:2 to 1:4, attributed to either monomeric or dimeric JAM-A binding to each Fab arm[43]. Comparing antibody-antigen binding measured by various techniques, including native MS, analytical ultracentrifugation (AUC), and SPR, has revealed that the binding stoichiometries of antibodies free in solution or immobilized on a surface can differ, suggesting the interaction depends on Fc flexibility[41,42]. From the structural point of view the antibody-antigen binding properties are also investigated using HDX-MS. However, the reader is referred to the section 9 of this chapter for further details on HDX-MS.

8. Antibody aggregation

An important consideration during antibody production and storage is the undesired formation of inactive high-order oligomers and/or non-specific aggregates. Several techniques, such as AUC and SEC, are routinely used to check for aggregation, however, it remains challenging to define the actual identity of the aggregation products. Because native MS combines the retention of non-covalent structures with mass-based identification, it is ideally suited for detection of higher-order oligomers. In this context, native ESI-MS has been applied to characterize aggregates formed when IgG1 is subjected to pH stress[44]. A valuable tool to investigate antibody aggregation from the structural point of view is hydrogen-deuterium exchange mass spectrometry (HDX-MS). For an in-depth discussion on this technology the reader is referred to section 9 of this chapter.

9. Structural conformation analysis

I. Ion mobility mass spectrometry (IMMS)

With the advance of native MS and the ability to preserve non-covalent interactions, the gas-phase structure of the studied species can be probed. Combining ion mobility spectrometry with MS (IMMS)[45] yields not only mass-based identification but also gas-phase collision cross-sections, which may provide insight to the gas-phase structure, albeit at low resolution. As already discussed, IMMS has been applied in the determination of the structural heterogeneity of both mAbs (IgG2) and antigen targets. Differences in disulfide bonds can affect not only the structure of a mAb but also its function. IMMS was used to successfully observe multiple gas-phase conformers of mAbs, which were then confirmed to be the result of different disulfide bond formation via point mutation of a cysteine in the hinge region[46]. IMMS also revealed disulfide bond heterogeneity of an antigen under native conditions; whereas a single population was observed by IMMS under reducing conditions, multiple conformers were observed under non-reducing conditions[43]. These studies illustrate how IMMS can rapidly assess structural variability of antibodies and their antigens. Flexibility between the Ig domains in the antibody molecule makes IMMS a challenging technique for absolute deduction of structure, however, it can easily be incorporated into MS workflows for comparability studies.

II. Hydrogen-Deuterium Exchange (HDX)

Changes in conformation, including misfolding, can significantly impact activity and stability of antibodies, and thus must be monitored in the development and

production of biopharmaceutical products. Due to the flexible linkers between the Ig domains in mAbs, structural characterization of these proteins tends to be challenging. mAbs are still too large for standard NMR procedures and also tend to be difficult to crystallize. Therefore, alternative techniques to elucidate structural information on mAbs are needed. Hydrogen-deuterium exchange mass spectrometry (HDX-MS) is a rapidly developing technique employed to investigate protein dynamics, conformational changes, and interaction interfaces[47-50]. HDX-MS exploits the different rates of exchange of hydrogens between protein backbone amide groups and the aqueous solvent, which depend on solvent exposure and secondary structure elements.

When HDX-MS is applied to the structural study of mAbs, continuous labelling conditions are typically used whereby the antibody is diluted into a deuterated buffer for a defined time. The rate of deuteration of the amide hydrogens is highly dependent on localized solvent exposure and hydrogen bonding kinetics, factors that can affect the rate of deuteration up to eight orders of magnitude. Decreasing the pH and temperature quenches the exchange reaction. Under the optimal quenching conditions of pH 2.7 and 0°C, the exchange reaction is reduced by five orders of magnitude[51-53], which is essential during sample analysis to minimize of back-exchange. The whole workflow of the HDX experiments has been subjected to progressive improvements during recent years. Initially, the labelling and proteolytic digestion steps were conducted manually off-line. Nowadays, the automation of both the labelling and digestion steps[54], has improved both speed and reproducibility[50,55]. Of great importance is automation at the level of data processing and analysis which significantly saves manpower and time[56,57]. With these recent improvements, HDX-MS is becoming popular and holds further potential, not only for studies on protein conformation and dynamics, but also in the analysis and quality control assays of biopharmaceuticals.

In a global HDX experiment, the mass of the intact protein is directly interrogated by MS, revealing the uptake of deuterium over time. Due to its simplicity and speed, this kind of experiment is preferable when extensive conformational changes are expected and spatial resolution of the exchange along the protein backbone is not required. Moreover, it represents a quick way to investigate the overall reactivity of the protein for D_2O uptake and is commonly used to optimize the labelling timescale prior to an HDX experiment involving proteolytic digestion.

Houde *et al.* described global HDX measurements comparing the structure of the Fab and Fc regions individually, as opposed to within the intact IgG1 molecule[58]. No significant difference in deuterium uptake was observed, and furthermore,

only one labelled population was observed, suggesting little conformational heterogeneity in the IgG1. Incomplete deuteration after four hours of labelling indicated the presence of some highly protected regions, and the overall increases in the extent of deuteration for the entire time frame studied confirmed the slow dynamic motions of these protected regions.

To achieve a more detailed picture of protein structural changes using HDX-MS, an enzymatic digestion step is incorporated to increase the spatial HDX resolution to the peptide level. As mentioned previously, digestion must be performed under optimal quench conditions, meaning that a protease active at acidic pH is necessary. Pepsin is the most commonly used protease for this purpose, as it is active at pH 2.5 and cleaves non-specifically. The generated proteolytic peptides are then analyzed by LC-MS, and the uptake of deuterium is measured for each individual peptide. With this type of experiment, conformational changes can be localized to small regions of the protein by comparing the deuterium uptake of a peptide produced from the protein under two or more different conditions. Several studies illustrate this, reporting on the use of HDX-MS to investigate conformational changes induced by various glycosylation profiles on mAbs[58,59].

The nature of the glycan chains on mAbs is highly dependent on the expression system, and glycosylation is known to influence the binding to the Fc receptor[59,60]. Houde *et al.* used HDX-MS to show that complete deglycosylation cause significant changes in the Fc region, while the rest of the protein did not show any changes in deuterium uptake (Figure 4)[58].

Subsequently, this group also performed HDX-MS on a number of IgG1 variants differing in the nature of the glycosylation, which generally showed changes in the structural dynamics of the CH2 domain.

The efforts in optimization of therapeutic antibodies to improve antibody properties, such as pharmacokinetics, pharmacodynamics, stability, and effector response, often lead to the development of products with single-point amino acid substitutions[61-64]. However, single mutations can bring dramatic conformational changes that can either positively or negatively affect the potential drug candidate. Rose *et al.* used HDX-MS to show how single-point mutations in the CH3 domain not only affect the conformation of the domain where the mutation is located but also induce allosteric changes in the CH2 domains. These allosteric changes occurred in parallel with significant changes in the attached glycan chain structure[65].

Besides glycosylation, other post-translational modifications can affect the overall molecular structure; methionine oxidation provides such an example affecting

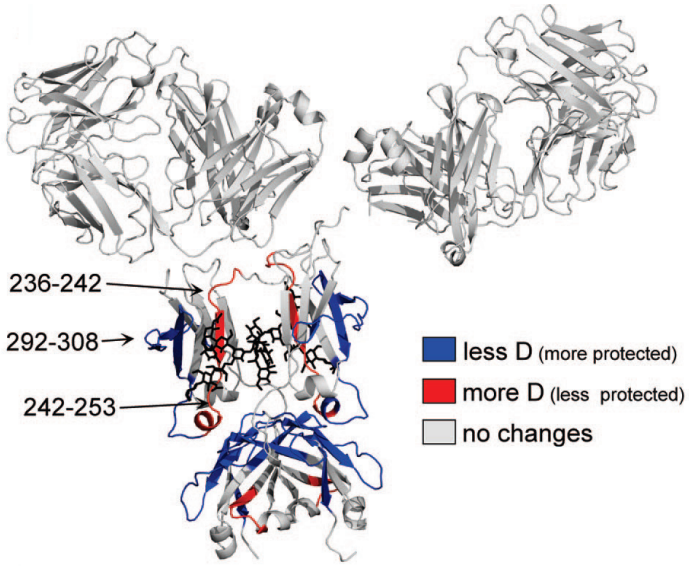

Figure 4. Comparison of deuterium levels in IgG1 with and without glycosylation. Crystal structure of an IgG1 antibody with the glycosylation indicated in black sticks. Parts colored blue indicate regions where the deglycosylated form had less deuterium (more protection from exchange). Parts colored red indicate regions where the deglycosylated form had more deuterium (less protection from exchange). Adapted from[58].

mAb stability. HDX-MS has been used to measure the overall conformational stability upon methionine oxidation, known to be significant when antibodies are stored for a long time, as in the case of therapeutic products. It was shown that methionine oxidation can not only structurally affect residues immediately surrounding the site of oxidation but also adjacent domains[59,66].

Aggregation is also often observed in antibody preparations, especially when mAbs are stored at high concentrations. HDX-MS can be used to detect regions involved in aggregation. Zhang *et al.* used HDX-MS to monitor antibody aggregation under both thermal and freeze-thaw stresses conditions. Aggregation resulted in destabilization of the interface between the light chain and heavy chain as indicated by an increase deuterium uptake. Additionally, the small regions which show decreased deuterium uptake after stress likely represent the aggregation interface[67].

One application of HDX-MS that is rapidly becoming popular is mapping the antigen epitope[68-70]. Characterization of the molecular determinants of the interaction between antibodies and their targets is essential when antibodies are used as therapeutic agents and also required for intellectual property reasons. An in-

teresting strategy to carry out this type of experiment is to perform the labelling on an immobilized antibody in order to selectively analyze the antigen. The successful application of this strategy has been demonstrated by Baerga-Ortiz *et al.* and by Coales *et al.*[71,72]. Also worthwhile mentioning is the application of a HDX dilution strategy to determine antibody-antigen binding affinities and residues involved in the epitope, as developed by Tu *et al.*[73].

III. Cross-linking

Chemical cross-linking combined with MS is a technique that makes use of reactive small molecules (cross-linkers) able to react with functional groups of the side chains of the amino acids. Their role is to link two functional groups of the same protein or two interacting proteins that are close in space in the native conformation. Digestion of the linked protein and MS analysis of the linked peptides allows the investigation of the three-dimensional structure of proteins, and characterization of protein interactions[74-76]. Chemical cross-linking with MS-based detection represents a useful alternative method for obtaining structural restraints for antibodies. For example, Bich *et al.* have demonstrated how newly developed cross-linkers can successfully be used in this manner to study structurally antibody-antigen interactions[77].

Conclusion

mAbs represent a fast emerging class of therapeutics in the biopharmaceutical field. As their development and expansion in the clinical arena have been very rapid during the last years, there is even further great expectation for the future. Along with the increasing complexity of new antibody entities, analytical techniques need to be optimized to thoroughly characterize these products. In this chapter, I have shown how MS already plays an important role in the in-depth characterization of multiple features of therapeutic mAbs. However, new technical challenges are expected with the increasing complexity of newly therapeutic entities.

References

1 Zhang, Z. Q., Pan, H. & Chen, X. Y. Mass Spectrometry for Structural Characterization of Therapeutic Antibodies. Mass Spectrom Rev 28, 147-176 (2009).

2 Beck, A., Wagner-Rousset, E., Ayoub, D., Van Dorsselaer, A. & Sanglier-Cianferani, S. Characterization of Therapeutic Antibodies and Related Products. Anal Chem 85, 715-736 (2013).

3 Zeck, A. et al. Low level sequence variant analysis of recombinant pro-

teins: an optimized approach. PLoS One 7, e40328 (2012).

4 Yang, Y. et al. Detecting low level sequence variants in recombinant monoclonal antibodies. MAbs 2, 285-298 (2010).

5 Wade, Y., Chapman, J. Protein and Peptide Analysis By Mass Spectrometry. J .Structural Analysis of Protein Variants in Molecular Biology, Humana Press Inc, Totowa 16, 101-113 (1996).

6 Rosati, S. et al. In-depth qualitative and quantitative analysis of composite glycosylation profiles and other micro-heterogeneity on intact monoclonal antibodies by high-resolution native mass spectrometry using a modified Orbitrap. MAbs 5 (2013).

7 Rosati, S., Yang, Y., Barendregt, A. & Heck, A. J. Detailed mass analysis of structural heterogeneity in monoclonal antibodies using native mass spectrometry. Nat Protoc 9, 967-976 (2014).

8 Thompson, N. J., Hendriks, L. J. A., de Kruif, J., Throsby, M. & Heck, A. J. R. Complex Mixtures of Antibodies Generated from a Single Production Qualitatively and Quantitatively Evaluated by Native Orbitrap Mass Spectrometry. MAbs 6 (2014).

9 Ambrogelly, A. et al. Characterization of antibody variants during process development: the tale of incomplete processing of N-terminal secretion peptide. MAbs 4, 701-709 (2012).

10 Creasy, D. M. & Cottrell, J. S. Error tolerant searching of uninterpreted tandem mass spectrometry data. Proteomics 2, 1426-1434 (2002).

11 Mao, Y., Valeja, S. G., Rouse, J. C., Hendrickson, C. L. & Marshall, A. G. Top-down structural analysis of an intact monoclonal antibody by electron capture dissociation-Fourier transform ion cyclotron resonance-mass spectrometry. Anal Chem 85, 4239-4246 (2013).

12 Fornelli, L., Ayoub, D., Aizikov, K., Beck, A. & Tsybin, Y. O. Middle-down analysis of monoclonal antibodies with electron transfer dissociation orbitrap fourier transform mass spectrometry. Anal Chem 86, 3005-3012 (2014).

13 Marino, K., Bones, J., Kattla, J. J. & Rudd, P. M. A systematic approach to protein glycosylation analysis: a path through the maze. Nat Chem Biol 6, 713-723 (2010).

14 Zauner, G. et al. Glycoproteomic analysis of antibodies. Mol Cell Proteomics 12, 856-865 (2013).

15 Scott, N. E. et al. Simultaneous glycan-peptide characterization using hydrophilic interaction chromatography and parallel fragmentation by CID, higher energy collisional dissociation, and electron transfer dissociation MS applied to the N-linked glycoproteome of Campylobacter jejuni. Mol Cell Proteomics 10, M000031-MCP000201 (2011).

16 Singh, C., Zampronio, C. G., Creese, A. J. & Cooper, H. J. Higher energy collision dissociation (HCD) product ion-triggered electron transfer dissociation (ETD) mass spectrometry for the analysis of N-linked glycoproteins. J Proteome Res 11, 4517-4525 (2012).

17 Ye, H., Boyne, M. T., 2nd, Buhse, L. F. & Hill, J. Direct approach for quali-

tative and quantitative characterization of glycoproteins using tandem mass tags and an LTQ Orbitrap XL electron transfer dissociation hybrid mass spectrometer. Anal Chem 85, 1531-1539 (2013).

18 Srebalus Barnes, C. A. & Lim, A. Applications of mass spectrometry for the structural characterization of recombinant protein pharmaceuticals. Mass Spectrom Rev 26, 370-388 (2007).

19 Olivova, P., Chen, W., Chakraborty, A. B. & Gebler, J. C. Determination of N-glycosylation sites and site heterogeneity in a monoclonal antibody by electrospray quadrupole ion-mobility time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 22, 29-40 (2008).

20 Kuribayashi, R., Hashii, N., Harazono, A. & Kawasaki, N. Rapid evaluation for heterogeneities in monoclonal antibodies by liquid chromatography/mass spectrometry with a column-switching system. J Pharm Biomed Anal 67-68, 1-9 (2012).

21 Wang, Y., Lu, Q., Wu, S. L., Karger, B. L. & Hancock, W. S. Characterization and comparison of disulfide linkages and scrambling patterns in therapeutic monoclonal antibodies: using LC-MS with electron transfer dissociation. Anal Chem 83, 3133-3140 (2011).

22 Uetrecht, C., Rose, R. J., van Duijn, E., Lorenzen, K. & Heck, A. J. Ion mobility mass spectrometry of proteins and protein assemblies. Chem Soc Rev 39, 1633-1655 (2010).

23 Chelius, D. et al. Structural and functional characterization of the trifunctional antibody catumaxomab. MAbs 2, 309-319 (2010).

24 Gunasekaran, K. et al. Enhancing antibody Fc heterodimer formation through electrostatic steering effects: applications to bispecific molecules and monovalent IgG. J Biol Chem 285, 19637-19646 (2010).

25 Xie, Z., Guo, N., Yu, M., Hu, M. & Shen, B. A new format of bispecific antibody: highly efficient heterodimerization, expression and tumor cell lysis. J Immunol Methods 296, 95-101 (2005).

26 Logtenberg, T. Antibody cocktails: next-generation biopharmaceuticals with improved potency. Trends Biotechnol 25, 390-394 (2007).

27 Rosati, S. et al. Qualitative and semiquantitative analysis of composite mixtures of antibodies by native mass spectrometry. Anal Chem 84, 7227-7232 (2012).

28 de Kruif, J. et al. Generation of stable cell clones expressing mixtures of human antibodies. Biotechnol Bioeng 106, 741-750 (2010).

29 May, C., Sapra, P. & Gerber, H. P. Advances in bispecific biotherapeutics for the treatment of cancer. Biochem Pharmacol 84, 1105-1112 (2012).

30 Sievers, E. L. & Senter, P. D. Antibody-Drug Conjugates in Cancer Therapy. Annu Rev Med (2012).

31 Alley, S. C., Okeley, N. M. & Senter, P. D. Antibody-drug conjugates: targeted drug delivery for cancer. Curr Opin Chem Biol 14, 529-537 (2010).

32 Senter, P. D. Potent antibody drug conjugates for cancer therapy. Curr Opin Chem Biol 13, 235-244 (2009).

33 Wagner-Rousset, E. et al. Antibody Drug Conjugate model fast characterization by LC-MS following IdeS proteolytic digestion. MAbs 6 (2013).

34 Valliere-Douglass, J. F., McFee, W. A. & Salas-Solano, O. Native intact mass determination of antibodies conjugated with monomethyl Auristatin E and F at interchain cysteine residues. Anal Chem 84, 2843-2849 (2012).

35 Rose, R. J. et al. Quantitative analysis of the interaction strength and dynamics of human IgG4 half molecules by native mass spectrometry. Structure 19, 1274-1282 (2011).

36 Labrijn, A. F. et al. Species-specific determinants in the IgG CH3 domain enable Fab-arm exchange by affecting the noncovalent CH3-CH3 interaction strength. J Immunol 187, 3238-3246 (2011).

37 Gramer, M. J. et al. Production of stable bispecific IgG1 by controlled Fab-arm exchange: Scalability from bench to large-scale manufacturing by application of standard approaches. MAbs 5 (2013).

38 Labrijn, A. F. et al. Efficient generation of stable bispecific IgG1 by controlled Fab-arm exchange. Proc Natl Acad Sci U S A 110, 5145-5150 (2013).

39 Labrijn, A. F. et al. Therapeutic IgG4 antibodies engage in Fab-arm exchange with endogenous human IgG4 in vivo. Nat Biotechnol 27, 767-771 (2009).

40 Tito, M. A. et al. Probing molecular interactions in intact antibody: antigen complexes, an electrospray time-of-flight mass spectrometry approach. Biophys J 81, 3503-3509 (2001).

41 Oda, M. et al. Effects of antibody affinity and antigen valence on molecular forms of immune complexes. Mol Immunol 47, 357-364 (2009).

42 Oda, M. et al. Regional and segmental flexibility of antibodies in interaction with antigens of different size. FEBS J 273, 1476-1487 (2006).

43 Atmanene, C. et al. Extending mass spectrometry contribution to therapeutic monoclonal antibody lead optimization: characterization of immune complexes using noncovalent ESI-MS. Anal Chem 81, 6364-6373 (2009).

44 Kukrer, B. et al. Mass spectrometric analysis of intact human monoclonal antibody aggregates fractionated by size-exclusion chromatography. Pharm Res 27, 2197-2204 (2010).

45 Uetrecht, C., Rose, R. J., van Duijn, E., Lorenzen, K. & Heck, A. J. R. Ion mobility mass spectrometry of proteins and protein assemblies. Chem Soc Rev 39, 1633-1655 (2010).

46 Bagal, D., Valliere-Douglass, J. F., Balland, A. & Schnier, P. D. Resolving disulfide structural isoforms of IgG2 monoclonal antibodies by ion mobility mass spectrometry. Anal Chem 82, 6751-6755 (2010).

47 Wales, T. E. & Engen, J. R. Hydrogen exchange mass spectrometry for the analysis of protein dynamics. Mass Spectrom Rev 25, 158-170 (2006).

48 Hoofnagle, A. N., Resing, K. A. & Ahn, N. G. Protein analysis by hydrogen exchange mass spectrometry. Annu Rev Biophys Biomol Struct 32, 1-25 (2003).

49 Garcia, R. A., Pantazatos, D. & Villarreal, F. J. Hydrogen/deuterium exchange mass spectrometry for investigating protein-ligand interactions. Assay

Drug Dev Technol 2, 81-91 (2004).

50 Engen, J. R. Analysis of protein conformation and dynamics by hydrogen/deuterium exchange MS. Anal Chem 81, 7870-7875 (2009).

51 Bai, Y. W., Milne, J. S., Mayne, L. & Englander, S. W. Primary Structure Effects on Peptide Group Hydrogen-Exchange. Proteins 17, 75-86 (1993).

52 Mayo, S. L. & Baldwin, R. L. Guanidinium Chloride Induction of Partial Unfolding in Amide Proton-Exchange in Rnase-A. Science 262, 873-876 (1993).

53 Smith, D. L., Deng, Y. & Zhang, Z. Probing the non-covalent structure of proteins by amide hydrogen exchange and mass spectrometry. J Mass Spectrom 32, 135-146 (1997).

54 Wales, T. E., Fadgen, K. E., Gerhardt, G. C. & Engen, J. R. High-speed and high-resolution UPLC separation at zero degrees Celsius. Anal Chem 80, 6815-6820 (2008).

55 Wu, Y., Engen, J. R. & Hobbins, W. B. Ultra performance liquid chromatography (UPLC) further improves hydrogen/deuterium exchange mass spectrometry. J Am Soc Mass Spectrom 17, 163-167 (2006).

56 Zhang, Z. Q., Zhang, A. & Xiao, G. Improved Protein Hydrogen/Deuterium Exchange Mass Spectrometry Platform with Fully Automated Data Processing. Anal Chem 84, 4942-4949 (2012).

57 Weis, D. D., Engen, J. R. & Kass, I. J. Semi-automated data processing of hydrogen exchange mass spectra using HX-Express. J Am Soc Mass Spectrom 17, 1700-1703 (2006).

58 Houde, D., Arndt, J., Domeier, W., Berkowitz, S. & Engen, J. R. Characterization of IgG1 Conformation and Conformational Dynamics by Hydrogen/Deuterium Exchange Mass Spectrometry. Anal Chem 81, 5966 (2009).

59 Houde, D., Peng, Y. C., Berkowitz, S. A. & Engen, J. R. Post-translational Modifications Differentially Affect IgG1 Conformation and Receptor Binding. Molecular & Cellular Proteomics 9, 1716-1728 (2010).

60 Krapp, S., Mimura, Y., Jefferis, R., Huber, R. & Sondermann, P. Structural analysis of human IgG-Fc glycoforms reveals a correlation between glycosylation and structural integrity. J Mol Biol 325, 979-989 (2003).

61 Beck, A., Wurch, T., Bailly, C. & Corvaia, N. Strategies and challenges for the next generation of therapeutic antibodies. Nat Rev Immunol 10, 345-352 (2010).

62 Moore, G. L., Chen, H., Karki, S. & Lazar, G. A. Engineered Fc variant antibodies with enhanced ability to recruit complement and mediate effector functions. MAbs 2, 181-189 (2010).

63 Kubota, T. et al. Engineered therapeutic antibodies with improved effector functions. Cancer Sci 100, 1566-1572 (2009).

64 Gunasekaran, K. et al. Enhancing Antibody Fc Heterodimer Formation through Electrostatic Steering Effects APPLICATIONS TO BISPECIFIC MOLECULES AND MONOVALENT IgG. J Biol Chem 285, 19637-19646 (2010).

65 Rose, R. J. et al. Mutation of Y407 in the CH3 Domain Dramatically Alters

Glycosylation and Structure of Human IgG. Proc Natl Acad Sci U S A (2012).

66 Burkitt, W., Domann, P. & O'Connor, G. Conformational changes in oxidatively stressed monoclonal antibodies studied by hydrogen exchange mass spectrometry. Protein Sci 19, 826-835 (2010).

67 Zhang, A., Singh, S. K., Shirts, M. R., Kumar, S. & Fernandez, E. J. Distinct aggregation mechanisms of monoclonal antibody under thermal and freeze-thaw stresses revealed by hydrogen exchange. Pharm Res 29, 236-250 (2012).

68 Pandit, D. et al. Mapping of discontinuous conformational epitopes by amide hydrogen/deuterium exchange mass spectrometry and computational docking. J Mol Recognit 25, 114-124 (2012).

69 Lu, J. et al. IL-1beta epitope mapping using site-directed mutagenesis and hydrogen-deuterium exchange mass spectrometry analysis. Biochemistry 44, 11106-11114 (2005).

70 Zhang, Q. et al. Epitope mapping of a 95 kDa antigen in complex with antibody by solution-phase amide backbone hydrogen/deuterium exchange monitored by Fourier transform ion cyclotron resonance mass spectrometry. Anal Chem 83, 7129-7136 (2011).

71 Baerga-Ortiz, A., Hughes, C. A., Mandell, J. G. & Komives, E. A. Epitope mapping of a monoclonal antibody against human thrombin by H/D-exchange mass spectrometry reveals selection of a diverse sequence in a highly conserved protein. Protein Sci 11, 1300-1308 (2002).

72 Coales, S. J., Tuske, S. J., Tomasso, J. C. & Hamuro, Y. Epitope mapping by amide hydrogen/deuterium exchange coupled with immobilization of antibody, on-line proteolysis, liquid chromatography and mass spectrometry. Rapid Commun Mass Spectrom 23, 639-647 (2009).

73 Tu, T. et al. Protein-peptide affinity determination using an h/d exchange dilution strategy: application to antigen-antibody interactions. J Am Soc Mass Spectrom 21, 1660-1667 (2010).

74 Sinz, A. Chemical cross-linking and mass spectrometry to map three-dimensional protein structures and protein-protein interactions. Mass Spectrom Rev 25, 663-682 (2006).

75 Tang, X. & Bruce, J. E. A new cross-linking strategy: protein interaction reporter (PIR) technology for protein-protein interaction studies. Mol Biosyst 6, 939-947 (2010).

76 Zhang, H. Z. et al. Identification of Protein-Protein Interactions and Topologies in Living Cells with Chemical Cross-linking and Mass Spectrometry. Molecular & Cellular Proteomics 8, 409-420 (2009).

77 Bich, C. et al. Reactivity and applications of new amine reactive cross-linkers for mass spectrometric detection of protein-protein complexes. Anal Chem 82, 172-179 (2010).

Chapter III

Qualitative and Semiquantitative Analysis of Composite Mixtures of Antibodies By Native Mass Spectrometry

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Abstract

Native mass spectrometry was evaluated for the qualitative and semiquantitative analysis of composite mixtures of antibodies representing biopharmaceutical products co-expressed from single cells. We show that by using automated peak fitting of the ion signals in the native mass spectra, we can quantify the relative abundance of each of the antibodies present in mixtures, with an average accuracy of 3%, comparable to a cation exchange chromatography based approach performed in parallel. Moreover, using native mass spectrometry we were able to identify, separate, and quantify nine antibodies present in a complex mixture of ten antibodies, whereas this complexity could not be unraveled by cation exchange chromatography. Native mass spectrometry presents a valuable alternative to existing analytical methods for qualitative and semiquantitative profiling of biopharmaceutical products. It provides both the identity of each species in a mixture by mass determination and the relative abundance through comparison of relative ion signal intensities. Native mass spectrometry is a particularly effective tool for characterization of heterogeneous biopharmaceutical products such as bispecific antibodies and antibody mixtures.

Introduction

Monoclonal antibodies represent a unique and promising category of therapeutic biomolecules. With the first therapeutic monoclonal antibody having been approved for clinical use in 1986, around 30 therapeutic monoclonal antibodies are now routinely used[1,2]. A considerable number of them target cancer and immunological diseases, while others focus on diseases such as Alzheimer's and various infectious diseases[1,3].

Naturally occurring human antibodies are divided into five classes according to their overall structure: IgA, IgD, IgE, IgG and IgM. IgGs are the most abundant in human and so far the only class used for therapeutic purposes. Wild-type IgGs consist of two identical heavy chains and two identical light chains (Figure 1a). Non-covalent dimerization between the two heavy chains is induced by the interaction of the CH3 domains[4,5]. The CH3 domain, together with the CH2 domain, forms the Fc region. A hinge region links the Fc with two Fab regions where the heavy chain interacts with the light chain. The complex of the two heavy chain-light chain pairs is stabilized by four inter-chain disulfide bridges: two of them are situated in the hinge region and link the two heavy chains, and two link each heavy chain to a light chain. Both the heavy chains and light chains have one variable domain (termed VH and VL, respectively) situated in the Fab region, which together are responsible for the antigen binding. Furthermore, the heavy chain contains three constant domains (termed CH1, CH2, CH3), whereas the light chain contains only one constant domain (CL).

Even though the clinical use of therapeutic antibodies is still not routine, their ability to specifically recognize and tightly interact with their target proteins has led to huge interest and large investment in their development. Murine antibodies were first explored as therapeutics, but soon after, the attention shifted towards humanized and human IgGs as these antibodies have better immunogenic profiles. Currently, several approaches are being explored to enhance the functionality of antibodies. The Fc regions of antibodies have been optimized to regulate their serum half-life and increase Fc receptor interactions and complement activation[6-8]. In particular, glycan profiles occurring on the antibodies have actively been altered, modulating inflammatory properties and influencing Fc receptor binding[9,10]. In addition, Fab fragments, antibody-drug conjugates, mixtures of recombinant monoclonal antibodies and bispecific antibodies are gaining significant interest as superior alternatives to standard monoclonal antibody therapeutics[4,11,12].

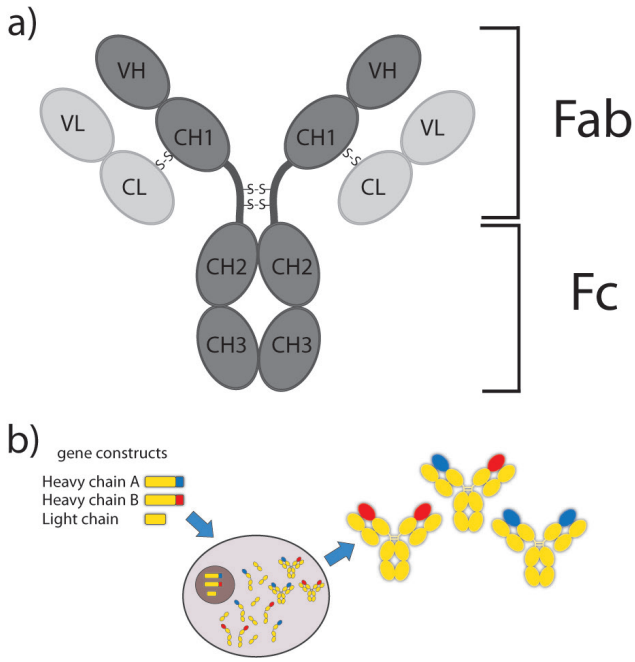

Figure 1. (a) Schematic structure of an IgG antibody. VH is the heavy chain variable domain; VL the light chain variable domain; CH1 the heavy chain constant domain1; CH2 the heavy chain constant domain2; CH3 the heavy chain constant domain3; CL light chain constant domain; Fab antigen binding fragment; Fc crystallizable fragment. (b) Schematic for the synthesis of a binary mixture of IgGs produced by co-expression of two heavy chain constructs together with a common light chain construct in a single cellular platform.

Bispecific antibodies only occur naturally in the IgG4 subclass[13,14], but there is intense interest in producing this class of hybrid antibodies as stable IgG1 molecules[4,15,16]. A unique characteristic of bispecific antibodies is their ability to target two different antigens simultaneously. This dual specificity can be exploited in various ways; for example, antibodies can be used against multiple targets to improve therapeutic efficacy, or they can be used as drug delivery vehicles[17,18].

Additionally, co-expression of multiple forms of monospecific antibodies within one cellular platform is being exploited[12,19]. These mixtures of antibodies can target different epitopes of the same antigen or different antigens involved in the same disease resulting in a synergistic effect with increased efficacy and potency[20]. Moreover, when mixtures of recombinant monoclonal antibodies are co-expressed, their improved pharmacodynamic properties come with a significant reduction of costs.

A downside of these developments is that there is a general increase in the mo-

lecular complexity of antibody products, making characterization even more challenging when compared to single monoclonal antibody products. Consequently, advanced bio-analytical tools are essential to characterize these new drug candidates in detail, and to ensure batch-to-batch consistency of the end-products. Cation exchange chromatography (CEX) represents a routine technology nowadays applied for both the separation of mixtures of antibodies and the analysis of isoforms within monoclonal antibody products[21-23]. In contrast, mass spectrometry is recently gaining interest in the analysis of therapeutic antibodies[24-29].

Here, we explore and evaluate a specific form of mass spectrometry, namely native mass spectrometry (native MS)[5,30], for the qualitative characterization and semiquantitation of composite mixtures of antibodies. Native MS has emerged as a valuable technique for the analysis of intact protein complexes[30-34], clearly distinguishing it from traditional mass spectrometry approaches, whereby proteins are first denatured or even digested prior to analysis. Here, native conditions were preferred over denaturing conditions because of the clearer spectra generated by fewer charge state peaks that not only concentrate the signal but also make the data processing faster (Supporting Information Figure S-1).

We use native MS to quantify each species present in composite mixtures containing both monospecific and bispecific antibodies. The monoclonal antibodies studied were produced in stably transfected mammalian cell lines, continuously overexpressing the antibodies[12]. We focused on constructs encoding two heavy chains differing only in the VH region that were co-expressed with a common light chain. Because the two constructs (termed A and B) share identical Fc regions, two parental monospecific antibodies (AA and BB) and a bispecific species (AB) are formed during expression (Figure 1b). Different clones will produce similar end products in which the relative abundances of the individual species can vary, due to differences in expression of the two heavy chains. We use native MS to measure these subtle differences and evaluate the results obtained against quantitative data obtained from the same samples analyzed by CEX. We additionally investigate a different bispecific IgG-containing antibody mixture and a very complex composite mixture of ten different antibodies. An attractive feature of our native MS method is that it does not require denaturation, reduction, alkylation, and digestion of the antibodies prior to analysis, diminishing sample handling and enabling the determination of the exact masses of all components present in their native state. The high resolving power and mass accuracy of an ESI-time-of-flight (ESI-TOF) instrument permits the simultaneous separation and identification of each component in a composite mixture of antibodies.

Experimental Section

Antibody production and purification. Antibody mixtures were produced either by transient transfection of Freestyle™ 293F cells or by fed-batch culture of PER. C6® clones or CHO-K1 clones stably producing the antibody mixtures of interest. Purification was performed using protein A binding, followed by neutralization. Samples were re-buffered to PBS before further sample preparation.

Sample preparation. All antibodies were analyzed in their fully deglycosylated form. In order to remove the N-linked glycosylation, a total amount of 50 µg of purified antibody mixture was incubated with 5 units of N-glycosidase F (PNGaseF; Roche Diagnostics, Mannheim, Germany) at 37°C overnight. Each mixture was then exchanged into 150 mM ammonium acetate buffer pH 7.5, using 10 kDa MWCO spin-filter columns (Vivaspin®500; Sartorius Stedim Biotech GmbH, Goettingen, Germany).

Native and denatured MS. 1 µl of a 3 µM (antibody tetramer equivalent) antibody sample was sprayed on an ESI-TOF mass spectrometer (LCT, Waters, Manchester, UK) using gold-coated borosilicate capillaries made in house (using a Sutter P-97 puller [Sutter instruments Co., Novato, CA, USA] and an Edwards Scancoat six sputter-coater [Edwards Laboratories, Milpitas, CA, USA]). Experiments under denaturing conditions were carried out by diluting the samples in 5% formic acid to a concentration of 3 µM. Source backing pressure was increased to 6.5 mbar[35]. Capillary voltage and sampling cone voltage were set to 1300 V and 150 V, respectively. Mass calibration was performed using 25 mg/ml CsI. All measurements were performed in triplicate.

Data analysis. MassLynx V4.1 (Waters, Manchester, UK) was used for experimental mass determination. Identification of antibody species within mixtures was carried out matching experimental masses with theoretical masses calculated from construct sequences. Igor Pro V 6.22A was used for peak fitting and peak area calculation. Individual peaks were fit using a Lorentzian function, peak width was automatically set according to the raw spectrum, and a constant baseline was selected. All charge states present in the native mass spectrum were included in these calculations. SOMMS, a software program developed in house by van Breukelen *et al.*[36] was used to simulate native mass spectra.

Cation exchange chromatography (CEX). CEX-HPLC was performed at ambient temperature on a Dionex HPLC system equipped with an SP STAT 7 µm column and a UV/Vis detector. 10 µg of sample was injected; a gradient of 25 mM phosphate buffer pH 6.0 with increasing NaCl concentration was used to separate the

antibodies. Data were analyzed using Chromeleon software.

Results and Discussion

Qualitative characterization and semiquantitation of binary mixtures of IgGs by native MS

Binary mixtures of two co-expressed IgG1 antibodies were produced in stable cell clones and purified as previously described by De Kruif *et al.*[12]. In our initial experiments, three stable PER.C6® cell clones were selected with favorable growth and antibody expression profiles. Each clone expressed two heavy chain constructs (termed A and B) along with a construct for a common light chain. Only the sequence for the VH domains varied, resulting in different antigen specificity. Antigen binding of each component of the mixtures is retained as all the heavy chain species associate with the identical light chain[20]. Dimerization of the heavy chains is facilitated by CH3 domain pairing[37-40], after which the two monomers are covalently linked via two inter-chain disulfide bridges. Identical sequences of the CH3 domain of the two heavy chain constructs (A and B) rules out any preferences in the formation of bispecific or monospecific species. As a consequence, the relative amount of the species formed by the dimerization follows a binomial distribution. For example, expression of both heavy chain constructs at equal amounts would result in an antibody mixture of AA : AB : BB at a 1 : 2 : 1 ratio. However, a number of factors can impact on expression levels. Productivity of the protein related to its sequence, the number of heavy and light chain expression cassettes incorporated by each clone, and their position in the cell genome all influence the expression level of each construct and therefore the relative composition of the mixtures often results in deviations from the standard binomial distribution.

After purification, the three monoclonal antibody mixtures were first fully enzymatically deglycosylated, as this facilitates the mass-separation of the antibodies. Antibodies differing in mass by as little as one single amino acid (*i.e.* ~ 0.1% mass difference on the intact antibody mass) can be resolved.

A nano-spray ESI-TOF analyzer was used for mass spectrometric analysis of the antibody mixtures under native conditions. A full native mass spectrum of one of the mixtures is shown in Figure 2a. It displays a narrow range of well resolved ion signals around m/z 6,000, whereby the total signal of each species is distributed over just seven charge states (z ranges from 22+ to 28+). These ion signals originate from the intact native AA, AB and BB constructs. When we inspect the 25+ charge state, these three distinct components are clearly present in the spectra exhibiting different intensities, as shown in Figure 2b. With a mass resolution over

1,000 at 6,000 m/z, the mass analyzer can separate all components, in this case with mass differences of 252 Da, or around 0.2% of the total molecular weight of an intact antibody of 145 kDa. The accurate mass assignments, within 0.003% of the theoretical mass, allow direct identification of the three species based on the theoretical mass calculated from the sequence. We used the software package Igor Pro V6.22A to automate the relative quantitation of the various species. Individual peaks were fit using a Lorentzian function and the area under the curve was calculated (Figure 2c). All charge states present in the native mass spectrum were included in these calculations.

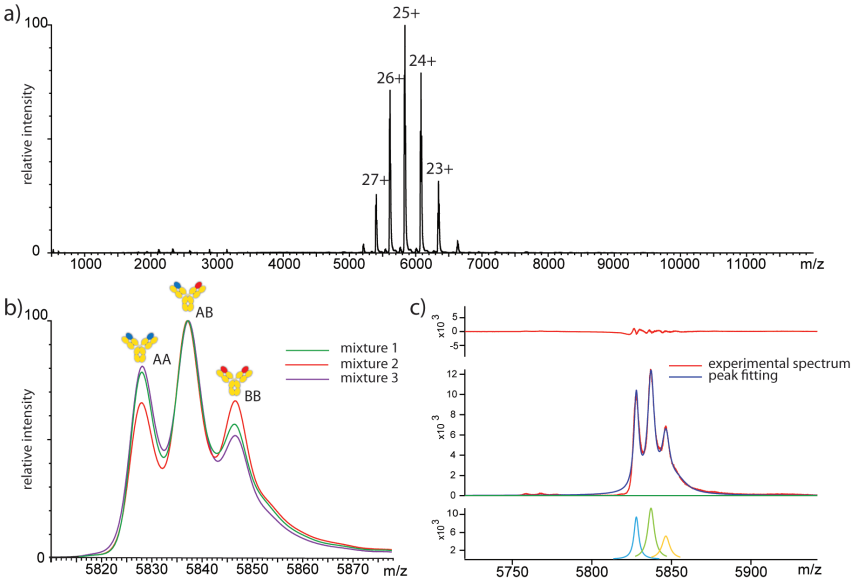

Figure 2. Native MS analysis of binary mixtures of monoclonal antibodies expressed in PER.C6® stable clones. (a) Full native ESI-MS spectrum of a binary mixture. The ion signals observed between 5,000 < m/z < 7,000 all originate from ions of the intact antibodies, corresponding to charge states 22+ to 28+. (b) Zoom-in on the 25+ charge state of three different mixtures of monoclonal antibodies. (c) Igor Pro based peak fitting of the 25+ charge state peaks: (top) residuals; (middle) composite peak fitting; (bottom) individual fitted peaks. Quantitation resulted from the analysis summed of all observed charge states.

As mentioned above, the mixtures were expected to be composed of three species: two monospecific antibodies AA and BB, and a bispecific antibody AB formed by the pairing of heavy chain A and heavy chain B. When comparing the samples obtained from the three cell cultures, there are small, albeit significant, differenc-

es observed in the overall relative abundances of the three species, revealing the different expression levels of the two heavy chains in each clone. In order to test the reproducibility of our quantitation method, standard deviations of the relative abundances were calculated based on three different technical replica experiments. The standard deviation in our experiments is around 3%, enabling the confident detection of even small changes in antibody abundances in these composite mixtures. Table 1 summarizes the determined relative abundances of AA, AB and BB in the samples obtained from each clone.

ID		Mass (Da)	Relative abundance	
			nMS	CEX
mixture 1	AA	145682.0 ± 3.7	22%	22%
	AB	145910.8 ± 4.4	38%	44%
	BB	146140.6 ± 5.1	40%	34%
mixture 2	AA	145682.6 ± 5.2	16%	16%
	AB	145909.8 ± 3.3	36%	40%
	BB	146144.7 ± 3.1	48%	44%
mixture 3	AA	145686.9 ± 3.5	26%	23%
	AB	145916.2 ± 3.5	42%	46%
	BB	146144.5 ± 4.6	31%	31%

Table 1. Mass determination and semiquantitation of the three components in the composite mixtures of antibodies expressed in three PER.C6® clones. nMS = native MS based quantitation, CEX = cation exchange based quantitation. For both techniques the standard deviation was between 3-4%.

Cross-validation of the native MS data with analysis by cation exchange chromatography

To validate our quantitative data, the same antibody mixtures were analyzed by CEX. Using CEX the antibodies AA, AB, and BB could also be separated effectively based on their charge (Supporting Information Figure S-2). We used the area under the curve in these chromatograms to quantify the antibody abundances, and found the standard deviation to be around 3-4% (Table 1). For all three clones, the relative abundance of the various antibodies measured by either native MS or CEX based quantitation was in agreement.

Qualitative and semiquantitative analysis of a CHO-K1 IgG1 mixture

In the field of therapeutic antibodies research, diverse cell lines are often used for antibody expression during the different stages of antibody development. The

type of cell line used has a great influence on the antibody production: for instance, post-translational modifications, especially glycosylation, can differ along with expression levels.

To evaluate the generic use of our native MS based quantitation approach, we analyzed an additional antibody mixture derived from a CHO-K1 cell line, which is frequently used for commercial manufacture of therapeutic antibodies. Again, CHO-K1 cells were transfected with two constructs (termed A and B) encoding for two different heavy chains and a common light chain, resulting in a binary mixture consisting of AA, AB and BB antibody species. We observed, however, that mixtures expressed in CHO-K1 cells show slightly more complex profiles due to the frequently observed post-translational cleavage of the heavy chain C-terminal lysine[41]. In the mass spectra, we can distinguish two peaks for each antibody species: one corresponds to the species where the C-terminal lysine is cleaved off from both of the heavy chains (Δ2Lys), and one corresponds to the species where the C-terminal lysine is cleaved off from only one of the two heavy chains (Δ1Lys). However, the species retaining both the C-terminal lysines were not detected in any case. This lack of detection might be due either to a total absence of these species or to low abundances that leave these peaks buried under the more intense peaks (Supporting Information Figure S-3).

Similar to mass spectrometry, six intense peaks are detected in the CEX chromatogram (Supporting Information Figure S-4). Relative quantitation of the identified components was carried out as described above. The relative quantitation results based on native MS and CEX were largely in agreement albeit not as closely as describe above for the species originating from the PER.C6®cells, most likely due to overlapping peaks (see Supporting Information Table S-1).

Qualitative and semiquantitative analysis of a more complex mixture of antibodies

Binary mixtures, and even more complex antibody mixtures, are of interest as possible therapeutic products targeting different antigens and involving different mechanisms of action[20]. For this reason, we tested our native MS based quantitation approach by analyzing more complex mixtures. For this purpose, we constructed an experimental sample by combining six mixtures, each consisting of two bivalent monospecific and one bispecific antibody, of known ratios. Because, in some mixtures, identical parental bivalent monospecific antibodies were used, the final experimental sample consisted of ten different antibody species (Supporting Information Figure S-5). As all the sequences and thus the masses of the

antibodies were known, we could simulate a theoretically predicted native MS spectrum using the software program SOMMS. Simulation of mass spectra also requires prior knowledge on the abundance of the individual species. Each single mixture of three co-expressed monoclonal antibodies was analyzed by native MS, and the relative abundances determined from these spectra were used. To simulate a theoretical spectrum, a peak width at half height of 7 Th was set to reproduce the experimental resolution observed in the analysis of the single mixtures. As shown in Figure 3a, in our simulation, six peaks can theoretically be resolved whereas two species, which differ in mass by 20 Da, are, with the achieved mass resolving power, indistinguishable (Supporting Information Table S-2). We analyzed the ten-component mixture experimentally by native MS (Figure 3b).

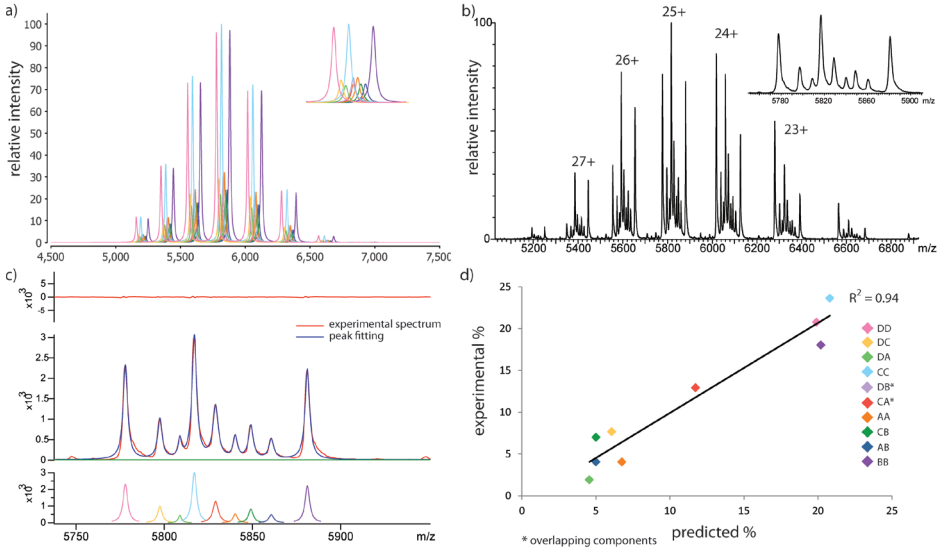

Figure 3. Native MS analysis of a composite mixture of ten monoclonal antibodies. (a) Simulation of the native ESI-MS spectrum obtained using SOMMS and zoom-in (inset) of the 25+ charge state peaks. (b) Full experimental native ESI-MS spectrum and zoom-in (inset) of the 25+ charge state peaks. (c) Igor Pro based peak fitting of the 25+ charge state peaks: (top) residuals; (middle) composite peak fitting; (bottom) individual fitted peaks. (d) Correlation between the predicted versus experimental species abundances.

Nine antibody species out of ten were detected and identified due to a higher resolution obtained experimentally compared to the one set for the simulation. Indeed, the two components, indistinguishable in the SOMMS simulation, were also found to overlap in the experimental spectrum. Using Igor Pro, we were able to achieve an effective peak fitting and thereby could quantify nine out of ten

components in this analytically challenging sample (Figure 3c). The experimentally derived ratios of the final composite mixture showed excellent agreement with the expected ratios calculated using the data from the analysis of the individual mixtures of the three co-expressed monoclonal antibodies (Figure 3d).

For comparison, this mixture of ten antibodies was also analyzed by CEX. In contrast to native MS, CEX could not resolve all antibody components in the mixture (Supporting Information Figure S-6) and overlapping peaks hampered accurate quantitation. In the analysis of these complex mixtures, native MS was clearly superior in resolving the individual components compared to CEX. Furthermore, it allowed for accurate identification of all but two of the components based on mass, a valuable qualitative feature not applicable with CEX analysis.

Conclusions

Here we show that native MS provides a reliable method for the qualitative and semiquantitative analysis of mixtures of antibodies allowing identification and relative quantitation of mixtures of mono- and bispecific antibodies. Its performance can complement more conventional approaches to profile antibodies, such as cation exchange chromatography, size exclusion chromatography and multi-angle light scattering. Native MS is a very robust and fast technique: after purification of the antibodies, sample preparation is limited to enzymatic deglycosylation, followed by buffer exchange, and only a few nanograms/picomoles of sample are required for the analysis. Component identification is achieved instantly by accurate mass measurements, while impurities and or modifications are easily identified by their specific mass-shifts. We show that native MS has reached a high level of reliability, and it is ready to be used for the routine characterization of therapeutic antibodies, and the advantages of the approach become even more clear when investigating very complex mixtures of antibodies. We propose that a wider introduction of this technique in antibody characterization will contribute to an improvement in reproducibility and analysis speed for all phases of biopharmaceutical drug innovation and development.

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References

1 Reichert, J. M. Antibody-based therapeutics to watch in 2011. MAbs 3, 76-99 (2011).

2 Reichert, J. M. Which are the antibodies to watch in 2012? MAbs 4, 1-3 (2012).

3 Boyiadzis, M. & Foon, K. A. Approved monoclonal antibodies for cancer therapy. Expert Opin Biol Ther 8, 1151-1158 (2008).

4 Gunasekaran, K. et al. Enhancing antibody Fc heterodimer formation through electrostatic steering effects: applications to bispecific molecules and monovalent IgG. J Biol Chem 285, 19637-19646 (2010).

5 Rose, R. J. et al. Quantitative analysis of the interaction strength and dynamics of human IgG4 half molecules by native mass spectrometry. Structure 19, 1274-1282 (2011).

6 Kaneko, E. & Niwa, R. Optimizing therapeutic antibody function: progress with Fc domain engineering. BioDrugs 25, 1-11 (2011).

7 Kubota, T. et al. Engineered therapeutic antibodies with improved effector functions. Cancer Sci 100, 1566-1572 (2009).

8 Moore, G. L., Chen, H., Karki, S. & Lazar, G. A. Engineered Fc variant antibodies with enhanced ability to recruit complement and mediate effector functions. MAbs 2, 181-189 (2010).

9 Nimmerjahn, F. & Lunemann, J. D. Expression and function of the inhibitory Fcgamma-receptor in CIDP. J Peripher Nerv Syst 16 Suppl 1, 41-44 (2011).

10 Beck, A., Wurch, T., Bailly, C. & Corvaia, N. Strategies and challenges for the next generation of therapeutic antibodies. Nat Rev Immunol 10, 345-352 (2010).

11 Sapra, P., Hooper, A. T., O'Donnell, C. J. & Gerber, H. P. Investigational antibody drug conjugates for solid tumors. Expert Opin Investig Drugs 20, 1131-1149 (2011).

12 de Kruif, J. et al. Generation of stable cell clones expressing mixtures of human antibodies. Biotechnol Bioeng 106, 741-750 (2010).

13 Labrijn, A. F. et al. Species-specific determinants in the IgG CH3 domain enable Fab-arm exchange by affecting the noncovalent CH3-CH3 interaction strength. J Immunol 187, 3238-3246 (2011).

14 Labrijn, A. F. et al. Therapeutic IgG4 antibodies engage in Fab-arm exchange with endogenous human IgG4 in vivo. Nat Biotechnol 27, 767-771 (2009).

15 Xie, Z., Guo, N., Yu, M., Hu, M. & Shen, B. A new format of bispecific antibody: highly efficient heterodimerization, expression and tumor cell lysis. J Immu-

nol Methods 296, 95-101 (2005).

16 Chelius, D. et al. Structural and functional characterization of the trifunctional antibody catumaxomab. MAbs 2, 309-319 (2010).

17 Beck, A. et al. The next generation of antibody-drug conjugates comes of age. Discov Med 10, 329-339 (2010).

18 Cao, Y. & Lam, L. Bispecific antibody conjugates in therapeutics. Adv Drug Deliv Rev 55, 171-197 (2003).

19 Rasmussen, S. K., Rasmussen, L. K., Weilguny, D. & Tolstrup, A. B. Manufacture of recombinant polyclonal antibodies. Biotechnol Lett 29, 845-852 (2007).

20 Logtenberg, T. Antibody cocktails: next-generation biopharmaceuticals with improved potency. Trends Biotechnol 25, 390-394 (2007).

21 Forrer, N., Butte, A. & Morbidelli, M. Chromatographic behavior of a polyclonal antibody mixture on a strong cation exchanger column. Part II: Adsorption modelling. J Chromatogr A 1214, 71-80 (2008).

22 Forrer, N., Butte, A. & Morbidelli, M. Chromatographic behavior of a polyclonal antibody mixture on a strong cation exchanger column. Part I: Adsorption characterization. J Chromatogr A 1214, 59-70 (2008).

23 Nielsen, L. S. et al. Single-batch production of recombinant human polyclonal antibodies. Mol Biotechnol 45, 257-266 (2010).

24 Zhang, Z., Pan, H. & Chen, X. Mass spectrometry for structural characterization of therapeutic antibodies. Mass Spectrom Rev 28, 147-176 (2009).

25 Atmanene, C. et al. Extending mass spectrometry contribution to therapeutic monoclonal antibody lead optimization: characterization of immune complexes using noncovalent ESI-MS. Anal Chem 81, 6364-6373 (2009).

26 Kukrer, B. et al. Mass spectrometric analysis of intact human monoclonal antibody aggregates fractionated by size-exclusion chromatography. Pharm Res 27, 2197-2204 (2010).

27 Beck, A., Cianferani, S. & Van Dorsselaer, A. Biosimilar, Biobetter, and Next Generation Antibody Characterization by Mass Spectrometry. Anal Chem (2012).

28 Valliere-Douglass, J. F., McFee, W. A. & Salas-Solano, O. Native intact mass determination of antibodies conjugated with monomethyl Auristatin E and F at interchain cysteine residues. Anal Chem 84, 2843-2849 (2012).

29 Persson, P., Engstrom, A., Rasmussen, L. K., Holmberg, E. & Frandsen, T. P. Development of mass spectrometry based techniques for the identification and determination of compositional variability in recombinant polyclonal antibody products. Anal Chem 82, 7274-7282 (2010).

30 Heck, A. J. Native mass spectrometry: a bridge between interactomics and structural biology. Nat Methods 5, 927-933 (2008).

31 van Duijn, E. et al. Monitoring macromolecular complexes involved in the chaperonin-assisted protein folding cycle by mass spectrometry. Nat Methods 2, 371-376 (2005).

32 Uetrecht, C., Barbu, I. M., Shoemaker, G. K., van Duijn, E. & Heck, A. J. Interrogating viral capsid assembly with ion mobility-mass spectrometry. Nat Chem

3, 126-132 (2011).

33 Kaddis, C. S. & Loo, J. A. Native protein MS and ion mobility large flying proteins with ESI. Anal Chem 79, 1778-1784 (2007).

34 Benesch, J. L., Ruotolo, B. T., Simmons, D. A. & Robinson, C. V. Protein complexes in the gas phase: technology for structural genomics and proteomics. Chem Rev 107, 3544-3567 (2007).

35 Tahallah, N., Pinkse, M., Maier, C. S. & Heck, A. J. The effect of the source pressure on the abundance of ions of noncovalent protein assemblies in an electrospray ionization orthogonal time-of-flight instrument. Rapid Commun Mass Spectrom 15, 596-601 (2001).

36 van Breukelen, B., Barendregt, A., Heck, A. J. & van den Heuvel, R. H. Resolving stoichiometries and oligomeric states of glutamate synthase protein complexes with curve fitting and simulation of electrospray mass spectra. Rapid Commun Mass Spectrom 20, 2490-2496 (2006).

37 McAuley, A. et al. Contributions of a disulfide bond to the structure, stability, and dimerization of human IgG1 antibody CH3 domain. Protein Sci 17, 95-106 (2008).

38 Thies, M. J. et al. Folding and association of the antibody domain CH3: prolyl isomerization preceeds dimerization. J Mol Biol 293, 67-79 (1999).

39 Thies, M. J. et al. Folding and oxidation of the antibody domain C(H)3. J Mol Biol 319, 1267-1277 (2002).

40 Thies, M. J., Kammermeier, R., Richter, K. & Buchner, J. The alternatively folded state of the antibody C(H)3 domain. J Mol Biol 309, 1077-1085 (2001).

41 Beck, A. et al. Characterization by liquid chromatography combined with mass spectrometry of monoclonal anti-IGF-1 receptor antibodies produced in CHO and NS0 cells. J Chromatogr B Analyt Technol Biomed Life Sci 819, 203-218 (2005).

Chapter IV

Exploring an Orbitrap Analyzer for the Characterization of Intact Antibodies by Native Mass Spectrometry

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chapter IV

Monoclonal antibodies (mAbs) represent a major class of therapeutic biomolecules being developed by the biopharmaceutical industry, their popularity largely due to their high specificity towards targets. Therapeutic mAbs are being developed for various diseases, with a strong focus on cancer and immunological disorders[1]. All antibody drugs currently approved for clinical use are based on the IgG class; these molecules are composed of two heavy chains and two light chains, connected by disulfide bonds, and with a molecular weight of approximately 150 kDa. Antibody-based biopharmaceuticals can be complex and heterogeneous molecules, and as such depend heavily on a range of analytical tools to characterize them fully.

In this context, mass spectrometry (MS) is gaining importance as a technique for the analysis of mAbs[2-4]. MS is versatile and can address numerous structural issues, including glycan profile characterization, disulfide bond mapping, determination of post-translational modification, and mapping epitopes[5-9]. For the majority of these applications, mass spectrometric analysis is performed at the peptide level, and therefore requires several sample preparation steps prior to analysis, including denaturation, reduction, alkylation, digestion, and release of glycan chains, any of which may introduce issues concerning reproducibility.

In principle, a more straightforward approach is the direct MS analysis of intact antibodies, either denatured or in their native form. Whilst MS analysis using denaturing conditions is now becoming routine[2,3], MS from non-denaturing conditions, *i.e.* native MS, also represents a viable alternative for accurate mass measurement of intact antibodies. Native MS, as defined previously[10], is used to probe the folded tertiary and quaternary structures of proteins and protein complexes. It may provide information inaccessible by other approaches, allowing for a more complete characterization of the antibody sample from a single analysis. Proven applications of native MS range from antibody-antigen binding studies[11], to evaluation of structural features, dynamics and interaction strengths[12], to qualitative and semi-quantitative analysis of mixtures of mAbs[13]. Analysis of native intact antibodies could benefit from increases in sensitivity, mass accuracy and resolving power, and robustness of the analytical platform. Towards that goal we recently pioneered native MS using a modified Orbitrap™ mass analyzer, resulting in significant improvements in performance compared to the TOF-instruments typically used for such measurements (supplementary figure S1)[2,14-17]. We initially proved the power of this new platform for the analysis of very large protein complexes such as *E. coli* GroEL (800 kDa) and the yeast 20S proteasome (730 kDa)[18]. Here, we further demonstrate the great versatility of this new Orbitrap platform for the

characterization of mAbs, enabling important needs of the biopharmaceutical industry to be met.

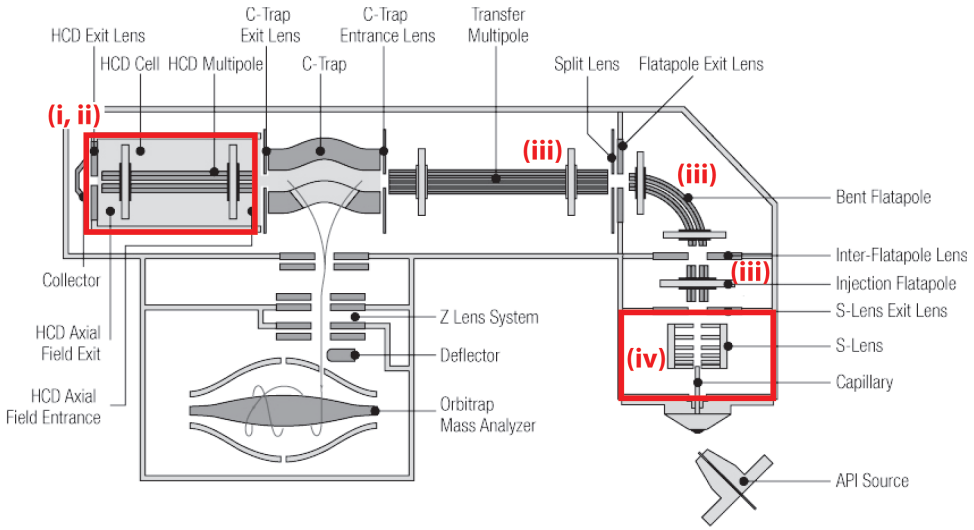

Figure 1. Schematic of the modified Exactive Plus™ instrument (ThermoFisher Scientific, Germany) as described in detail in[18]. Modifications included: (i) storing ions in the HCD cell, rather than trapping in the C-trap, allowing more efficient trapping and increased desolvation, (ii) manual regulation of the N_2 pressure in the HCD cell, (iii) altering the voltage offset on the flatapoles and transfer multipole and (iv) applying in-source dissociation energy, and (v) modifying the software and applying maximum RF voltages to all RF multipoles (including the C-trap) to enable m/z up to 30,000 to be measured.

One of the challenges of characterizing IgG is the presence of an N-linked glycosylation site in each heavy chain. The nature of this glycan chain has been found to influence antibody Fc receptor binding and serum half-life[19,20], and is highly dependent on the cell line used for protein expression. Therefore, an exhaustive characterization of the different glycan chains present is crucial for therapeutic mAbs. Native mass spectra of a deglycosylated and a glycosylated IgG (~146 kDa) acquired using static nano-electrospray ionization on a modified Exactive Plus instrument (Figure 1, see Experimental Section) are shown in Figure 2. As is typically observed in native ESI-MS spectra, the signal is spread over only a few charge-state peaks, primarily 23+ to 27+. Different glycoforms of the antibody are clearly baseline-resolved, allowing accurate assignments of the glycan identity (ΔMw = 162 Da, relating to different numbers of hexose (galactose) units present, Figure 2b). At the resolution obtained in this spectrum, mass differences down to 25 Da can be sufficiently resolved, allowing the identification of different modifications such as glycosylation, C-terminal lysine cleavage or primary sequence mutations. To illustrate this, we resolved and assigned glycoforms for an antibody-based con-

struct with a very heterogeneous glycan pattern, as shown in Figure 2c. Furthermore, these data can be collected from a single analysis, in a matter of seconds, typically consuming a few femtomoles of sample. In these spectra a mass spectral resolution could be achieved up to 12000 at m/z 6000; in combination with a high mass accuracy (< 1.5 Da/ 10 ppm *i.e.* 0.001%), thus allowing for confident assignment of modifications to the antibody. For denatured antibodies at lower m/z even higher resolution could be achieved using a FT-ICR instrument, albeit at the expense of sensitivity and detection speed[21,22].

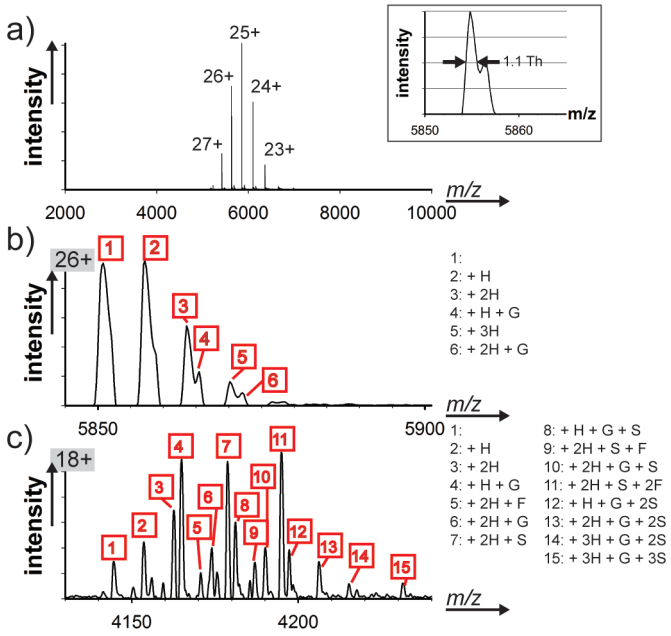

Figure 2. Antibody glycoform profiling by Orbitrap native MS. Native mass spectra of IgG with increasingly complex glycosylation profiles. (a) Full native mass spectrum of a deglycosylated IgG, showing that all ion signals accumulate in 5 main charge states ($23 \leq z \leq 27$). The accurate mass determined from this spectrum (146,352.4 Da) was within 2 ppm of the expected mass (146,352.6 Da). Inset: 25+ charge state, showing a peak width of 1.1 Th, and a shoulder peak at + 1.5 Th. (b,c) Native mass spectrum of one charge state of a glycosylated intact IgG (b) or a highly glycosylated IgG half-molecule (c), with different glycoforms baseline resolved. Individual glycoforms were assigned based on the differences in m/z between peaks, corresponding to 162 Da (hexose (galactose), H), 203 Da (GlcNAc, G), 146 Da (fucose, F) or 291 Da (sialic acid, S), as indicated by the lists on the right of each spectrum.

Besides the structural heterogeneity caused by glycosylation and other modifications, further complexity may arise due to the pharmaceutical industry moving towards artificial constructs of increased intricacy, *e.g.* co-expressed mixtures and antibody-drug conjugates, or antibody-DNA conjugates for diagnostic purposes.

As potential new therapeutic entities with synergic effects for minimal extra costs, mixtures of monoclonal antibodies are gaining momentum in the pharmaceutical industry, requiring dedicated novel analytical tools[13,23,24]. To explore the capabilities of the Orbitrap analyser in this regard, a complex mixture of ten monoclonal antibodies was analyzed after deglycosylation (Figure 3a). For each of the charge state peaks (see Figure 3a, 3b), nine distinct peaks could be observed; these can clearly be assigned to the individual components, by measuring their accurate mass. Only two components, differing in mass by just 20 Da (equivalent to < 0.8 Th), could not be fully resolved (note the width of the isotopic distribution for an antibody is approximately 25 Da[2]). The experimental resolving power of the Orbitrap also allows mixtures of glycosylated mAbs to be analyzed, as shown in Figure 3c, 3d. Each of four IgG species is seen to result in five well-resolved peaks, attributed to five different glycan combinations. From the spectrum, it is also clear that all the antibodies share the same glycan modifications in equal abundances. Thus, both the relative amounts of the four antibody components, and their glycosylation profile, can be obtained from a single Orbitrap native MS analysis.

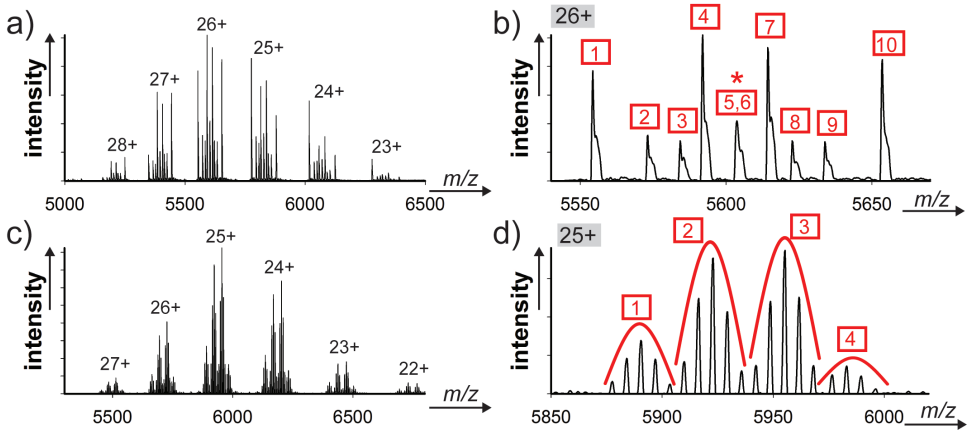

Figure 3. Composite mixtures of antibodies analyzed by Orbitrap native MS. (a) Full native mass spectra of a mixture of ten distinct deglycosylated IgG antibodies and (b) zoom-in on 26+ charge state. The well-resolved ion signals and accurate masses measured enable the unambiguous assignment and relative quantification of eight out of the ten compounds, the asterisk marks signals arising from two components, the molecular weight of which differs by only 20 Da (0.8 Th). (c) Full native mass spectra of a mixture of four glycosylated antibodies and (d) zoom-in on 25+ single charge state. Again, the well-resolved ion signals and accurate mass measurements enable the unambiguous identification and relative quantification of all 4 antibodies, revealing that their glycosylation patterns are highly similar.

Similar data as described above can be achieved on Q-TOF platforms, albeit at a lower resolving power under "native" conditions (Supplementary Figure S1)[13-17]. We attribute the higher resolving power largely to superior desolvation of the ions on the Orbitrap platform, taking place in the ion source and the Xe-filled HCD cell. One potential benefit of native MS, is that the signal is concentrated into fewer charge states, reducing the likelihood of overlapping ion signals from other protein species present. Moreover, using native MS, it is also possible to measure non-covalent interactions simultaneously, such as protein-protein and protein-ligand interactions. In the context of mAbs, this includes monitoring antibody-antigen binding[11,25,26], non-covalently assembled antibody structures[12], and oligomerization or aggregation[27]. Demonstrating that such analyses are now also possible on the Orbitrap platform, we next analyzed non-covalent antibody species and antibody-antigen interactions.

IgG4 molecules can exist as non-covalently bound dimers of heavy chain-light chain pairs. Removal of the hinge region (IgG4Δhinge), where disulfide bonds can form, enhances this effect, and the strength of heavy chain association can be further altered by single-point mutations[12]. Here, we analyzed on an Orbitrap instrument, one IgG4Δhinge variant with a weak binding constant (K_D > 100 μM) and one where the heavy chains bind with a much higher affinity (K_D < 1 nM). The Orbitrap mass spectra reveal a single species of 75 kDa for the former construct (Supplementary Figure S2a), and a dimeric species with a molecular weight of 150 kDa for the latter construct (Supplementary Figure S2b). These represent the 'half' and 'intact' structures, as expected, and prove that the non-covalent association of the heavy chains is completely retained during Orbitrap analysis. Antigen-binding studies are clearly also of pivotal importance for therapeutic antibody development. By analyzing proteins using native MS, their three-dimensional structures, and as a consequence their binding properties, can be at least partially preserved[25,26]. After incubation of a monoclonal anti IL6-IgG1 antibody with its antigen, an Orbitrap native mass spectrum of the mixture was recorded. Three charge-state envelopes are observed, resulting from the unbound antibody, the antibody bound to a single molecule of IL6, and the antibody bound to two molecules of IL6 (Figure 4). Thus, the interaction between these two proteins is evidently maintained during the analysis, and the charge-state envelopes from the different complexes are clearly resolved. Native MS on the Orbitrap is therefore an effective and efficient technique for monitoring antibody-antigen binding, providing information about each individual binding state, as opposed to the average amount of binding determined by other techniques.

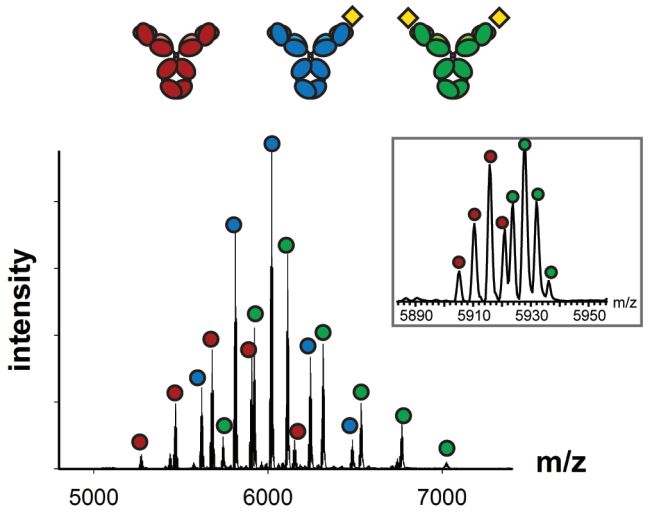

Figure 4. Monitoring antibody-antigen binding by Orbitrap native MS. Native mass spectrum of an anti-IL6 antibody incubated with the IL6 antigen. Three well-resolved charge-state envelopes are observed, relating to unbound mAb (red circles, 147,639.2 Da), mAb-IL6 (blue circles, 168,547.5 Da) and mAb-(IL6)2 (green circles, 189,452.8 Da), as indicated by the schematic structures. Inset: zoom-in of m/z 5890-5950, showing resolution of glycoforms of species with similar m/z values. Masses quoted are for the most abundant glycoform.

From an experimental point of view, performing native MS analyses of mAbs on Orbitrap analyzers is fast and sensitive. Starting from a purified protein solution, further sample preparation is limited to just a desalting or buffer-exchange step, and data acquisition can be complete within a minute. As an indication of the sensitivity of our technique, a sample of an IgG1 monoclonal antibody was successfully analyzed at concentrations down to 1 nM. Figure S3 shows an example raw spectrum of a 5 nM solution, acquired over 15 seconds, and with a clear charge-state envelope and signal-to-noise greater than 10. Estimating the flow-rate from the nano-electrospray capillaries as 1 $\mu L \cdot h^{-1}$, this corresponds to approximately 20 attomoles.

These highly sensitive and experimentally simple analyses represent a very powerful method for protein characterization. The improved experimental resolution at high m/z achievable using the Orbitrap analyzer is very advantageous for studying such naturally heterogeneous proteins as antibodies. Baseline separation of different species with small mass differences allows confident qualitative and potentially even quantitative characterization of mixtures of antibodies, different glycosylation states of a single monoclonal antibody, non-covalent interactions and antibody-antigen binding. The technology introduced here, by allowing anal-

ysis of very complex mixtures of compounds, could also be exploited to analyze, for example, impurities and degradation of mAbs, other post-translational modifications occurring on mAbs and mAb-drug conjugates. Orbitrap native MS can thus be extremely powerful for the characterization of antibodies, and in particular we believe this can be exploited to enhance significantly the analysis of therapeutic antibodies within the pharmaceutical industry.

Experimental Section

Materials

Antibody samples, produced in HEK293T cells, were kindly provided by Merus and Genmab. All purified proteins were exchanged into ammonium acetate (100mM or 150mM, pH 7 or 7.5) using either 10 or 5 kDa MWCO spin-filter columns (Vivaspin®500; Sartorius Stedim Biotech GmbH, Goettingen, Germany). Protein concentration was measured by UV absorbance at 280 nm, and adjusted to 2 µM. Enzymatic deglycosylation of the antibodies was performed, when needed, by incubating 25 µg of protein with 1 units of N-glycosidase F (PNGaseF; Roche Diagnostics, Mannheim, Germany) at 37°C overnight prior to buffer exchange. For antibody-antigen binding experiment a molecular ratio of 1 antibody to 4 antigen was used. For sensitivity tests, serial dilutions of deglycosylated IgG1 down to 1 nM were made.

ESI-MS Analysis

Approximately 1-2 µl of each sample was loaded into a gold-plated glass nano-electrospray capillary (made in-house) to which a capillary voltage of between 1.2 and 1.6 kV was applied. Data were acquired on a slightly modified Exactive PlusTM instrument[13] over the m/z range 400 - 30,000 Th. Ions were stored in the HCD cell before return to the C-trap for increased desolvation and higher sensitivity. The pressure of nitrogen gas in the HCD cell was increased as required with a manual pressure regulator. The voltage offsets on the flatapoles and transport octapole were manually tuned to increase the transmission of the larger protein ions. Resolution settings were adjusted as required, between 9,000 and 70,000 at m/z 200. In-source dissociation energy was applied (100-200V) to aid in desolvation. Data were calibrated using clusters of ammonium hexafluorophosphate or caesium iodide.

Acknowledgements

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References

1 Reichert, J. M. Antibody-based therapeutics to watch in 2011. MAbs 3, 76-99 (2011).

2 Zhang, Z., Pan, H. & Chen, X. Mass spectrometry for structural characterization of therapeutic antibodies. Mass Spectrom Rev 28, 147-176 (2009).

3 Beck, A., Cianferani, S. & Van Dorsselaer, A. Biosimilar, Biobetter, and Next Generation Antibody Characterization by Mass Spectrometry. Anal Chem (2012).

4 Bondarenko, P. V., Second, T. P., Zabrouskov, V., Makarov, A. A. & Zhang, Z. Mass measurement and top-down HPLC/MS analysis of intact monoclonal antibodies on a hybrid linear quadrupole ion trap-Orbitrap mass spectrometer. J Am Soc Mass Spectrom 20, 1415-1424 (2009).

5 Mauko, L. et al. Glycan profiling of monoclonal antibodies using zwitterionic-type hydrophilic interaction chromatography coupled with electrospray ionization mass spectrometry detection. Anal Biochem 408, 235-241 (2011).

6 Huang, S. Y. et al. Automatic Disulfide Bond Assignment Using a(1) Ion Screening by Mass Spectrometry for Structural Characterization of Protein Pharmaceuticals. Anal Chem (2012).

7 Bagal, D., Valliere-Douglass, J. F., Balland, A. & Schnier, P. D. Resolving disulfide structural isoforms of IgG2 monoclonal antibodies by ion mobility mass spectrometry. Anal Chem 82, 6751-6755 (2010).

8 Wu, S. L. et al. Mass spectrometric determination of disulfide linkages in recombinant therapeutic proteins using online LC-MS with electron-transfer dissociation. Anal Chem 81, 112-122 (2009).

9 Sundaram, S. et al. An innovative approach for the characterization of the isoforms of a monoclonal antibody product. MAbs 3, 505-512 (2011).

10 Heck, A. J. Native mass spectrometry: a bridge between interactomics and structural biology. Nat Methods 5, 927-933 (2008).

11 Atmanene, C. et al. Extending mass spectrometry contribution to therapeutic monoclonal antibody lead optimization: characterization of immune complexes using noncovalent ESI-MS. Anal Chem 81, 6364-6373 (2009).

12 Rose, R. J. et al. Quantitative analysis of the interaction strength and dynamics of human IgG4 half molecules by native mass spectrometry. Structure 19, 1274-1282 (2011).

13 Rosati, S. et al. Qualitative and semiquantitative analysis of composite mixtures of antibodies by native mass spectrometry. Anal Chem 84, 7227-7232 (2012).

14 Wagner-Rousset, E. et al. The way forward, enhanced characterization of therapeutic antibody glycosylation: comparison of three level mass spectrometry-based strategies. J Chromatogr B Analyt Technol Biomed Life Sci 872, 23-37 (2008).

15 Valliere-Douglass, J. F., McFee, W. A. & Salas-Solano, O. Native intact mass determination of antibodies conjugated with monomethyl Auristatin E and F at interchain cysteine residues. Anal Chem 84, 2843-2849 (2012).

16 Siemiatkoski, J., Lyubarskaya, Y., Houde, D., Tep, S. & Mhatre, R. A comparison of three techniques for quantitative carbohydrate analysis used in characterization of therapeutic antibodies. Carbohydr Res 341, 410-419 (2006).

17 Damen, C. W. et al. Electrospray ionization quadrupole ion-mobility time-of-flight mass spectrometry as a tool to distinguish the lot-to-lot heterogeneity in N-glycosylation profile of the therapeutic monoclonal antibody trastuzumab. J Am Soc Mass Spectrom 20, 2021-2033 (2009).

18 Rose, R. J., Damoc, E., Denisov, E., Makarov, A. & Heck, A. J. High-sensitivity Orbitrap mass analysis of intact macromolecular assemblies. Nat Methods 9, 1084-1086 (2012).

19 Houde, D., Peng, Y., Berkowitz, S. A. & Engen, J. R. Post-translational modifications differentially affect IgG1 conformation and receptor binding. Mol Cell Proteomics 9, 1716-1728 (2010).

20 Zalevsky, J. et al. Enhanced antibody half-life improves in vivo activity. Nat Biotechnol 28, 157-159 (2010).

21 Fornelli, L. et al. Top-down analysis of monoclonal antibody IgG1 by electron transfer dissociation Orbitrap FTMS. Mol Cell Proteomics (2012).

22 Valeja, S. G. et al. Unit mass baseline resolution for an intact 148 kDa therapeutic monoclonal antibody by Fourier transform ion cyclotron resonance mass spectrometry. Anal Chem 83, 8391-8395 (2011).

23 Logtenberg, T. Antibody cocktails: next-generation biopharmaceuticals with improved potency. Trends Biotechnol 25, 390-394 (2007).

24 de Kruif, J. et al. Generation of stable cell clones expressing mixtures of human antibodies. Biotechnol Bioeng 106, 741-750 (2010).

25 Tito, M. A. et al. Probing molecular interactions in intact antibody: antigen complexes, an electrospray time-of-flight mass spectrometry approach. Biophys J 81, 3503-3509 (2001).

26 Oda, M. et al. Effects of antibody affinity and antigen valence on molecular forms of immune complexes. Mol Immunol 47, 357-364 (2009).

27 Kukrer, B. et al. Mass spectrometric analysis of intact human monoclonal antibody aggregates fractionated by size-exclusion chromatography. Pharm Res 27, 2197-2204 (2010).

Chapter V

In-Depth Qualitative and Quantitative Analysis of Composite Glycosylation Profiles and Other Micro-Heterogeneity on Intact Monoclonal Antibodies by High-Resolution Native Mass Spectrometry Using a Modified Orbitrap

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Abstract

Here, we describe a fast, easy-to-use and sensitive method to profile in-depth structural micro-heterogeneity, including intricate N-glycosylation profiles, of monoclonal antibodies at the native intact protein level by means of mass spectrometry using a recently introduced modified Orbitrap Exactive Plus mass spectrometer. We demonstrate the versatility of our method to probe structural micro-heterogeneity by describing the analysis of three types of molecules: a) a non-covalently bound IgG4 hinge deleted full-antibody in equilibrium with its half-antibody, b) IgG4 mutants exhibiting highly complex glycosylation profiles and c) antibody-drug conjugates. Using the modified instrument, we obtain baseline separation and accurate mass determination of all different proteoforms that may be induced, for example by glycosylation, drug loading and partial peptide backbone-truncation. We show that our method can handle highly complex glycosylation profiles, identifying more than 20 different glycoforms per monoclonal antibody preparation, and more than 30 proteoforms on a single highly purified antibody. In analysing antibody-drug conjugates, our method also easily identifies and quantifies more than 15 structurally different proteoforms that may result from the collective differences in drug loading and glycosylation. The method presented here will aid in the comprehensive analytical and functional characterization of protein micro-heterogeneity, which is crucial for successful development and manufacturing of therapeutic antibodies.

Introduction

Controlling and understanding the protein micro-heterogeneity of monoclonal antibodies (mAbs) both in a qualitative and quantitative manner represents one of the main focuses in the development and manufacturing of this class of therapeutics. Post-translational modification (PTM) on mAbs needs to be minutely characterized because it may affect antibody structure, efficacy and potency, and its potential antigenicity or immunogenicity. The most widespread PTM occurring on mAbs is N-glycosylation. The nature of the glycan chains influences Fc-effector function and serum half-life[1,2]. In particular, the lack of core fucosylation enhances antibody dependent cellular cytotoxicity (ADCC)[3,4], while the presence of (α2-6)-linked sialic acids (N-acetylneuraminic acids) may be beneficial for anti-inflammatory activity[5-7]. MAb glycosylation can be very diverse in nature, leading to an extensive molecular heterogeneity of the glycoprotein. Wild-type mAbs typically exist as a mixture of 3-5 different glycoforms, with their nature and abundance highly dependent on the cell line and expression system used. Human antibodies expressed in non-human cell lines can bear non-human carbohydrate chains (*e.g.*, containing N-glycolylneuraminic acid, the galactose(α1-3)galactose epitope or xylose) that may trigger undesired immunogenic responses. Therefore, regulatory authorities require a thorough qualitative and quantitative analysis of mAb glycosylation for the preparation of medicinal products.

Other modifications frequently occurring on mAbs are disulfide pairings, N- and C-terminal modifications such as pyroGlu, Lys and Gly clipping[8], which also require qualitative and quantitative analysis. At present, it is not possible to analyze all this micro-heterogeneity using a single method. For the most part, efforts so far have been directed toward the analysis of mAbs glycosylation. Several analytical approaches to characterize antibody N-glycosylation targeted at different molecular levels, *i.e.*, the glycan, the glycopeptide and the glycoprotein level, have been developed. One of the dominant approaches currently used is analysis at the glycan level, whereby the glycan chains are first enzymatically released from the protein. Subsequently, these glycans, eventually derivatized with a fluorescent label at the reducing side (reductive amination), are analyzed using chromatographic methods, such as normal-phase liquid chromatography (NP-LC), hydrophilic interaction liquid chromatography (HILIC), and high-performance anion-exchange chromatography (HPAEC), regularly combined with intermediate exo-glycosidase digestions[9-12]. Alternatively, mass spectrometry (MS) can also be used for the analysis at the glycan level, mostly by using matrix-assisted laser desorption ionization (MALDI) in combination with time of flight (TOF) analyzers. A general drawback of

analyzing only at the glycan level is that the connectivity to the protein of origin is lost, which becomes a problem if the protein to be analyzed is not very pure. An additional drawback of using MALDI-MS for the analysis of glycans is represented by the relatively poor ionization efficiency, especially of sialylated glycans, that severely hampers their detection, and thus quantitative analysis[13-15].

Analysis by MS at the glycopeptide level has recently become more perceptible, partly to overcome some of the aforementioned shortcomings[16]. In this sort of analysis, the connectivity issue is evidently solved; however, analysis on the glycopeptide level is still immature, needing more advanced and dedicated chromatographic approaches. Moreover, in this case proteolysis (*e.g.*, by trypsin) needs to be complete and reproducibly controlled, which typically becomes more difficult when a protein is highly glycosylated. Additionally, glycopeptide fragmentation in MS has not always been sufficiently informative. This last issue may be (partly) solved as it has recently been shown that glycopeptides can be more efficiently fragmented and characterized using in series collision-induced dissociation (CID) and electron transfer dissociation (ETD), which target the structures of the glycans and peptide backbone, respectively, and can also provide information on their structural branching [17-19].

Antibody glycosylation can also be investigated at the intact protein level through a combination of chromatography and MS. Investigations at the intact protein level have the advantage of requiring less sample handling, but glycan analyses of intact proteins by chromatography and MS are still in their infancy compared to measurements at the peptide and glycan level. Most commonly, these mass spectrometric analyses are performed under denaturing conditions, with the protein eluted in an acidified organic/water mixture using LC-MS[20-22], prior to ionization by electrospray. Current TOF mass analyzers provide sufficient resolving power to resolve (less complex) glycan profiles on the intact proteins in a qualitative and relative quantitative manner. Alternatively, mAbs can be mass analyzed by native MS, wherein the protein is ionized in an aqueous ammonium acetate buffer[23]. Native MS seems to retain the protein in a more folded structure, whereby the proteins also become substantially less charged in the ionization process. This implies, however, that they need to be detected at high m/z values, which so far has been little explored on ion traps or Orbitraps.

In this work, we aim for the detailed characterization of highly complex micro-heterogeneity, including glycosylation profiles, on intact native mAbs. We use the recently described Orbitrap Exactive Plus (ThermoFisher Scientific) that has been modified to perform native MS[24]. We show that this instrument is capa-

ble, through its high sensitivity, mass accuracy and resolving power, of providing baseline separation of the different proteoforms on intact half- (~75 kDa) and full-mAbs (~150 kDa). The analysis by native MS on the Orbitrap at the intact protein level provides a number of advantages. Most importantly, a single highly resolved profile of all protein micro-heterogeneity could be obtained within a few minutes using a few femto-mole of sample, making it a time- and cost-efficient tool for routine analysis. Very little sample preparation is required, as the direct injection into the mass spectrometer excludes the need for a chromatographic step prior to MS analysis. Additionally, differences in the chemical nature of the glycan chains do not substantially affect the ionization efficiency of the intact protein, allowing the relative quantification of all proteoforms/glycoforms, including highly sialylated glycans. The detailed qualitative and quantitative profiles we observe reveal, in some cases, more than 30 different proteoforms of a single mAb[25], extending the depth of structural characterization usually obtained by current technologies.

Results

Benchmarking the performance of native MS using an Orbitrap mass analyzer in the characterization of protein micro-heterogeneity of intact full-length mAbs.

To test the performance and demonstrate the versatility of the new analysis workflow, we selected three different samples: I) a full-length (150 kDa), hinge deleted, IgG4 that exists in equilibrium with its half-antibody (75 kDa); II) IgG4 mutants exhibiting highly complex glycosylation profiles; and III) an IgG1 antibody-drug conjugate (ADC).

We benchmarked our approach evaluating the glycosylation profile on a wild-type, hinge-deleted IgG4 antibody (ΔhingeIgG4). The deletion of the hinge region excludes inter-molecular disulfide bonds between the two heavy chains, making the dimerization of the two half-antibodies occur solely through non-covalent interactions[26]. The full native mass spectrum of the ΔhingeIgG4WT antibody is shown in Figure 1A. Notably, as described earlier[27] this spectrum can be generated in a matter of a few minutes, consuming just a few femtomoles of sample. The native MS spectrum provides a glimpse of the equilibrium, caused by the deletion of the hinge region, that exists between the half- and full-antibody in solution at the particular concentration used, from which dimerization constants can be determined[26]. This feature enables the dedicated analysis of the glycosylation profile at both the half- and full-antibody level in a single spectrum. The mAb protein micro-heterogeneity caused by the diverse glycosylation becomes apparent when

zooming-in on a single charge state (Figure 1A in-sets). Multiple peaks corresponding to the different glycoforms are easily baseline-resolved at high S/N levels, allowing very accurate mass measurement and, therefore, reliable proteoform assignment.

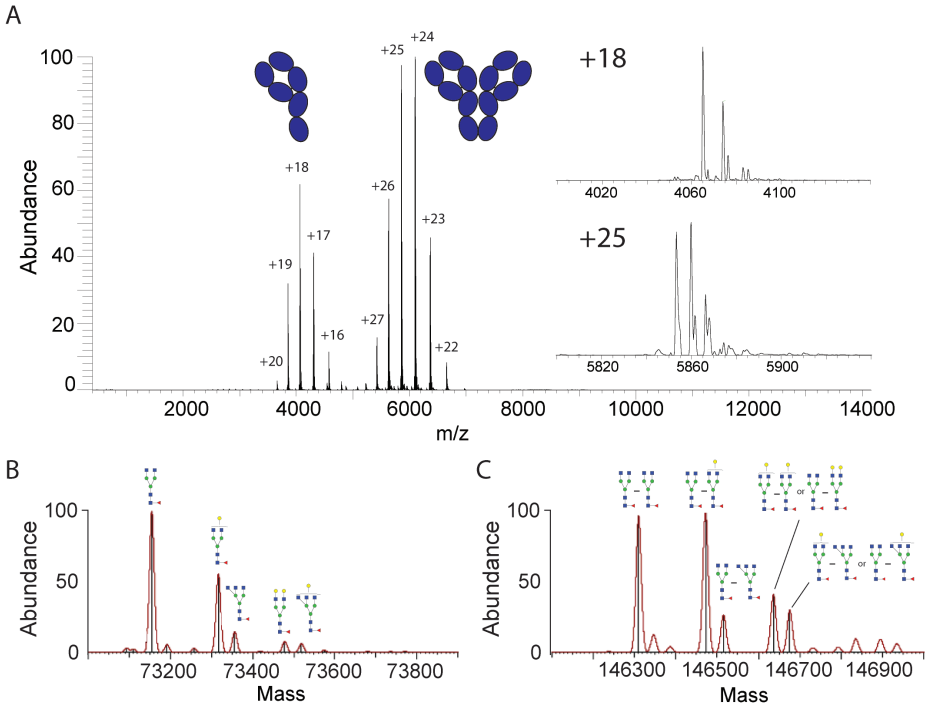

Figure 1. Antibody glycosylation analysis at the intact protein level by native Orbitrap MS. In A) the full native mass spectrum of an ΔhingeIgG4WT antibody is shown, revealing two charge-states envelopes originating from the half- (~m/z 4000) and full-antibody (~m/z 6000) being in equilibrium. The in-sets show enlarged single charge state spectra for both the half- and full-antibody, highlighting the micro-heterogeneity caused by glycosylation. The convoluted zero-charge mass spectra and glycan assignments, of the major components, are shown for the half-antibody in (B) and the full-antibody in (C). In case of more than one possible schematic structure for a $(Neu5Ac_a)(Gal_b)Man_cGlcNAc_dFuc_e$ composition, only one isoform is included; additional isomeric structures are displayed in Figure S4.

First, we evaluated the micro-heterogeneity profile on the half-antibody (~75 kDa). Therefore, the spectra were convoluted to zero-charge, taking the signals at all detected charge states into account, which facilitated mass assignment (Figure 1B). The masses found experimentally were in very good agreement with the theoretical masses of the protein sequence with the addition of glycan structures typically observed when expressing mAbs in a human (HEK-293F) cell line (Ta-

ble S1). After identification and assignment of the most naive glycan (G0F in this case), the subsequent peaks in the spectrum can be easily assigned by examination of the mass shifts, which correspond to the addition of single successive monosaccharide residues.

Additionally, convolution to a zero charge mass spectrum provides a means to measure peak intensities, which are based on the sum of the intensities over all detected charge states peaks. Therefore, we also have quantitative information on the abundance of the different proteoforms largely caused by the micro-heterogeneity in the glycosylation of the protein backbone. The likely confidence of this quantitative data is very high, as it may be hypothesized that different glycan structures have negligible influence on the ionization efficiency of the much larger protein backbones. We used the quantitative experimental information of the different glycovariants of the half-antibody to predict the identity and the abundance of the glycovariants present in the full-antibody (~150 kDa) (Figure 1C). Because the full-antibody consists of a pair of two half-antibodies, all glycan combinations are possible, including asymmetric species, *i.e.*, full-antibodies carrying two different glycans on the two halves. We used a statistical model to predict the abundances of all possible pair combinations based on the abundances of the glycans on the half-antibody. A comparison between the predicted and experimental glycan profiles is given in Figure S1. The quantitative similarity between the predicted and measured spectrum is excellent, providing evidence that, at the ensemble level, the glycan profiles are alike on both halves of the antibody.

Glycosylation profiles and further micro-heterogeneity on ΔhingeIgG4 antibody mutants.

We next analyzed several ΔhingeIgG4 single-point mutants that are known to display more complex glycosylation profiles than the wild-type ΔhingeIgG4[28]. Recently, Rose *et al.* revealed that specific point mutations in the CH3 domain of ΔhingeIgG4 antibodies affect both the CH3-CH3 interaction strength, and therefore their dimerization constant, and their glycosylation profiles[28]. The change in glycosylation is a surprising finding because the glycosylation site is quite distant from the CH3 domain. Among all samples previously analyzed with more conventional methods such as HPAEC-PAD and MALDI-TOF, the Y407E, Y407A, Y407Q and Y407K mutants showed high predominance of the half-antibody form, as well as the most diverse glycosylation profiles.

The zero-charge convoluted spectra of Y407E, Y407A, Y407Q and Y407K are displayed in Figure 2. The high resolving power (see also Figure S2 and S3) allows the

discrimination of a plethora of peaks that are quite close in mass. The mass and structural assignment is rather straightforward, even for the lower abundant species in the very congested spectra.

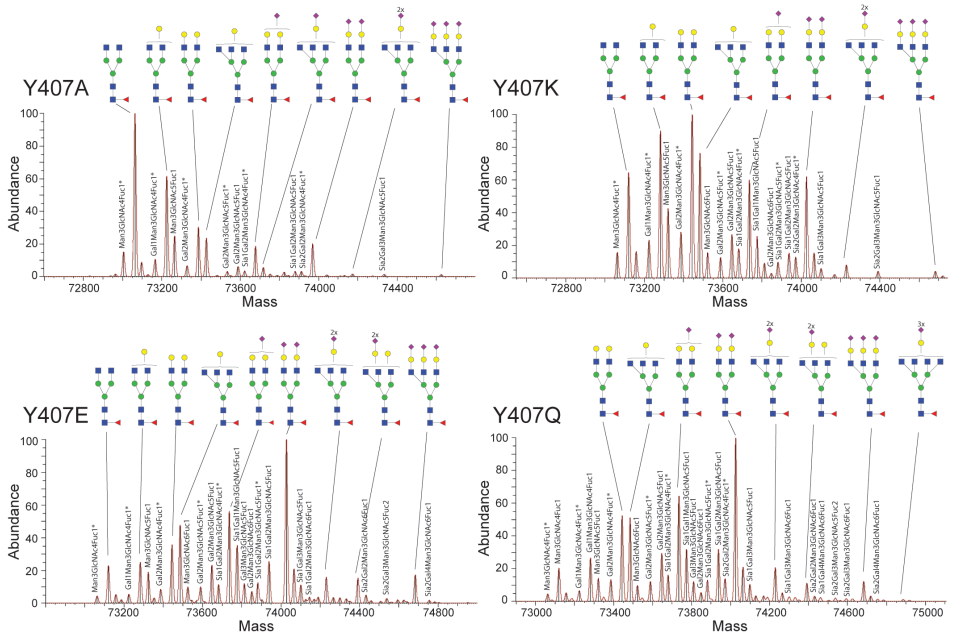

Figure 2. Overview of glycosylation profiles at the intact protein level of four IgG4 half-antibody mutants. As shown previously26, mutants at Y407 in ΔhingeIgG4 induce a dramatic change in the half- and full-antibody equilibrium and substantially alter the glycosylation pattern. From top left to bottom right, the spectra obtained for Y407A, Y407K, Y407E and Y407Q are shown. Compared to the WT, these mutants display a relatively high sialic acid content. The schematic structures of the most abundant assigned N-glycans, taking into account the N-glycan biosynthetic pathways in human and the known N-glycosylation patterns of human mAbs, are displayed. In case of more than one possible schematic structure for a $(Neu5Ac_a)(Gal_b)Man_cGlcNAc_dFuc_e$ composition, only one isoform is included; additional isomeric structures are displayed in Figure S4. *Antibody species bearing the glycine truncation.

Based on the MS compositional analysis in terms of hexose, N-acetylhexosamine, deoxyhexose and sialic acid (Table S1), more than 20 different complex-type N-glycan compositions could be assigned within a single spectrum. Our data reveals a considerable increase in the number of N-glycan species compared to previous studies[28]. Most notably, next to the glycan structures most frequently observed on mAbs expressed in human cell lines, we could identify lower abundant glycans that occur less frequently, but that are still allowed considering the N-glycan biosynthetic pathways typical of the human (HEK) cells. Notably, we could

even detect tetra-antennary and trisialylated glycan structures, these being most prominent in the Y407K, Y407E and Y407Q mutants, and a structure with two Fuc residues that seemed to hint at the presence of an H- or Lewis-type antigen, which is not frequently detected in mAbs.

A glimpse of some of the most extended glycan structures assigned is provided in Figure 3, while a summary of all assigned structures is given in Figure S4. The glycan mass data were used to determine the stoichiometry of the Man, Gal, GlcNAc, Fuc and Neu5Ac building blocks (Table S1), translated into the most likely N-glycan structures, based on literature data. Our analysis reveals even more clearly than previously reported that there is an intricate interplay between the mutations of Y407 in the CH3 domain and the glycosylation at N297 distantly located in the CH2 domain[28]. A more detailed investigation of the data disclosed re-occurring lower abundant satellite peaks that appeared at the lower mass-side of each peak and had a mass consistently 57 Da lighter, most likely corresponding to the truncation of the C-terminal glycine. The amount of truncated antibody was consistently 13±5%. If we also take these species into account, some of the recorded spectra displayed more than 30 proteoforms, due to the combination of glycosylation and Gly-truncation. Generally, the glycosylation profile was alike for the intact and the Gly-truncated antibody. This further demonstrates the advantage of the new method of analyzing intact antibodies by native MS on the modified Orbitrap, as all co-occurring micro-heterogeneities are observed in a single analysis.

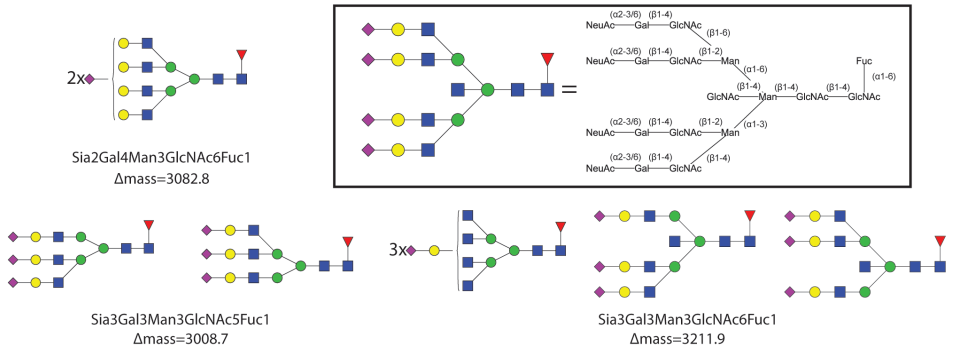

Figure 3. Overview of some of the most extended glycan structures identified on the IgG4 mutant antibodies. Typical examples of (sialylated) tri- and tetra-antennary N-glycans are presented. The in-set shows the relationship between the structure of a tetra-sialylated tetra-antennary N-glycan and the used symbolic notation for such a structure, clarifying also the symbolic notation of diantennary and triantennary structures. The Δmass, *i.e.*, the mass shift of the glycosylated form compared to the deglycosylated form, is also indicated. See Figure S4 for an overview of all glycan structures identified and structurally assigned.

Next, we subjected the ΔhingeIgG4 Y407E mutant, which we found to be particularly rich in sialic acids, to enzymatic desialylation using the enzyme neuraminidase. Removal of the (α2-3)/(α2-6)-linked sialic acid residues could be achieved, resulting in a much more simplified native MS spectrum (Figure S5). As a consequence of the loss of sialylated glycoforms, the relative abundances of the non-sialylated species do change as each sialylated species turns into its corresponding non-sialylated version. The increase of peak intensities in the desialylated sample was found to exactly match the intensities of their corresponding sialylated versions, confirming that both glycan identification and quantitation were correct.

Mass Resolution and Mass Accuracy

Mass accuracy and resolution often decrease with increasing mass of the analyte, impeding the analysis of intact proteins, especially when compared to lower molecular weight species such as glycopeptides and released glycans. This may be due to the general lower instrumental resolution at high m/z and the incomplete desolvation of the analyte in ESI caused by salt or solvent adducts. To probe the accuracy of our method, we assessed the mass accuracy for all samples analyzed. The theoretical masses are compared with the experimental ones in Table S1. Theoretical masses were obtained from the protein and glycan sequences; experimental masses were assessed from well-calibrated native MS spectra. A systematic mass error of around +3 Da was observed for the half-antibodies, while this error doubles to 6 Da for the full-antibody (Figure 4). From our experiments on the calibrant, *i.e.*, CsI clusters generated by ESI[24], we know that the instrumental mass accuracy is two orders of magnitude better at 6000 m/z range (±0.02 Da). Therefore, the shift in the peak maximum cannot be correlated to instrumental mass accuracy, but it might be attributed to an incomplete desolvation or substitution of one or more protons acquired by the mAb in the ESI process by Na^+ or K^+. In its current settings, our Orbitrap cannot fully resolve such very small mass differences. To explore this hypothesis, we simulated spectra of the wild-type ΔhingeIgG4 antibody: completely desolvated, or with one Na^+, K^+ or H_2O adduct, taking also into account the natural isotopic peak widths. These simulations, as displayed in Figure 4, clearly reveal that the majority of mAb ions are completely “naked”, *i.e.*, completely desolvated, while likely a very small fraction bears a single small molecule or cation adduct, causing a slight shift of the peak maximum (the 6 Da mentioned above) and a peak shoulder.

ΔhingeIgG4WT	half-antibody (G0F)	full-antibody (G0F/G0F)
theoretical mass (Da)	73152.2	146304.3
experimental mass (Da)	73155.3	146310.5
Δ exp-th (Da)	3.1	6.2

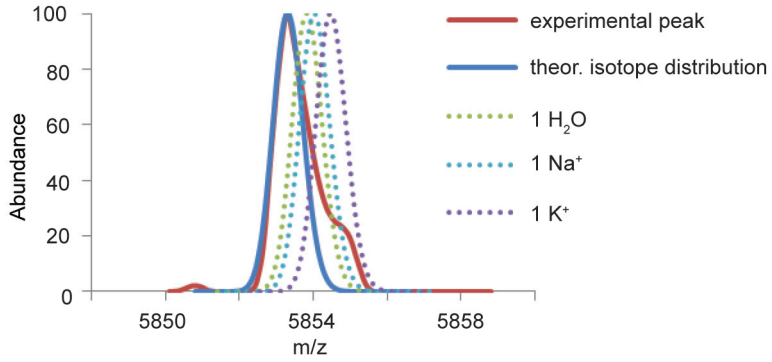

Figure 4. Limitations in accurate mass measurements in native Orbitrap MS of intact proteins. Comparison of the theoretical (blue line) and the experimental (red line) peak signal at a single charge state peak. The simulated peak of the ΔhingeIgG4WT antibody is displayed in solid blue when the ions are entirely desolvated (theoretical isotope distribution). Modelling into this peak the partial presence of a single water molecule, sodium (Na^+) or potassium (K^+) ion broadens the signal, leading to an increase in the experimentally measured mass.

Analyzing micro-heterogeneity in antibody-drug conjugates

The method described here may easily be extended to study induced protein micro-heterogeneity caused, for instance, by chemically-induced modifications. To illustrate this, we analyzed brentuximab vedotin (ADCETRIS®). This ADC is of particular interest because it carries a potent cytotoxic drug, monomethyl auristatin E, covalently attached via a maleimidecaproyl linker to cysteine residues of the IgG1 mAb that are usually involved in inter-molecular disulfide bridges. To allow drug conjugation, disulfide bridges in the IgG1 are first (partially) reduced, where after the drug can be coupled. Because each reduced disulfide bridge exposes two free cysteine residues, it is expected that two drug molecules are conjugated per reduced disulfide bridge. Figure 5A depicts the native MS spectrum of the brentuximab vedotin ADC following deglycosylation by PNGase-F. The peaks arising originate from the intact mAb (150 kDa), but clearly show a diverse conjugation profile of the drug molecules. Species originating from the different drug-loads

are clearly baseline resolved. Binding of the drug induces a mass shift of 2636 Da, corresponding to the binding of two drug molecules (one to each available cysteine residue). These spectra clearly indicate that the ADC product is not homogeneous in terms of drug-load, and antibodies carrying 0, 2, 4, 6 and 8 drug molecules co-exist. The average load could be semi-quantified as ~4.4, which is in agreement with previous findings[29,30]. To examine whether deglycosylation by PGNase-F was really indispensable to characterization of the ADC, we also recorded the native MS spectrum of the unprocessed ADC (Figure 5B). These spectra clearly demonstrate that the glycosylation-induced protein micro-heterogeneity profile of this ADC can be monitored in parallel with the drug-load in a single analysis.

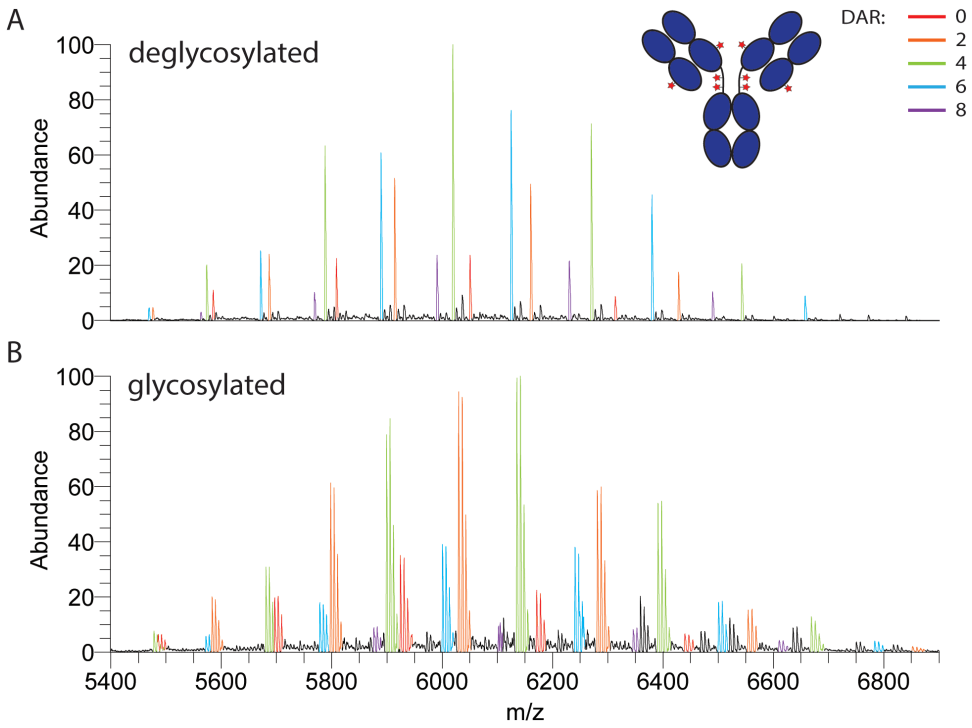

Figure 5. Analysis of the antibody-drug conjugate brentuximab vedotin (ADCETRIS®) at the intact protein level by native Orbitrap MS. Native spectra of the A) deglycosylated and B) glycosylated ADC. The differentially colored charge-state envelopes correspond to the different amount of drug molecules loaded onto the antibody. The drug loading clearly increases in steps of two, linked to the two accessible cysteine amino acids when a disulfide bridge is reduced.

Discussion

Here, we describe a method to minutely characterize at the intact protein level micro-heterogeneity originating from complex N-glycosylation, truncations and chemically- or biologically-induced modifications on mAbs. Performing native MS using an Orbitrap Exactive Plus instrument (ThermoFisher Scientific), we were able to identify, assign and quantify up to 25 different glycan structures present on IgG4 antibodies produced in human (HEK-293F) cells. We recently described the modifications made on the Orbitrap Exactive Plus instrument making it amenable for native MS[24,27]. The high sensitivity, resolving power and mass accuracy make this instrument particularly interesting for the characterization of protein micro-heterogeneities. Hence, we focus here primarily on protein glycosylation. Our investigation on mass accuracy revealed that a large majority of the ions generated in the electrospray source reach the detector completely "naked" and, thus provide clear evidence that complete desolvation is feasible in the native state for 150 kDa protein assemblies. As a result, experimental mass measurements were found to be in agreement with the theoretical masses obtained from the mAb primary sequence and glycan structures. Incomplete desolvation and salt adducts (*e.g.* Na^+ and K^+) have always been a limitation in the analysis of intact proteins, especially in native MS where aqueous buffers are used. Water molecules and salt adducts cause broadening of the peaks and the shift of the peak maximum, both of which typically hamper exact mass measurements on larger proteins[31]. As argued before, we hypothesize that the slightly harsher conditions in the source region of the Exactive Plus instrument ensure more efficient desolvation[24], thereby still retaining non-covalent protein-protein interactions.

The improved instrumentation performances led to an increase of the number of identified species, with special regard to the low abundant ones. In particular, referring to the analysis performed by Rose *et al.,* we were now able to detect, besides other glycoforms, low abundant trisialylated glycoforms in mutant ΔhingeIgG4-Y407A and ΔhingeIgG4-Y407K, which were not observed previously. Glycosylation studies of highly sialylated antibodies using MS can be rather challenging, especially when the analysis is performed on released glycans, because the presence of a negatively charged carboxyl group severely affects their ionization efficiency. This issue is solved when the analysis is performed at the intact protein level. Efficient ionization is ensured by the protonation of basic residues of the protein, while the glycan chains remain neutral. This is confirmed by the fact that a native mass spectrum of a glycosylated antibody and a deglycosylated antibody show alike charge-state distributions. More importantly, because the

electrospray-induced charging process primarily involves the protein backbone, which is identical in all components (or very similar in case of sequence variants), the ionization efficiency is not substantially affected by the differences in the glycan chains. This aspect is crucial to obtain (semi-) quantitative data.

A thorough characterization of glycosylation, both qualitatively and quantitatively, is imperative, especially for therapeutic antibodies. Such characterization may help in the analysis of batch-to-batch variability, and in biosimilar/reference product comparability exercises[32]. The data acquired enable relative quantitation[33], directly from the native MS spectra.

As previously demonstrated by Rose *et al.*[28] and confirmed by our analysis, the IgG4 glycosylation profile is highly influenced by a single point mutation in the CH3 domain, even though the glycosylation site is located in the CH2 domain. We previously showed using H/D exchange MS that single mutations can significantly alter the tertiary structure, which may be a cause for the observed change in glycosylation[28].

Some obvious advantages of performing protein micro-heterogeneity analysis by using native MS are the ease of sample preparation and the overall analysis speed. After antibody purification, just a buffer exchange step is required prior to mass spectrometric analysis. This normally requires only a couple of minutes per sample[31,34]. Moreover, by using native MS, non-covalently assembled proteins, such as ΔhingeIgG4 antibodies and cysteine-conjugated ADCs (brentuximab vedotin in our case), can be still be maintained and analyzed in their native quaternary state. This allows the assessment of the drug-antibody ratio (DAR) for cysteine-linked ADCs[29,35].

An obvious remaining limitation is represented by the inability to deduce monosaccharide stereoisomers, linkages, anomeric configurations, and glycan branching, which would still require dedicated glycan analysis by MS/MS or, for instance, NMR spectroscopy or LC combined with exo-glycosidases and methylation analysis[9,17,36-38].

In conclusion, the results described here show the amount of information that can be obtained from a single native mass spectrum on the modified Orbitrap mass analyzer. A detailed picture of the most complex antibody glycosylation profiles can be drawn, but the presence of sequence variants can also be detected. In addition, quantitative data of all species identified can be easily obtained from peak intensities. In our opinion, biotechnology and biopharmaceutical companies working on therapeutic antibodies or other types of biotherapeutics can benefit substantially from the accuracy and the speed of this method. This kind of analy-

sis can also be seen as a high-resolution fingerprint that can be used, for instance, for batch-to-batch comparisons or for comparability studies between biosimilar antibodies and their reference products.

Materials and Methods

Sample preparation. A ΔhingeIgG4WT antibody and related mutants ΔhingeIgG4Y407A, ΔhingeIgG4Y407E, ΔhingeIgG4Y407Q and ΔhingeIgG4Y407K were expressed in HEK-293F cells and purified as previously described[28]. After purification, 25 µg of each sample were buffer-exchanged into 150 mM ammonium acetate pH 7.5, using 10 kDa MWCO centrifugal filter units (Amicon® Ultra, Millipore). Enzymatic desialylation of the ΔhingeIgG4Y407E sample was performed using the neuraminidase (sialidase) enzyme (Roche). After digestion, the desialylated sample was buffer-exchanged prior to mass spectrometric analysis. Brentuximab vedotin (Seattle Genetics) was dissolved in sterile water. 25 µg were buffer-exchanged and analyzed as described.

Native MS. After buffer exchange, approximately 1 µl of 3 µM sample (antibody tetramer equivalent) was directly injected into the mass spectrometer using a gold-coated borosilicate capillary made in-house using a Sutter P-97 puller (Sutter Instrument Co.) and an Edwards Scancoat six sputter-coater (Edwards Laboratories). All samples were analyzed using a slightly modified Exactive Plus instrument (ThermoFisher Scientific). Modifications and main settings were previously described[24]. In particular, ions were trapped in the HCD cell filled with nitrogen at a pressure of 5 e^{-10} mbar. Resolution was set at 35,000, scans were acquired for a few minutes, combining 10 microscans.

Data Analysis. Protein Deconvolution V2.0 (ThermoFisher Scientific) was used to convolute raw spectra and for mass assignment and relative quantitation.

Acknowledgements

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References

1 Jefferis, R. Glycosylation of recombinant antibody therapeutics. Biotechnol Prog 21, 11-16 (2005).

2 Raju, T. S. Terminal sugars of Fc glycans influence antibody effector functions of IgGs. Curr Opin Immunol 20, 471-478 (2008).

3 Shinkawa, T. et al. The absence of fucose but not the presence of galactose or bisecting N-acetylglucosamine of human IgG1 complex-type oligosaccharides shows the critical role of enhancing antibody-dependent cellular cytotoxicity. J Biol Chem 278, 3466-3473 (2003).

4 Ferrara, C. et al. Unique carbohydrate-carbohydrate interactions are required for high affinity binding between FcgammaRIII and antibodies lacking core fucose. Proc Natl Acad Sci U S A 108, 12669-12674 (2011).

5 Kaneko, Y., Nimmerjahn, F. & Ravetch, J. V. Anti-inflammatory activity of immunoglobulin G resulting from Fc sialylation. Science 313, 670-673 (2006).

6 Anthony, R. M. et al. Recapitulation of IVIG anti-inflammatory activity with a recombinant IgG Fc. Science 320, 373-376 (2008).

7 Anthony, R. M., Wermeling, F., Karlsson, M. C. & Ravetch, J. V. Identification of a receptor required for the anti-inflammatory activity of IVIG. Proc Natl Acad Sci U S A 105, 19571-19578 (2008).

8 Beck, A., Wagner-Rousset, E., Ayoub, D., Van Dorsselaer, A. & Sanglier-Cianferani, S. Characterization of therapeutic antibodies and related products. Anal Chem 85, 715-736 (2013).

9 Tharmalingam, T., Adamczyk, B., Doherty, M. A., Royle, L. & Rudd, P. M. Strategies for the profiling, characterisation and detailed structural analysis of N-linked oligosaccharides. Glycoconj J 30, 137-146 (2013).

10 Zauner, G., Deelder, A. M. & Wuhrer, M. Recent advances in hydrophilic interaction liquid chromatography (HILIC) for structural glycomics. Electrophoresis 32, 3456-3466 (2011).

11 Zaia, J. On-line separations combined with MS for analysis of glycosaminoglycans. Mass Spectrom Rev 28, 254-272 (2009).

12 van Leeuwen, S. S., Schoemaker, R. J., Timmer, C. J., Kamerling, J. P. & Dijkhuizen, L. N- and o-glycosylation of a commercial bovine whey protein product. J Agric Food Chem 60, 12553-12564 (2012).

13 Harvey, D. J. Structural determination of N-linked glycans by matrix-assisted laser desorption/ionization and electrospray ionization mass spectrometry. Proteomics 5, 1774-1786 (2005).

14 Gil, G. C., Iliff, B., Cerny, R., Velander, W. H. & Van Cott, K. E. High throughput quantification of N-glycans using one-pot sialic acid modification and matrix assisted laser desorption ionization time-of-flight mass spectrometry. Anal Chem 82, 6613-6620 (2010).

15 Tep, S., Hincapie, M. & Hancock, W. S. A MALDI-TOF MS method for the simultaneous and quantitative analysis of neutral and sialylated glycans of CHO-expressed glycoproteins. Carbohydr Res 347, 121-129 (2012).

16 Dell, A. & Morris, H. R. Glycoprotein structure determination by mass spectrometry. Science 291, 2351-2356 (2001).

17 Scott, N. E. et al. Simultaneous glycan-peptide characterization using hydrophilic interaction chromatography and parallel fragmentation by CID, higher energy collisional dissociation, and electron transfer dissociation MS applied to the N-linked glycoproteome of Campylobacter jejuni. Mol Cell Proteomics 10, M000031-MCP000201 (2011).

18 Singh, C., Zampronio, C. G., Creese, A. J. & Cooper, H. J. Higher energy collision dissociation (HCD) product ion-triggered electron transfer dissociation (ETD) mass spectrometry for the analysis of N-linked glycoproteins. J Proteome Res 11, 4517-4525 (2012).

19 Ye, H., Boyne, M. T., 2nd, Buhse, L. F. & Hill, J. Direct approach for qualitative and quantitative characterization of glycoproteins using tandem mass tags and an LTQ Orbitrap XL electron transfer dissociation hybrid mass spectrometer. Anal Chem 85, 1531-1539 (2013).

20 Srebalus Barnes, C. A. & Lim, A. Applications of mass spectrometry for the structural characterization of recombinant protein pharmaceuticals. Mass Spectrom Rev 26, 370-388 (2007).

21 Olivova, P., Chen, W., Chakraborty, A. B. & Gebler, J. C. Determination of N-glycosylation sites and site heterogeneity in a monoclonal antibody by electrospray quadrupole ion-mobility time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 22, 29-40 (2008).

22 Kuribayashi, R., Hashii, N., Harazono, A. & Kawasaki, N. Rapid evaluation for heterogeneities in monoclonal antibodies by liquid chromatography/mass spectrometry with a column-switching system. J Pharm Biomed Anal 67-68, 1-9 (2012).

23 Heck, A. J. Native mass spectrometry: a bridge between interactomics and structural biology. Nat Methods 5, 927-933 (2008).

24 Rose, R. J., Damoc, E., Denisov, E., Makarov, A. & Heck, A. J. High-sensitivity Orbitrap mass analysis of intact macromolecular assemblies. Nat Methods 9, 1084-1086 (2012).

25 Smith, L. M. & Kelleher, N. L. Proteoform: a single term describing protein complexity. Nat Methods 10, 186-187 (2013).

26 Rose, R. J. et al. Quantitative analysis of the interaction strength and dynamics of human IgG4 half molecules by native mass spectrometry. Structure 19, 1274-1282 (2011).

27 Rosati, S. et al. Exploring an orbitrap analyzer for the characterization of intact antibodies by native mass spectrometry. Angew Chem Int Ed Engl 51, 12992-12996 (2012).

28 Rose, R. J. et al. Mutation of Y407 in the CH3 domain dramatically alters glycosylation and structure of human IgG. MAbs 5, 219-228 (2013).

29 Valliere-Douglass, J. F., McFee, W. A. & Salas-Solano, O. Native intact mass determination of antibodies conjugated with monomethyl Auristatin E and F at interchain cysteine residues. Anal Chem 84, 2843-2849 (2012).

30 Sievers, E. L. & Senter, P. D. Antibody-drug conjugates in cancer therapy. Annu Rev Med 64, 15-29 (2013).

31 Lorenzen, K. & van Duijn, E. Native mass spectrometry as a tool in structural biology. Curr Protoc Protein Sci Chapter 17, Unit17 12 (2010).

32 Beck, A., Sanglier-Cianferani, S. & Van Dorsselaer, A. Biosimilar, biobetter, and next generation antibody characterization by mass spectrometry. Anal Chem 84, 4637-4646 (2012).

33 Rosati, S. et al. Qualitative and semiquantitative analysis of composite mixtures of antibodies by native mass spectrometry. Anal Chem 84, 7227-7232 (2012).

34 Thompson, N. J., Rosati, S. & Heck, A. J. Performing native mass spectrometry analysis on therapeutic antibodies. Methods (2013).

35 Chen, J., Yin, S., Wu, Y. & Ouyang, J. Development of a native nanoelectrospray mass spectrometry method for determination of the drug-to-antibody ratio of antibody-drug conjugates. Anal Chem 85, 1699-1704 (2013).

36 Hokke, C. H., Bergwerff, A. A., Van Dedem, G. W., Kamerling, J. P. & Vliegenthart, J. F. Structural analysis of the sialylated N- and O-linked carbohydrate chains of recombinant human erythropoietin expressed in Chinese hamster ovary cells. Sialylation patterns and branch location of dimeric N-acetyllactosamine units. Eur J Biochem 228, 981-1008 (1995).

37 Llop, E. et al. Evaluation of protein N-glycosylation in 2-DE: Erythropoietin as a study case. Proteomics 7, 4278-4291 (2007).

38 Hocking, H. G. et al. Structure of the O-glycosylated conopeptide CcTx from Conus consors venom. Chemistry 19, 870-879 (2013).

chapter V

chapter V

Chapter VI

Detailed Mass Analysis of Structural Heterogeneity in Monoclonal Antibodies Using Native Mass Spectrometry

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Abstract

The molecular complexity of biopharmaceuticals puts severe demands on bioanalytical techniques required for their comprehensive structural characterization. Mass spectrometry (MS) has gained importance in the analysis of biopharmaceuticals, taking different complementary approaches ranging from peptide based sequencing to direct analysis on intact proteins and protein assemblies. In this protocol we describe procedures optimized to perform the analysis of monoclonal antibodies (mAbs) at the intact protein level under pseudo-native conditions, using native MS. Some of the strengths of native MS in the analysis of biopharmaceuticals are the analysis speed, sensitivity and specificity: for most experiments the whole protocol requires one working day, whereby tens of samples can be analyzed in a multiplexed manner, making it suitable for high throughput analysis. The method can be used for different applications such as the analysis of mixtures of mAbs, drug-antibody conjugates and the analysis of mAb post-translational modifications, including the qualitative and quantitative analysis of mAbs glycosylation.

Introduction

More than 30 monoclonal antibody–based therapeutics have been approved in the last 25 years. While the majority of clinically used monoclonal antibodies (mAbs) have been developed for the treatment of different cancer types[1], there are also some applications for infectious and immunological diseases. In this protocol we describe our method for characterizing intact mAbs using native mass spectrometry (native MS).

Antibody-based therapeutics

Of the five classes of human antibodies, IgGs are, up to now, the dominant antibody class employed as therapeutics. Naturally occurring IgGs are 150 kDa four-component systems, resulting from the dimerization of two identical light-heavy chain pairs. Their typical Y-shape structure (Figure 1) harbors three distinct regions: two antigen binding fragments (Fabs) and the crystallizable fragment (Fc) linked together via the so-called hinge region. Each of the two Fabs consist of one light chain and the VH and CH1 domains of one heavy chain. Complementary determining regions (CDRs), *i.e.*, the regions accounting for the antigen binding, normally involve both light and heavy chains and are situated at the extremity of the Fabs. The Fc tail is involved in the dimerization of the two light-heavy chain pairs whereby especially the CH2 and CH3 domains of each heavy chain are involved in the interaction.

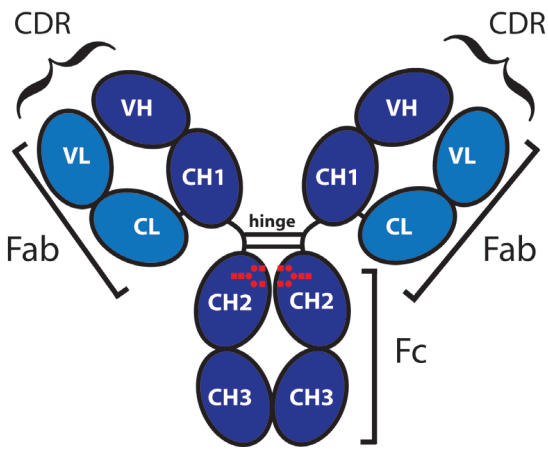

Figure 1. Schematic structure of an IgG1 antibody. Three main regions can be distinguished in the antibody structure: the Fc region, involved in the dimerization between the two heavy chains (dark blue), and two Fab regions, which result from the interaction between one light chain (light blue) and one heavy chain typically coupled by disulfide bridges. The two Fabs are connected with the Fc via the hinge region, where inter-chain disulfide bridges between the two heavy chains occur. Glycosylation, the dominant PTM on IgGs, occurs in the CH2 domain, while variable domains (VL=variable light; VH=variable heavy) determine antibody specificity and contain the antigen binding site (CDR, complementary determining regions).

Structurally, IgG antibodies are highly dynamic and flexible. Such flexibility hampers their crystallization, but through a few available high-resolution X-ray structures the general concepts of the structure of antibodies are known[2,3]. The structural complexity of IgG is amplified by a variety of post-translational modifications (PTMs), which increases their molecular heterogeneity substantially, giving rise to a mixture of different proteoforms/glycoforms for a single mAb product[4]. The main source of molecular heterogeneity is due to N-glycosylation. IgGs are extensively (*i.e.* stoichiometrically) glycosylated at the Asn297 of the CH2 domain of each heavy chain while, more rarely, an additional glycosylation site can be present in the Fab regions. Most mAbs, expressed in mammalian cell lines, consists of a mixture of 3 to 5 abundant glycoforms[5-8]. Other modifications, either resulting from cellular processing or introduced by sample handling or storage, can contribute to sample complexity. These include disulfide bridges between cysteine residues, N-terminal glutamine cyclization, C-terminal lysine clipping, oxidation, deamidation, non-enzymatic glycation, sequence truncation and/or single amino acid substitutions[9-13].

To enhance therapeutic potential, complexity is sometimes also intentionally further increased. For instance, an emerging field in antibody-based therapy involves mixtures of mAbs. The combination of different mAbs in a single therapeutic product with different molecular targets or mechanisms of action can result in improved pharmacological profiles compared to the single mAb[14,15], although this evidently comes at the expense of increase product heterogeneity. Another emerging field is the use of conjugates between antibodies and small molecule drugs; the antibody may for instance be used to target the coupled toxic anti-cancer drug to the tumor cells[16,17].

The importance of Quality Control

For the use of mAbs as therapeutic products, it is important to characterize their composition and molecular heterogeneity in detail as each component can affect the safety and efficiency of the product. A thorough structural characterization is required not only in the development stage of the antibodies, but also in the production stage to control inter-batch consistency. Such analysis also helps to distinguish biosimilars from the original product[18]. To accomplish a detailed structural characterization, a wide range of techniques are used, and chromatographic techniques and mass spectrometry dominate such workflows[19-21]. In the next section, we will describe in more detail one of the emerging approaches, *i.e.* native MS, and highlight the unique and/or complementary features this method brings to the analysis of therapeutic mAbs.

Native MS for the analysis of mAbs

With the progressive improvements in sensitivity, resolution and mass accuracy of mass spectrometers and subsequent development of new MS-based methods, MS is gaining rapidly momentum in the analysis of mAbs and other protein therapeutics. The mass spectrometric analysis of mAbs at the intact protein level is already routine in many pharmaceutical and biotechnology laboratories. It is used for antibody identification by accurate mass measurements, to assess purity, but also to profile antibody glycosylation[22]. Yet, for this type of analysis, denaturing conditions are still most commonly used, often in combination with liquid chromatography. This approach, although fast and sensitive, does not preserve non-covalent interactions and native-folded structures, and occasionally biopharmaceutical products are less stable and may aggregate under the conditions used for LC-MS.

Native MS is a particular mass spectrometric technique that allows the analysis of intact proteins and protein complexes under more native conditions[23-25]. The use of aqueous buffers and nano-electrospray ionization (nESI) allows the retention of non-covalent interactions and the folded native conformation, broadening the range of applications for the analysis of mAbs. Moreover, native MS possesses some advantages over the conventional denaturing approach even in those cases where the retention of non-covalent interaction is not strictly necessary. Due to the folded conformation of the protein, the resulting native mass spectrum of a mAb is characterized by a smaller charge-state envelope that simplifies the spectra and also condenses the ion signals into fewer peaks increasing the signal to noise ratio.

During the last few years, others and we have described how native MS can be applied in the structural characterization of intact mAbs[18,26-28]. Most recently, we introduced a modified Orbitrap-based mass spectrometer (Exactive Plus, ThermoFisher) to the field of native MS that enables measurements with improved mass resolving power and accuracy[29,30]. This higher resolving power allows the identification of multiple co-occurring PTMs and other minor modifications in mAbs. As the use of such a high resolution instrument is ideal, but not always strictly required, in this protocol, we describe the procedures making use of two different instrumental platform: time-of-flight (TOF) based mass spectrometers, which are still more routinely used for native MS, and the recently introduced Orbitrap-based instrument (Figure 2). We also provide recommendations on the type of applications that can be performed on the TOF instruments, and those that require the higher resolving power attained by the Orbitrap (Table 1).

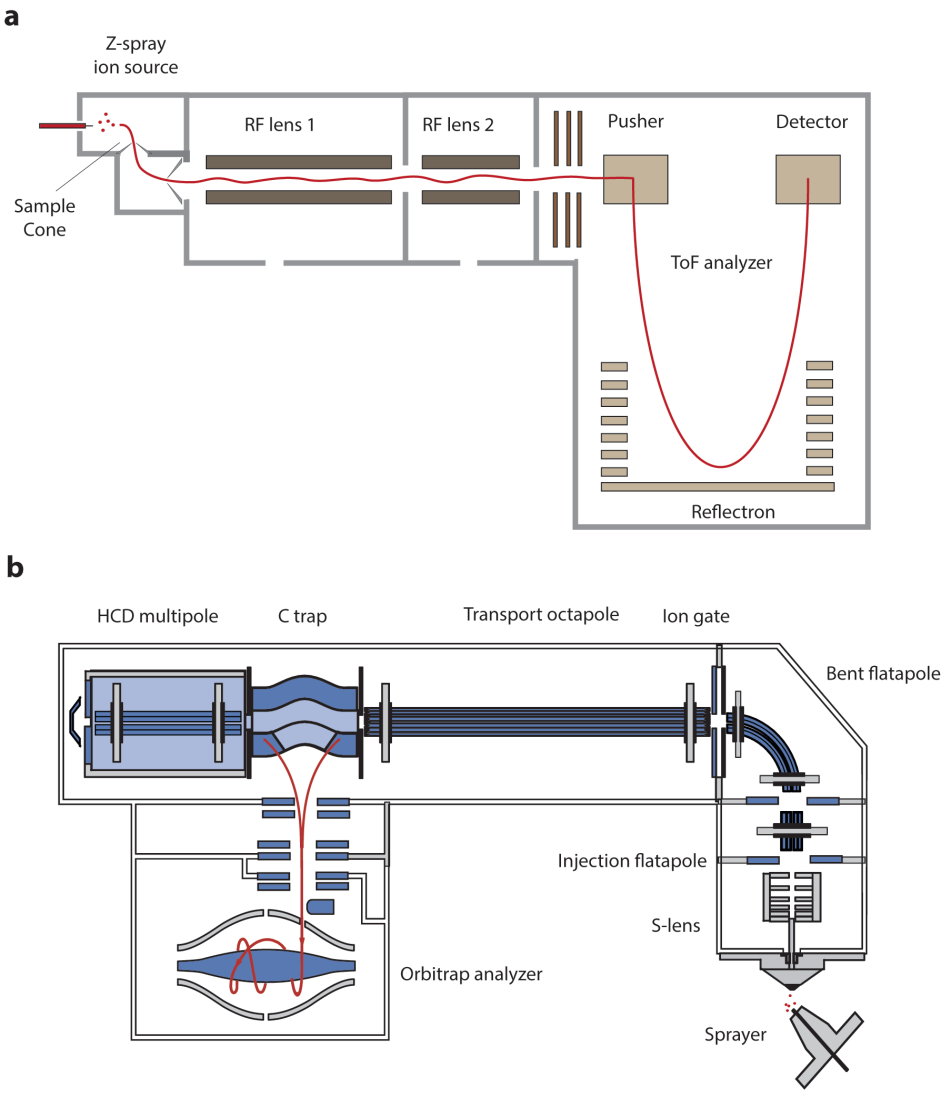

Figure 2. Schematic of two mass spectrometers applied in the native analysis of mAbs. (a) time-of-flight based instrument; the LCT (Waters) and (b) an Orbitrap based instrument; Exactive Plus (ThermoFisher Scientific). (a) The LCT mass spectrometer comprises of a Z-spray ion source where intact mAbs are ionized in their native-like folded conformation using nESI. The ions enter the instrument through the sample cone and are transmitted and focused by two hexapoles (RF lens 1 and RF lens 2). The pusher sends packages of ions in the TOF for their m/z measurements. (b) The Exactive Plus mass spectrometer consists of a nESI source wherein, similarly to the LCT, ions are generated. The ions are focused and transmitted through a bent flatapole, transport octapole and C-trap until they enter the HCD cell. In the HCD cell, the ions are trapped and eventually sent to the Orbitrap for accurate mass analysis.

	Inherent resolving power at 6000 m/z	Experimentally observed resolving power at 6000 m/z	Optimal concentration range	MS/MS capability	Cost
Orbitrap	~20,000	~4,000 - 5,000	3 - 1 µM	NO (AIF)*	●●○
Q-TOF	~5,000 - 20,000	~1,500 - 1,700	5 - 3 µM	YES	●●○ – ●●●**
LCT	~5,000	~1,500 - 1,700	5 - 2 µM	NO	●○○

* All ion fragmentation (AIF) can be performed in the HCD cell.
** The price depends on vendors and instrumental features.

Table 1. Instrumental features of analyzers used for native MS analysis of intact mAbs. The first column describes the inherent mass resolution of the mass analyzer, which is typically only reached when measuring mono-isotopic ions, such as CsI clusters, which thus can be best used for calibration purposes. For more biological relevant molecules such as the mAbs the experimentally observed resolving power is much lower and affected by the natural isotope envelope and incomplete desolvation and possibly suboptimal detection efficiency. We report here both the inherent instrumental mass resolution and the experimental resolving power as full-width at half maximum (FWHM) at 6000 m/z. Furthermore, optimal analyte concentrations are given. Concentrations higher than 25 µM should be avoided, whereas spectra have been demonstrated recorded with 20 nM of mAbs. Lower concentrations can be detected but at the cost of lower signal-to-noise ratios and robustness. Due to the presence of the quadrupole, the Q-TOF allows ion selection and, therefore, it has tandem-MS capabilities. The Exactive Plus Orbitrap instrument does not (yet) allow ion selection, but on the other hand all ions can be fragmented in the HCD cell (AIF).

As already mentioned, native MS has a broad range of applications[27], from the identification of a single mAb by accurate mass measurement to the qualitative and quantitative assessment of molecular heterogeneity. Recently, we showed how native MS can be also used for the analysis of complex mixtures of mAbs[31]. The protocol presented here can be used not only to identify and relatively quantify all mixture components, but is also useful for the analysis of bispecific antibodies, where, in early stages of the development, the bispecific species often contains residual monospecific species. Although not discussed here, we believe that this protocol can be further adapted for the analysis of other mAb-based therapeutics, such as such as Fc-fusion proteins and peptides[32-34] and tri-functional antibodies[35]. Other types of studies where the native conditions have been strictly required are: the analysis of dimer formation in engineered CH3 domains[36]; antibody aggregation[37]; and antibody antigen-binding[38].

As the aforementioned types of investigation do not require a very high resolving power, both TOF- and Orbitrap-based instruments can be used equally well.

On the other hand, when the objective of the study focuses on small modifications, such as a variety of glycosylations and/or sequence variance, or mixtures of mAbs having very small mass differences (less than 0.1% of the total mass), a higher resolving power becomes compulsory. Besides higher mass accuracy for antibody identification, the Orbitrap-based instrument facilitates the characterization of glycosylation profiles, and the identification of sequence truncations such as C-terminal lysine clipping, or other modifications such as the N-terminal glutamine cyclization[39]. In order to validate the identity of these various modifications, specific enzymes such as neuraminidase (sialidase), β1,4-galactosidase, glutaminyl–peptide cyclotransferase and carboxypeptidase B can be used[40,41]. Moreover, the enhanced resolving power allows the characterization of higher complexity mixtures of antibodies up to 15 species[42], and the qualitative and quantitative analysis of heterogeneous antibody-drug conjugates (ADCs)[39].

Advantages and limitations

We argue that native MS exhibits some clear advantages. The measurements can be done rapidly (minutes for one analysis) at high sensitivity (requiring only pmole of mAbs) and they require a minimum in sample preparation, which make it suitable for routine high-throughput analyses. However, there are evidently also some limitations. To name a few; smaller mass modifications such as caused by deamidation (+1 Da) cannot (yet) be detected. Moreover, apart from the C-terminal lysine clipping, N-terminal glutamine cyclization and glycosylation that represent well-known modifications, PTMs cannot be easily site-localized. For this type of investigations, other approaches, for instance bottom-up peptide mapping or top-down fragmentation, are still needed. Although accurate qualitative and quantitative mass spectrometric data can be gathered on the various glycan structures attached to the mAbs[39], tandem MS and NMR are still needed to convert these masses to correct glycan structures, including the appropriate linkages in between the sugar moieties.

Experimental Design

In the following we describe the main steps in the experimental workflow, giving, based on our experience, our point of view on how it can be adapted to specific sample characteristics and aims of the experiments (Figure 3). Moreover, we indicate clearly steps that allow a wider flexibility, giving the user the possibility to alter the protocol depending on equipment and software availability.

Deglycosylation. Sample characteristics and the goal of the experiment dictate

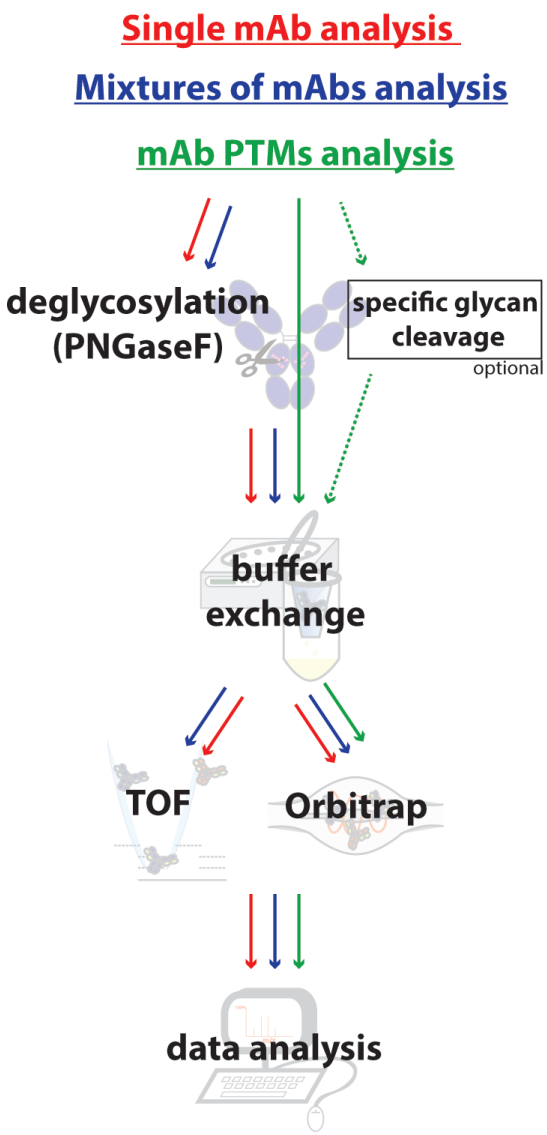

Figure 3. Typical workflows for native MS analysis of mAbs. Depending on sample characteristics and goal of the experiments, the workflow can be adapted. For single mAb identification (red arrows) or characterization of mAb mixtures (blue arrows), a deglycosylation step is preferable followed by buffer exchange and analysis with either TOF or Orbitrap based instruments. For mAb PTMs analysis (green arrows) the workflow starts directly with the buffer exchange, and the analysis benefits from the use of an Orbitrap based instrument. Additionally, extra experiments, where enzymes for the cleavage of specific carbohydrate residues are used instead of PNGaseF, can be performed to confirm and validate glycoform assignments (see also Figure 4).

whether it is beneficial to remove the glycans prior to the mass analysis on the intact mAbs. In general, whenever the antibody glycans are not the objective of the study, it is valuable to include this step in the workflow for a number of reasons. Firstly, after deglycosylation the mass spectra will become considerably simplified. Secondly, the collapse of all peaks arising from different glycosylation states into a single peak leads to an increase in signal-to-noise and thus sensitivity. Thirdly, whenever the resolution of the instrumentation does not allow baseline separation of the different glycoforms, the deglycosylation step can be seen as a stratagem to "sharpen" peaks, thus contributing to more accurate mass determinations. Also when dealing with antibody mixtures and/or antibody-drug conjugates, the deglycosylation step is also beneficial as it reduces the likelihood of overlapping ion signals in the mass spectra. However, mixtures of lower complexity can be analyzed in their native glycosylated state when using higher resolution instruments.

Evidently, when the goal of the investigation is the study of antibody glycosylation, samples are directly analyzed in their native glycosylated state. However, to further confirm the assignments of glycan structures, the analysis can be repeated treating the sample with deglycosylation enzymes that cleave specific glycan residues. For instance, the enzymes neuraminidase (sialidase) and β1,4-galactosidase can be used to assess the presence of sialic acids and free galactoses, respectively (Figure 4).

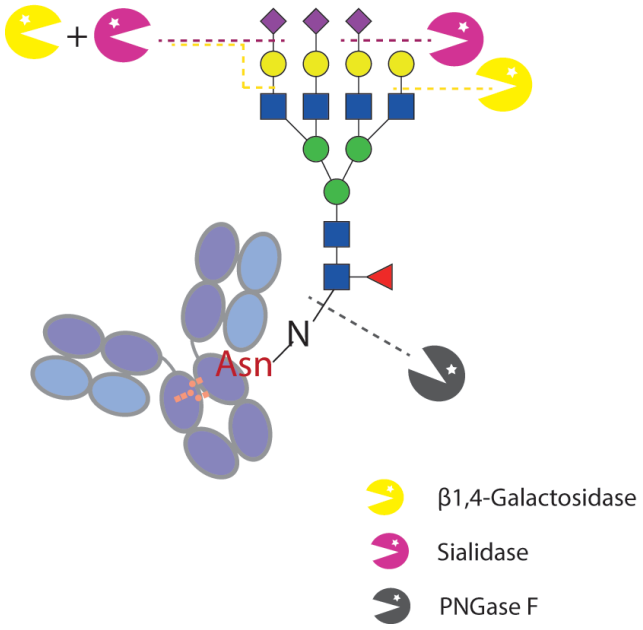

Figure 4. Specific glycan digestion under non-denaturing conditions on intact mAbs. For glycan analysis, after the analysis of the glycosylated mAbs, additional experiments can be performed using enzymes for the specific cleavage of glycan residues such as β1,4-galactosidase and/or neuraminidase (see also Figure 7).

Buffer exchange. This is an unavoidable important step for native MS. Although it has been shown that the required buffer exchange can be performed online using size-exclusion chromatography (SEC)[26], more commonly the analyte is directly infused into the mass spectrometer without any online chromatographic steps. Therefore, it is necessary to exchange the original buffer into an MS compatible one preferably shortly before the analysis, as samples may be less stable in the pseudo-buffers used in native MS. Such an aqueous ammonium acetate solution does not provide that much buffering capacity, but most proteins (and protein complexes) remain bioactive in such a solution, and in their native structural

conformation. There are various ways to exchange the buffer; most often centrifugal-filter concentrators are used because they allow a fast buffer exchange (1-2 hours), and they give the possibility to concentrate the sample. Alternatively, the sample can be dialyzed with the advantage of a higher recovery, at the cost of lower speed and, often, sample dilution. Regarding the molecular weight cut-off (MWCO) of the filter (or membrane), for intact mAbs any MWCO lower than 150 kDa can be used. However, it might be a good habit to use a much lower MWCO, such as 10 kDa, that allows you to assess both the purity of the sample, and whether mAb fragments are present, *e.g.* the free light chain or dissociation of the two halves of the antibody. We suggest using a buffer of 150 mM ammonium acetate at pH 7.5. Both the ionic strength and pH can, however, be varied without significantly affecting the analysis (ionic strength 50-200 mM; pH 6.5-8).

Native MS analysis. TOF and Orbitrap based mass spectrometers can be used to perform native MS (Figure 2). In particular for TOF instruments, both simple ESI-TOFs as well as more extensive hybrid Q-TOFs are suitable for the analysis of mAbs. However, it is worth bearing in mind that Q-TOF instruments used for native MS require some special instrument modifications, including a low frequency quadrupole that allows transmission of ions at high m/z's[43,44]. Such modifications have been implemented on dedicated instruments by the original vendor (*e.g.* Waters), moreover, a few dedicated companies (*e.g.* MSVision) offer such options now commercially on existing platforms. Likewise, also the Orbitrap instrument dedicated to native MS requires some minor adjustments. This new configuration has now (September 2013) been made commercially available. In our view nano-electrospray ionization (nESI) is essential for native MS, for efficient volatilization of aqueous buffers and for higher sensitivity[45,46]. Similarly for TOFs and Orbitraps, samples can be manually infused into the instrument using gold-coated capillaries (home-made but also commercially available); otherwise, auto samplers for direct infusion represent an attractive alternative especially for high throughput analysis[47,48].

Data analysis. The data can be processed in various ways, depending on the structural complexity of the samples, using a few available software programs. Unfortunately, the type of instrument dictates to a large extent which software may be used for data analysis. Both qualitative and quantitative data analysis can be performed either directly from the raw spectrum or by implementing a zero-charge deconvolution of the data using software programs such as Protein Deconvolution (Thermo) or MaxEnt (Waters). Various tools are available for the specific data analysis of mAbs, such as BPLX or BiopharmaLynx (Waters).

Materials

Reagents

- Ammonium acetate (Sigma-Aldrich, CAS no. 631-61-8)
- High–purity water obtained from a Milli-Q purification system (Millipore)
- Cesium iodide (Sigma-Aldrich, Cat. no. 203033)
- PNGaseF (Roche, REF. 11365193001)
- Neuraminidase (Sialidase) (Roche, REF. 10269611001)
- β1,4-Galactosidase (Millipore, Cat no. 345806-50MIU)

Reagent Setup

- Purified mAbs sample. Store it at 4 °C for a maximum of 1 year.
- 150 mM ammonium acetate buffer pH 7.5. Dilute the 7.5 M stock solution of ammonium acetate 50 times using high-purity water. Adjust pH with ammonia. Store the buffer at 4 °C for a maximum of one month.
- Cesium iodide (CsI), 25 mg/ml. Dissolve 1.25 g of cesium iodide in 50 ml of high-purity water. Store at 20 °C for a maximum of 1 year.
- PNGaseF. Dissolve the whole content into 250 µl of high-purity water. Store it at 4 °C for a maximum of 6 months.

Equipment

- Thermomixer (Eppendorf, Thermomixer comfort)
- Milli-Q purification system (Millipore)
- Amicon Ultra-0.5 ml Centrifugal Filters 10 kDa MWCO (Millipore, REF. UFC501096)
- Centrifuge (Eppendorf, centrifuge 5417R)
- Borosilicate glass capillaries (World Precision Instruments, Inc., Cat no. 1B120F-4)
- Sutter P-97 puller (Sutter Instrument Co.)
- Petri dish (Sterilin Ltd, Cat no: 101VR20)
- Edwards Scancoat six sputter-coater (Edwards Laboratories)
- Syringe (SGE analytical science, Cat no: 002108)
- Fused silica with 250 µm i.d. and 360 µm o.d. (TSP-FS-Tubing, TSP-250350)
- LCT mass spectrometer (Waters)
- Exactive Plus mass spectrometer (ThermoFisher Scientific)

Procedure

DEGLYCOSYLATION (optional)

1 | As discussed in the introduction, glycosylation of mAbs at the intact protein level can be studied by native MS. To perform glycoanalysis by native MS, the deglycosylation step using PNGaseF (described in option A), is initially skipped and thus, glycosylated antibodies are directly buffer exchanged, infused into the mass spectrometer and analyzed.

There are also are a number of enzymes available that are able to (specifically) cleave particular carbohydrate residues. Additional mass analysis can be performed using these enzymes, instead of PNGaseF, to further confirm glycan structural assignments.

A procedure using neuraminidase (sialidase) and β1,4-galactosidase on intact mAbs under native conditions is described in option B (see also Figure 4). While the first enzyme cleaves both α2,3 and α2,6 acylneuraminic acids (Neu5Ac), the latter enzyme cleaves only free β1,4-galactoses. Therefore, a mixture of the two enzymes is necessary to cleave galactoses substituted with a sialic acid (Neu5Ac).

A **PNGaseF reaction** ● **TIMING** overnight

i. Mix 4 units of PNGase F with 25 µg of purified antibody sample (conc. ~0.5 mg/ml).
ii. Incubate at 37 °C overnight.

? TROUBLE SHOOTING

Δ CRITICAL STEP Check the manufacturer's instructions of the enzyme product whether the buffer of your antibody is compatible with the reaction. Alternatively, exchange your sample buffer into a compatible buffer indicated in the instructions (see steps 3-5 for buffer exchange).

■ **PAUSE POINT** After deglycosylation, samples can be stored at 4 °C for weeks or even months, depending on the nature of the initial buffer.

B **Neuraminidase (sialidase) and β1,4-galactosidase reaction** ● **TIMING** overnight

i. Neuraminidase (sialidase). Mix 10 mU with 25 µg of purified antibody sample (conc. ~0.5 mg/ml).
ii. β1,4-galactosidase. Mix 5 mU with 25 µg of purified antibody sample (conc. ~0.5 mg/ml).

iii. Incubate the mixture at 37 °C overnight.

BUFFER EXCHANGE ● **TIMING** 1-2 h

2 | Rinse 10 kDa MWCO centrifugal filters: load 0.5 ml of ammonium acetate pH 7.5 and centrifuge for approximately 5 min at 10,000 g. Discard flow-through. For a cleaner procedure discard also the buffer left in the filter by using a gel loading pipet tip or differently if the centrifugal filter device allows an alternative method of recovery.

3 | Fill centrifugal filters with fresh ammonium acetate pH 7.5 and sample. Centrifuge at 4 °C for approximately 10 min at 10,000 g.

4 | Discard flow-through, refill with fresh ammonium acetate pH 7.5 and centrifuge again. Repeat this step until the concentration of the initial buffer reaches the nM range (typically, 5-6 rounds). At last round, concentrate to (at least) around 50 µl.

■ **PAUSE POINT** Buffer-exchanged samples can be stored at 4 °C typically for a few weeks to months.

CAPILLARY PREPARATION ● **TIMING** 30 min for 25 capillaries

5 | Follow instructions of the capillary puller to pull borosilicate capillaries. For optimal spray, capillaries employed for nESI end with a 10-1 µm diameter tip. (See ref[46,49] for further details).

■ **PAUSE POINT** Capillaries can be kept in a Petri-dish, fixed on a double-sided adhesive tape.

6 | Put the Petri-dish in the coating chamber and follow instructions of the coater.

Δ CRITICAL STEP Always handle capillaries with tweezers or gloves to keep them clean before coating, and to avoid removal of the gold after coating.

■ **PAUSE POINT** Coated capillaries can be kept in the Petri-dish for maximum 1 month.

SAMPLE LOADING ● **TIMING** 2-5 min

7 | If needed, dilute your sample down to a concentration between 5 and 1 µM (see Table 1 for optimal concentration) using ammonium acetate buffer pH 7.5.

8 | With the help of a syringe connected with a fused silica capillary (Figure 5a), load about 2 µl of sample into the capillary (Figure 5a).

Δ CRITICAL STEP Handle the capillary with care to avoid to damage the capillary tip.

9 | Place the capillary in the capillary holder.

(A) TOF

i. Slide from the back of the capillary a small conductive rubber between the two parts of the capillary holder to ensure conductivity (Figure 5b).
ii. Place the capillary onto the stage and position it at 90° compared to the direction of the cone.
iii. Open the capillary by touching the side of the cone or using the tweezers under a microscope (Figure 5c).
iv. Position the capillary in front of the cone (Figure 5d).

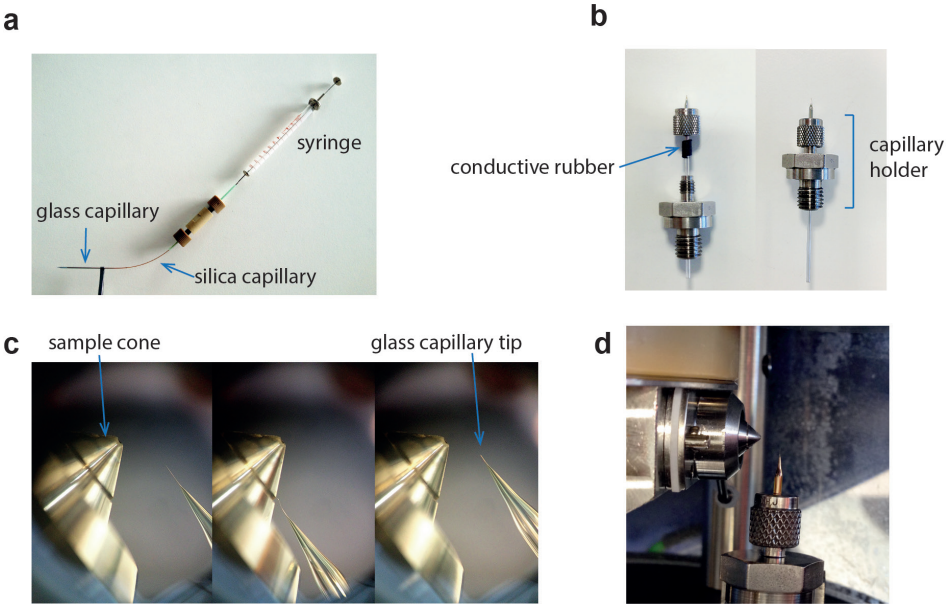

Figure 5. Pictures displaying in detail sample loading before injection. When performing native MS samples are typically injected into the mass spectrometer via direct infusion. This figure shows pictures of the main steps of the sample loading into an ESI-TOF instrument (LCT, Waters). (a) A couple of microliters of sample, containing mAbs at µM concentration, are loaded in the capillary using a syringe. (b) The capillary is inserted in the capillary holder. (c) The capillary is first placed on the capillary stage, then the tip is opened and finally it is positioned in front of the sample cone. (d) Different views of the capillary positioned in front of the sample cone.

(B) Orbitrap

i. Open the capillary using the tweezers under the microscope.
ii. Slide the capillary inside the capillary holder.
iii. Place the capillary onto the stage and position it at a couple of millimeters away from the inlet.

SPECTRUM ACQUISITION ● **TIMING** 1-5 min

10 | Apply capillary voltage and start acquisition by referring to the table below for instrument settings. The acquisition time can be adjusted depending on the S/N.

(A) TOF	(B) Orbitrap
Backing pressure 6.5- 7.5 mbar	Ultra high vacuum (UHV) ~ $5.0e^{-10}$
Capillary voltage 1.2-1.5 kV	Capillary voltage 1.3-1.4 kV
Cone voltage 150-200 V	Source fragmentation 200 V
Source temperature 80 °C	Extended trapping 10 V

(?) TROUBLE SHOOTING

CALIBRATION ● **TIMING** 10-30 min

11 | Perform calibration using 25 mg/ml CsI solution.

(A) TOF

i. Acquire a spectrum of CsI clusters making sure to cover the whole mass range that has been used during experiments (see steps 9-11).
ii. If you are using a Waters TOF instrument, calibration can be done either before or after sample measurements. Make a calibration file (.scl) using csiesi.ref as reference file and apply the calibration file to all acquired spectra.

(B) Orbitrap

i. Spray the 25mg/ml CsI solution without acquiring. Note that for Orbitrap instruments calibration needs to be performed before sample measurements.
ii. Once you have stable spray, start mass calibration (positive mode) making

sure you are using CsI as reference. A mass accuracy below 2 ppm would be preferable.

DATA PROCESSING ● **TIMING** 1-8 h per sample

Qualitative data analysis

12 | Calculate masses of all species present in the spectra. When protein sequence is known, mAb identification is done comparing the measured masses with expected masses.

Δ CRITICAL STEP When calculating expected masses from protein sequence, do not forget to take into account protein modifications (C-terminal lysine clipping, N-terminal glutamine cyclization, etc.) when these are known.

(A) TOF

i. Combine all scans acquired in your chromatogram or, alternatively, combine all scans subtracting those you prefer to discard (for example scans with very low S/N).
ii. Smooth and center peaks according to S/N and peak shape, respectively.
iii. Assign masses to all species present in the spectrum.

Δ CRITICAL STEP Make sure the assignment of the charge state envelope is correct. Find a compromise between using a small error window (as low as 0.5 Da is preferable) to have the least standard deviation, and assigning all most abundant charge states peaks, we recommend no fewer than 4.

(B) Orbitrap

i. For mass assignments, deconvolute your spectra using Protein Deconvolution using the “isotopically unresolved” experiment type.
ii. Combine all scans acquired in your chromatogram or, alternatively, combine a preferred region of the chromatogram.
iii. Define your parameters according to sample characteristics and start the deconvolution process.

Δ CRITICAL STEP When you have a mixture of antibodies or different proteoforms for a single antibody, the deconvoluted spectrum should closely resemble the spectrum of a single charge state of the raw spectrum. Make sure that all deconvoluted peaks are present in the raw spectrum and, on the contrary, that the deconvoluted spectrum is not missing high abundant peaks due too stringent parameters and or erroneous thresholds.

Quantitative data analysis

13 | When the mAb sample consists of a mixture of different antibodies and/or proteoforms, perform a relative quantitation of all different species.

(A) TOF or Orbitrap

i. Use Igor Pro (or any other program that allows peak fitting) to fit raw peaks and to calculate the area under the peaks. Your input into Igor Pro will be a peak list of the spectrum.
ii. Sum areas of all most abundant charge state peaks for each species.
iii. Manually calculate the relative abundances of all mAb species present in the mixture.

(B) Orbitrap

i. If you have already used Protein Deconvolution for qualitative analysis, the software automatically provides you with the relative abundance (based on peak intensities of all charge state peaks) of all species present in the deconvoluted spectrum.

TROUBLESHOOTING

Troubleshooting guidelines can be found in Table 2.

Table 2. Troubleshooting.

STEP	PROBLEM	POSSIBLE REASON	SOLUTION
1	Glycosylation is detected	Sample buffer is not compatible with enzymatic reaction	Check the instructions of the enzyme product and exchange your sample into a compatible buffer
11	No signal is detected	Capillary tip is closed	Open capillary tip
		Capillary tip diameter is too big (big droplets)	Change capillary
		Spray is not optimal	Apply some air pressure from the back of the capillary with a syringe
		Sample is too concentrated	Dilute sample
	Broad peaks; mass is higher than expected	Presence of salt adducts due to inefficient buffer exchange	Repeat the buffer exchange
	Low signal is detected	Poor ionization	Increase the capillary voltage

TIMING

Deglycosylation PNGaseF: overnight

Buffer exchange: 1-2 h

Capillary preparation: 30 min for 25 capillaries

Sample loading: 2-5 min

Spectrum acquisition: 1-5 min per sample

Calibration: 10-30 min

Data processing: 1-8 h per sample

Anticipated Results

To illustrate the applicability of the protocols, we describe two examples of mass analysis performed on mAbs following two, TOF and Orbitrap based, instrumental approaches presented in the protocol. First, we show data from a qualitative and quantitative analysis of a mAb mixture consisting of four different mAbs. Because of the particular goal of the experiment being the quantitative characteristics of the mixture, the sample was first fully deglycosylated using PNGaseF and the analysis was done using, as far as mass resolving power is concerned, a relatively low-end ESI-TOF instrument (LCT, Waters). Figure 6a shows a native mass spectrum of the aforementioned mixture. Reliable identification of the products in the mixture is ensured by accurate mass measurements, while relative quantitation, performed taking peak areas averaged over all charge states into account, revealed the presence of the four species in different amounts (Figure 6b).

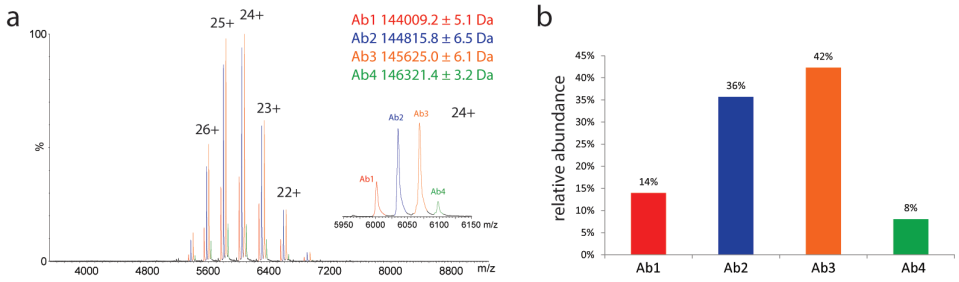

Figure 6. Qualitative and quantitative analysis of a mixture of 4 different mAbs. (a) The qualitative analysis is obtained by accurate mass measurements of each mixture component. The native ESI mass spectrum consists of six charge states appearing at around 6000 m/z. The inset shows the zoom-in of a single charge state (24+). Each charge state consists of four peaks arising from the four mAbs present in the mixture that may be distinguished by their mass. (b) The quantitative analysis is performed by calculating peaks areas, averaged over all detected charge states, using dedicated software such as Igor Pro, and directly reveals differences in the abundances of the four mAbs in the mixture.

The second example focuses on the glycosylation analysis at the intact protein level of an IgG1Y407E mutant, which exhibits a rather complex and extended glycosylation profile, and is mainly present as half antibody (*i.e.* one light chain-heavy chain pair, 75 kDa). Because of the close similarities in mass of the various glyco-

forms, an Orbitrap-based instrument (Exactive Plus, ThermoFisher Scientific) was used for this analysis. As, in this case, the glycans represented the objective of the study, no deglycosylation step was performed prior to the analysis (Figure 7a). However, to further validate glycan assignments, the analysis was repeated using specific deglycosylation enzymes used for the specific cleavage of particular carbohydrate residues from the native intact mAbs.

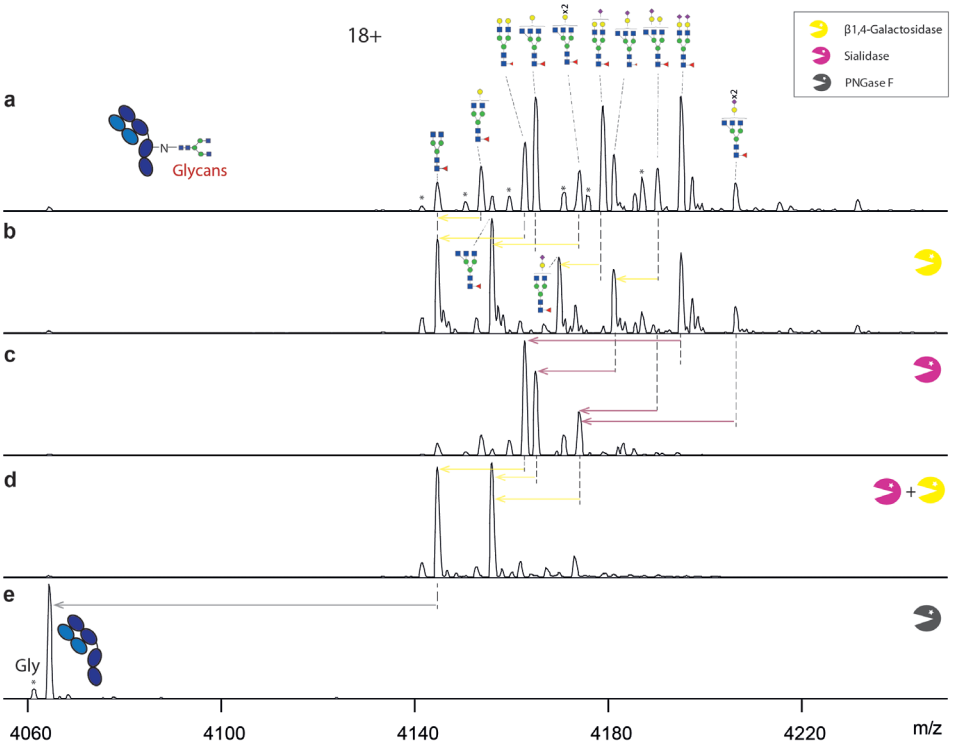

Figure 7. Detailed mass analysis of heterogeneous glycosylation occurring on an IgG1 Y407E mutant antibody. As an example of the detailed glycoanalysis possible an IgG1 Y407E mutant antibody was chosen as it exhibits a quite complex and heterogeneous glycosylation profile. From top to bottom, the panels show the decrease of the glycosylation profile complexity upon treatment with various enzymes that cleaves specific glycan residues. (a) native ESI mass spectra of the untreated IgG1 Y407E sample, and spectra obtained from the same sample, but then treated with (b) β1,4-galactosidase, (c) neuraminidase (sialidase), (d) β1,4-galactosidase and neuraminidase, (e) PNGaseF. *Antibody species bearing glycine truncation.

First, the sample was incubated with a β1,4-galactosidase enzyme to cleave galactose residues. The resulting mass spectra were slightly simplified, as only peaks corresponding to glycoforms having free galactoses disappeared from the initial spectrum obtained for the unprocessed IgG1Y407E mutant, while glycans bear-

ing galactoses substituted with a sialic acid were still present in the spectrum suggesting that the enzyme is only active on free galactoses (Figure 7b). Next, a second experiment was performed prior to mass analysis using a neuraminidase (sialidase) enzyme to cleave all α2,3 and α2,6 sialic acids. As a result, peaks corresponding to sialylated glycoforms disappeared from the mass spectra, whereas concomitantly an increase of some ion signals corresponding to non-sialylated species is detected (Figure 7c). An additional experiment was performed where the sample has been incubated with both enzymes simultaneously. This resulted in a largely simplified spectrum exhibiting only two main glycoforms (Figure 7d). Finally, the IgG1Y407E mutant sample was treated with PNGaseF resulting in the removal of the entire glycan from the protein backbone. The resulting mass spectrum shows a single peak corresponding to the mass of the deglycosylated mAb (Figure 7e).

Acknowledgements

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References

1 Reichert, J. M. Marketed therapeutic antibodies compendium. MAbs 4, 413-415 (2012).

2 Harris, L. J., Skaletsky, E. & McPherson, A. Crystallographic structure of an intact IgG1 monoclonal antibody. J Mol Biol 275, 861-872 (1998).

3 Saphire, E. O. et al. Crystal structure of a neutralizing human IGG against HIV-1: a template for vaccine design. Science 293, 1155-1159 (2001).

4 Liu, H., Gaza-Bulseco, G., Faldu, D., Chumsae, C. & Sun, J. Heterogeneity of monoclonal antibodies. J Pharm Sci 97, 2426-2447 (2008).

5 Jefferis, R. Glycosylation of recombinant antibody therapeutics. Biotechnol Prog 21, 11-16 (2005).

6 Beck, A. et al. Trends in glycosylation, glycoanalysis and glycoengineering of therapeutic antibodies and Fc-fusion proteins. Curr Pharm Biotechnol 9, 482-501 (2008).

7 Huhn, C., Selman, M. H., Ruhaak, L. R., Deelder, A. M. & Wuhrer, M. IgG glycosylation analysis. Proteomics 9, 882-913 (2009).

8 Flynn, G. C., Chen, X., Liu, Y. D., Shah, B. & Zhang, Z. Naturally occurring

glycan forms of human immunoglobulins G1 and G2. Mol Immunol 47, 2074-2082 (2010).

9 Brady, L. J., Martinez, T. & Balland, A. Characterization of nonenzymatic glycation on a monoclonal antibody. Anal Chem 79, 9403-9413 (2007).

10 Diepold, K. et al. Simultaneous assessment of Asp isomerization and Asn deamidation in recombinant antibodies by LC-MS following incubation at elevated temperatures. PLoS One 7, e30295 (2012).

11 Liu, H. & May, K. Disulfide bond structures of IgG molecules: structural variations, chemical modifications and possible impacts to stability and biological function. MAbs 4, 17-23 (2012).

12 Li, X. et al. Disulfide bond assignment of an IgG1 monoclonal antibody by LC-MS with post-column partial reduction. Anal Biochem 436, 93-100 (2013).

13 Pace, A. L., Wong, R. L., Zhang, Y. T., Kao, Y. H. & Wang, Y. J. Asparagine deamidation dependence on buffer type, pH, and temperature. J Pharm Sci 102, 1712-1723 (2013).

14 Logtenberg, T. Antibody cocktails: next-generation biopharmaceuticals with improved potency. Trends Biotechnol 25, 390-394 (2007).

15 Robak, T. The emerging therapeutic role of antibody mixtures. Expert Opin Biol Ther 13, 953-958 (2013).

16 Pro, B. & Perini, G. F. Brentuximab vedotin in Hodgkin's lymphoma. Expert Opin Biol Ther 12, 1415-1421 (2012).

17 Sievers, E. L. & Senter, P. D. Antibody-drug conjugates in cancer therapy. Annu Rev Med 64, 15-29 (2013).

18 Beck, A., Sanglier-Cianferani, S. & Van Dorsselaer, A. Biosimilar, biobetter, and next generation antibody characterization by mass spectrometry. Anal Chem 84, 4637-4646 (2012).

19 Beck, A., Wagner-Rousset, E., Ayoub, D., Van Dorsselaer, A. & Sanglier-Cianferani, S. Characterization of therapeutic antibodies and related products. Anal Chem 85, 715-736 (2013).

20 Gahoual, R. et al. Rapid and multi-level characterization of trastuzumab using sheathless capillary electrophoresis-tandem mass spectrometry. MAbs 5 (2013).

21 Kang, X., Kutzko, J. P., Hayes, M. L. & Frey, D. D. Monoclonal antibody heterogeneity analysis and deamidation monitoring with high-performance cation-exchange chromatofocusing using simple, two component buffer systems. J Chromatogr A 1283, 89-97 (2013).

22 Zhang, Z., Pan, H. & Chen, X. Mass spectrometry for structural characterization of therapeutic antibodies. Mass Spectrom Rev 28, 147-176 (2009).

23 Sharon, M. & Robinson, C. V. The role of mass spectrometry in structure elucidation of dynamic protein complexes. Annu Rev Biochem 76, 167-193 (2007).

24 Heck, A. J. Native mass spectrometry: a bridge between interactomics and structural biology. Nat Methods 5, 927-933 (2008).

25 Konijnenberg, A., Butterer, A. & Sobott, F. Native ion mobility-mass spec-

trometry and related methods in structural biology. Biochim Biophys Acta 1834, 1239-1256 (2013).

26 Valliere-Douglass, J. F., McFee, W. A. & Salas-Solano, O. Native intact mass determination of antibodies conjugated with monomethyl Auristatin E and F at interchain cysteine residues. Anal Chem 84, 2843-2849 (2012).

27 Thompson, N. J., Rosati, S., Rose, R. J. & Heck, A. J. The impact of mass spectrometry on the study of intact antibodies: from post-translational modifications to structural analysis. Chem Commun (Camb) 49, 538-548 (2013).

28 Zhang, H., Cui, W. & Gross, M. L. Mass spectrometry for the biophysical characterization of therapeutic monoclonal antibodies. FEBS Lett (2013).

29 Rosati, S. et al. Exploring an orbitrap analyzer for the characterization of intact antibodies by native mass spectrometry. Angew Chem Int Ed Engl 51, 12992-12996 (2012).

30 Rose, R. J., Damoc, E., Denisov, E., Makarov, A. & Heck, A. J. High-sensitivity Orbitrap mass analysis of intact macromolecular assemblies. Nat Methods 9, 1084-1086 (2012).

31 Rosati, S. et al. Qualitative and semiquantitative analysis of composite mixtures of antibodies by native mass spectrometry. Anal Chem 84, 7227-7232 (2012).

32 Beck, A. & Reichert, J. M. Therapeutic Fc-fusion proteins and peptides as successful alternatives to antibodies. MAbs 3, 415-416 (2011).

33 Lynaugh, H., Li, H. & Gong, B. Rapid Fc glycosylation analysis of Fc fusions with IdeS and liquid chromatography mass spectrometry. MAbs 5, 641-645 (2013).

34 Peters, R. T. et al. Biochemical and functional characterization of a recombinant monomeric factor VIII-Fc fusion protein. J Thromb Haemost 11, 132-141 (2013).

35 Chelius, D. et al. Structural and functional characterization of the trifunctional antibody catumaxomab. MAbs 2, 309-319 (2010).

36 Rose, R. J. et al. Quantitative analysis of the interaction strength and dynamics of human IgG4 half molecules by native mass spectrometry. Structure 19, 1274-1282 (2011).

37 Kukrer, B. et al. Mass spectrometric analysis of intact human monoclonal antibody aggregates fractionated by size-exclusion chromatography. Pharm Res 27, 2197-2204 (2010).

38 Atmanene, C. et al. Extending mass spectrometry contribution to therapeutic monoclonal antibody lead optimization: characterization of immune complexes using noncovalent ESI-MS. Anal Chem 81, 6364-6373 (2009).

39 Rosati, S. et al. In-depth qualitative and quantitative analysis of composite glycosylation profiles and other micro-heterogeneity on intact monoclonal antibodies by high-resolution native mass spectrometry using a modified Orbitrap. MAbs 5 (2013).

40 Shi, Y., Li, Z., Qiao, Y. & Lin, J. Development and validation of a rapid capillary zone electrophoresis method for determining charge variants of mAb. J Chro-

matogr B Analyt Technol Biomed Life Sci 906, 63-68 (2012).

41 Xu, W. et al. Method to convert N-terminal glutamine to pyroglutamate for characterization of recombinant monoclonal antibodies. Anal Biochem 436, 10-12 (2013).

42 Thompson, N. J., Hendriks, L. J. A., de Kruif, J., Throsby, M. & Heck, A. J. R. Complex Mixtures of Antibodies Generated from a Single Production Qualitatively and Quantitatively Evaluated by Native Orbitrap Mass Spectrometry. MAbs 6 (2014).

43 van den Heuvel, R. H. et al. Improving the performance of a quadrupole time-of-flight instrument for macromolecular mass spectrometry. Anal Chem 78, 7473-7483 (2006).

44 Sobott, F., Hernandez, H., McCammon, M. G., Tito, M. A. & Robinson, C. V. A tandem mass spectrometer for improved transmission and analysis of large macromolecular assemblies. Anal Chem 74, 1402-1407 (2002).

45 Fenn, J. B., Mann, M., Meng, C. K., Wong, S. F. & Whitehouse, C. M. Electrospray ionization for mass spectrometry of large biomolecules. Science 246, 64-71 (1989).

46 Wilm, M. & Mann, M. Analytical properties of the nanoelectrospray ion source. Anal Chem 68, 1-8 (1996).

47 Almeida, R. et al. Coupling of fully automated chip-based electrospray ionization to high-capacity ion trap mass spectrometer for ganglioside analysis. Anal Biochem 378, 43-52 (2008).

48 Zhang, S., Van Pelt, C. K. & Henion, J. D. Automated chip-based nanoelectrospray-mass spectrometry for rapid identification of proteins separated by two-dimensional gel electrophoresis. Electrophoresis 24, 3620-3632 (2003).

49 Hernandez, H. & Robinson, C. V. Determining the stoichiometry and interactions of macromolecular assemblies from mass spectrometry. Nat Protoc 2, 715-726 (2007).

Chapter VII

Summary And Future Outlook

Nederlandse Samenvatting

Supplementary Information

Summary And Future Outlook

This thesis highlights some of the data I gathered in the last 4 years during my period as PhD candidate. However, the PhD journey is not merely about scientific results, it comes concomitantly with a personal and professional development made up by achievements which don't appear as nice looking spectra, from one day to another, but can only be appreciated when viewed with retrospect in a longer time frame. Unfortunately, these achievements that have deepened my personal life, cannot be put easily in words in the thesis, still this thesis couldn't have been written without them.

Summarizing the work of four years in a couple of pages is a daunting task. It gets even more difficult when it is the biased PhD student him- or herself that is asked to perform this, as he/she is proud of the work and eager to minutely describe every single experiment. Squeezing all the work in a few lines where there is no room for details is just not fair. Still, by the end of their studies, PhD students are well trained in facing serious challenges, so here comes my summary!

The first chapter of my thesis introduces the reader to the two main subjects discussed in this thesis: native mass spectrometry and therapeutic monoclonal antibodies. After a brief discussion on the general features and applications of native mass spectrometry, the first section of this chapter takes the reader into the details of the instrumentation used in native mass spectrometry with particular focus of the type of mass analyzers used for the realization of this work. In the second part, the discussion is focused on therapeutic monoclonal antibodies. Here a taste of the pharmacological properties and therapeutic relevance of this relatively new class of therapeutics is provided.

Subsequently, in chapter II the two main subjects of the thesis, discussed separately in the introduction, are brought together. Here the discussion digs into the numerous applications of mass spectrometry for the analysis of monoclonal antibodies. The main challenges encountered during the antibody development process often originate from protein heterogeneity and from new engineered products that put a strain on the analysis, often requiring new and more advanced analytical techniques for their characterization. Different mass spectrometric approaches can be used to address these issues. This chapter covers the approaches that are most relevant for the characterization of therapeutic monoclonal antibodies.

The following chapters highlight some of the innovative research applications performed during my PhD studies. In chapter III native mass spectrometry was used

for the qualitative and semi-quantitative analysis of composite mixtures of antibodies representing biopharmaceutical products co-expressed from single cells. By using automated peak fitting of the ion signals in the native mass spectra, the relative abundance of each of the antibodies present in complex mixtures could be quantified with an average accuracy of 3%, comparable to a more conventional cation exchange chromatography based approach performed in parallel. Moreover, using native mass spectrometry nine antibodies present in a complex mixture of ten antibodies could be qualitatively and quantitatively characterized whereas this complexity could not be unraveled by cation exchange chromatography. This chapter shows how native mass spectrometry presents a valuable alternative to existing analytical methods for qualitative and quantitative profiling of biopharmaceutical products. It provides both the identity of each species in a mixture by mass determination and the relative abundance through comparison of relative ion signal intensities.

An innovative instrumental improvement that characterized the rest of my thesis is described in chapter IV. With this work, for the first time, a modified Orbitrap Exactive Plus™ instrument is used for the analysis of monoclonal antibodies under native conditions. The improved experimental resolution at high m/z achievable using the Orbitrap analyzer is very advantageous for studying such naturally heterogeneous proteins as antibodies. Baseline separation of different species with small mass differences allows confident qualitative and potentially even quantitative characterization of mixtures of antibodies, different glycosylation states of a single monoclonal antibodies, non-covalent interactions and antibody-antigen binding.

Building further on these early successes gathered by high-resolution native MS, I focused my interest on antibody post-translational modifications (PTMs). Chapter V describes a fast, easy-to-use and sensitive method to profile in-depth structural micro-heterogeneity, including intricate N-glycosylation profiles, of monoclonal antibodies at the native intact protein level by means of mass spectrometry using a modified Orbitrap mass spectrometer. This chapter highlights the versatility of our method to probe structural micro-heterogeneity by describing the analysis of three types of molecules: a) a non-covalently bound IgG4 hinge deleted full-antibody in equilibrium with its half-antibody, b) IgG4 mutants exhibiting highly complex glycosylation profiles and c) antibody-drug conjugates. Using the modified instrument, baseline separation and accurate mass determination of all different proteoforms are obtained. It shows that our method can handle highly complex glycosylation profiles, identifying more than 20 different glycoforms per mono-

clonal antibody preparation, and more than 30 proteoforms on a single highly purified antibody. In analysing antibody-drug conjugates, our method also easily identifies and quantifies more than 15 structurally different proteoforms that may result from the collective differences in drug loading and glycosylation.

I finally conclude my PhD research with a protocol that describes procedures optimized to perform the analysis of monoclonal antibodies (mAbs) at the intact protein level under pseudo-native conditions, using native MS. Some of the strengths of native MS in the analysis of biopharmaceuticals are the analysis speed, sensitivity and specificity: for most experiments the whole protocol requires one working day, whereby tens of samples can be analyzed in a multiplexed manner, making it suitable for high throughput analysis. The method can be used for different applications such as the analysis of mixtures of mAbs, drug-antibody conjugates and the analysis of mAb post-translational modifications, including the qualitative and quantitative analysis of mAbs glycosylation.

Seen the current trend of strategies adopted in development of mAbs formats, a prosperous future for this class of therapeutic proteins can be foreseen. It is to be expected that new engineering strategies will be adopted in the attempt to further optimize both pharmacodynamic and pharmacokinetic properties. Many companies are now focusing their strengths on the development of antibody-drug conjugates (ADC) products characterized by more homogeneous profiles and greater stability. Hopefully, in the near future a number of these products will be ready for clinical use. Moreover, with the expiration of the first patents, biosimilars mAbs will swiftly enter the clinic as they benefit of facilitated approval procedures compared to new mAbs products.

As mAb platform development is rapidly evolving, analytical techniques need to keep pace with this evolution in order to be able to face the new challenges raised by these new products. With regard to mass spectrometry, significant progression has been made during the last years, as also described in this thesis. Along with native MS, the method of choice in this thesis, a variety of different mass spectrometric approaches have been used to this end. Approaches where separation techniques compatible with native conditions are coupled to mass spectrometry, such as capillary electrophoresis (CE) or size-exclusion chromatography (SEC), are very promising[1-3]. Recently, also top-down tandem mass spectrometry on intact proteins and larger peptides is gaining popularity. By using different fragmentation techniques such as collision-induced dissociation (CID), electron-transfer induced dissociation (ETD) or electron-capture induced dissociation (ECD) top-down approaches swiftly combine the advantage of avoiding digestion steps with

detailed sequence analysis[4-7]. Finally, in the last decade also hydrogen-deuterium exchange mass spectrometry (HDX-MS) underwent a number of significant technical improvements, *e.g.* the implementation of ETD and ECD in the fragmentation of the exchanged peptides ensures reduced deuterium migration, potentially increasing the resolution from the peptide level to the single amino acid level [8,9].

References

1 Valliere-Douglass, J. F., McFee, W. A. & Salas-Solano, O. Native intact mass determination of antibodies conjugated with monomethyl Auristatin E and F at interchain cysteine residues. Anal Chem 84, 2843-2849 (2012).

2 Gahoual, R., Busnel, J. M., Wolff, P., Francois, Y. N. & Leize-Wagner, E. Novel sheathless CE-MS interface as an original and powerful infusion platform for nanoESI study: from intact proteins to high molecular mass noncovalent complexes. Anal Bioanal Chem 406, 1029-1038 (2014).

3 Wagner-Rousset, E. et al. Antibody-drug conjugate model fast characterization by LC-MS following IdeS proteolytic digestion. MAbs 6, 173-184 (2014).

4 Bondarenko, P. V., Second, T. P., Zabrouskov, V., Makarov, A. A. & Zhang, Z. Mass measurement and top-down HPLC/MS analysis of intact monoclonal antibodies on a hybrid linear quadrupole ion trap-Orbitrap mass spectrometer. J Am Soc Mass Spectrom 20, 1415-1424 (2009).

5 Fornelli, L. et al. Top-down analysis of monoclonal antibody IgG1 by electron transfer dissociation Orbitrap FTMS. Mol Cell Proteomics (2012).

6 Mao, Y., Valeja, S. G., Rouse, J. C., Hendrickson, C. L. & Marshall, A. G. Top-down structural analysis of an intact monoclonal antibody by electron capture dissociation-Fourier transform ion cyclotron resonance-mass spectrometry. Anal Chem 85, 4239-4246 (2013).

7 Fornelli, L., Ayoub, D., Aizikov, K., Beck, A. & Tsybin, Y. O. Middle-down analysis of monoclonal antibodies with electron transfer dissociation orbitrap fourier transform mass spectrometry. Anal Chem 86, 3005-3012 (2014).

8 Rand, K. D., Zehl, M., Jensen, O. N. & Jorgensen, T. J. Protein hydrogen exchange measured at single-residue resolution by electron transfer dissociation mass spectrometry. Anal Chem 81, 5577-5584 (2009).

9 Huang, R. Y., Garai, K., Frieden, C. & Gross, M. L. Hydrogen/deuterium exchange and electron-transfer dissociation mass spectrometry determine the interface and dynamics of apolipoprotein E oligomerization. Biochemistry 50, 9273-9282 (2011).

Nederlandse Samenvatting

Het eerste hoofdstuk van mijn proefschrift introduceert de lezer in de twee belangrijkste onderwerpen die besproken zullen worden: natieve massa spectrometrie en therapeutische monoclonale antilichamen. Na een korte discussie van de algemene eigenschappen en toepassingen van natieve massa spectrometrie gaat het eerste gedeelte van dit hoofdstuk gedetailleerd in op de instrumentatie die wordt gebruikt waarbij de aandacht vooral ligt bij de verschillende massa analysatoren die tijdens dit werk zijn gebruikt. In het tweede gedeelte ligt de aandacht vooral bij de therapeutische monoclonale antilichamen waarbij een voorproefje wordt gegeven van de farmacologische eigenschappen en therapeutische relevantie van deze relatief nieuwe klasse medicijnen.

De twee onderwerpen die apart zijn besproken in de introductie zullen in hoofdstuk II worden gecombineerd. Hier worden de verschillende toepassingen van massa spectrometrie voor de analyse van monoclonale antilichamen bediscussieerd. De belangrijkste uitdagingen in het ontwikkelen van de antilichamen liggen voornamelijk in de heterogeniteit van de eiwitten en de nieuw ontworpen producten, waar nog geen standaard analyse voor bestaat. Daardoor zijn er voor deze antilichamen vaak relatief nieuwe en geavanceerde technieken nodig. Dit hoofdstuk gaat in op de meest relevante methoden voor de karakterisatie van therapeutische monoclonale antilichamen.

De rest van het proefschrift bevat verschillende innovatieve onderzoeken die gedaan zijn tijdens mijn promotie. In hoofdstuk III wordt natieve massa spectrometrie gebruikt voor de kwalitatieve en semi-kwantitatieve analyse van oplossingen met verschillende antilichamen die representatief zijn voor de biofarmaceutische producten en die tot co-expressie komen tijdens de antilichaam productie in cellen. Door gebruik te maken van geautomatiseerde piek analyse in de massa spectra worden de verhouding van de verschillende antilichamen in de complexe mengsels gemeten met een gemiddelde nauwkeurigheid van 3%, vergelijkbaar met de conventionele kation uitwisselingschromatografie die in parallel werd uitgevoerd. Sterker nog, met behulp van natieve massa spectrometrie konden negen verschillende antilichamen in een mengsel van tien antilichamen op deze manier worden geanalyseerd terwijl de complexiteit van het monster dit niet toeliet bij kation uitwisselingschromatografie. Dit hoofdstuk laat zijn hoe natieve massa spectrometrie een waardevol alternatief is voor al bestaande technieken voor de analyse van kwalitatieve en kwantitatieve aspecten van biofarmaceutische producten. Het levert niet alleen de identiteit van alle componenten in een oplossing via de bepaling van hun massa maar ook hun verhouding door

vergelijking van de relatieve signaal intensiteiten.

Een innovatieve instrumentele verbetering die een belangrijke rol speelt in de rest van mijn proefschrift wordt beschreven in hoofdstuk IV. Daar wordt voor het eerst een gemodificeerd Orbitrap Exactive Plus™ instrument gebruikt voor de analyse van monoclonale antilichamen onder natieve condities. De verbeterde resolutie bij hoge m/z die dit instrument mogelijk maakt geeft een belangrijk voordeel bij het bestuderen van natuurlijke heterogene eiwitten zoals antilichamen. Het onderscheiden van componenten met zeer kleine verschillen in massa tot op de basislijn vergroot het gemak waarop oplossingen met verschillende antilichamen kunnen worden onderzocht, kwalitatief en kwantitatief. Daarnaast verbetert het ook de analyse van monoclonale antilichaam glycosylering, non-covalente interacties en antilichaam-antigen binding.

Op deze successen behaald met hoge-resolutie natieve MS wordt voortgebouwd in hoofdstuk V, waar de nadruk ligt op de analyse van post-translationele modificaties (PTMs) van antilichamen. In dit hoofdstuk wordt een snelle, gebruiksvriendelijke en gevoelige methode beschreven die tot in details de structurele microheterogeniteit, waaronder de uitgebreide N-glycosylerings profielen, van monoclonale antilichamen op het natieve intacte eiwit niveau kan analyseren met behulp van een gemodificeerde Orbitrap massa spectrometer. De toepasbaarheid van de methode wordt belicht met de analyse van drie verschillende typen moleculen: a) niet covalent gebonden IgG4 zonder IgG scharnier die in evenwicht is met het halve antilichaam, b) IgG4 mutanten met zeer complexe glycosylerings profielen en c) antilichaam-drug conjugaten. Door gebruik te maken van het gemodificeerde instrument worden de verschillende eiwit vormen gescheiden tot de basislijn en hun massa bepaald. Dit laat zien dat de beschreven methode zeer complexe glycosylerings profielen aan kan, en resulteert in identificatie van 20 verschillend geglycosyleerde eiwitten per monoclonale antilichaam bereiding en meer dan 30 verschillende eiwit varianten van een enkel zuiver antilichaam. Bij de analyse van de antilichaam-drug conjugaten was onze methode ook met gemak in staat om 15 verschillende vormen resulterende van verschillen in drug lading en glycosylering te identificeren en kwantificeren.

Ik beëindig mijn promotieonderzoek met een protocol die geoptimaliseerde stappen beschrijft voor de analyse van monoclonale antilichamen (mAbs) op het intact eiwit niveau onder pseudo-natieve condities met behulp van natieve MS. Enkele voordelen van natieve MS in de analyse van biofarmaceutische moleculen zijn de analyse snelheid, gevoeligheid en specificiteit: voor de meest experimenten is maar een werkdag nodig, waarbij tientallen monsters in parallel kunnen

worden geanalyseerd wat de techniek toegankelijk maakt voor zeer grote hoeveelheden monsters. De methode kan worden gebruikt voor verschillende applicaties waaronder de analyse van mAb mengsels, antilichaam-drug conjugaten en mAb post-translationele modificaties en glycosylering op kwalitatief en kwantitatief niveau.

Supplementary Information

Chapter III

Qualitative and Semiquantitative Analysis of Composite Mixtures of Antibodies By Native Mass Spectrometry

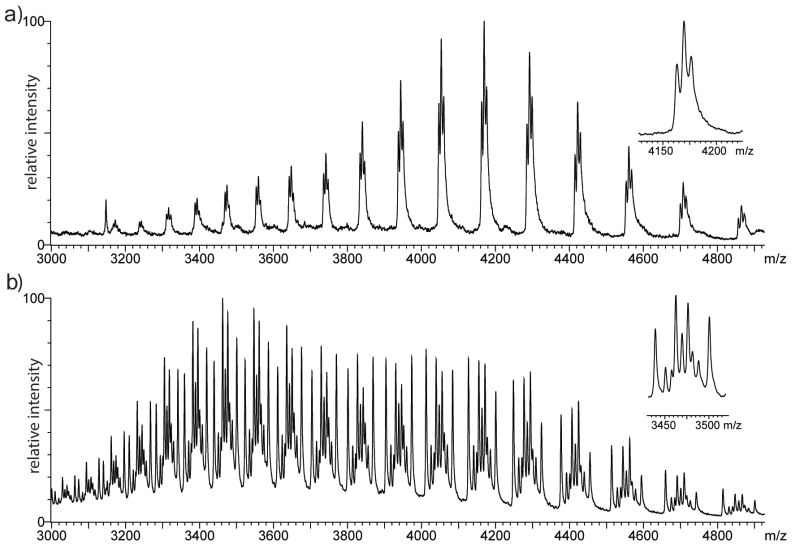


Figure S-1. Mixtures of monoclonal antibodies analyzed under denatured conditions. (a) Denatured ESI-MS spectrum of a binary mixture of monoclonal antibodies. Inset: zoom-in of a single charge state peak. (b) Denatured ESI-MS spectrum of composite mixture of ten monoclonal antibodies. Inset: zoom-in of a single charge state peak.

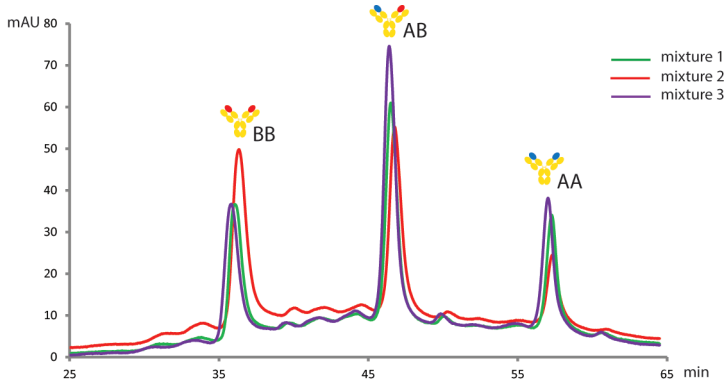


Figure S-2. CEX chromatograms of the three binary mixtures of monoclonal antibodies expressed in PER.C6® stable clones.

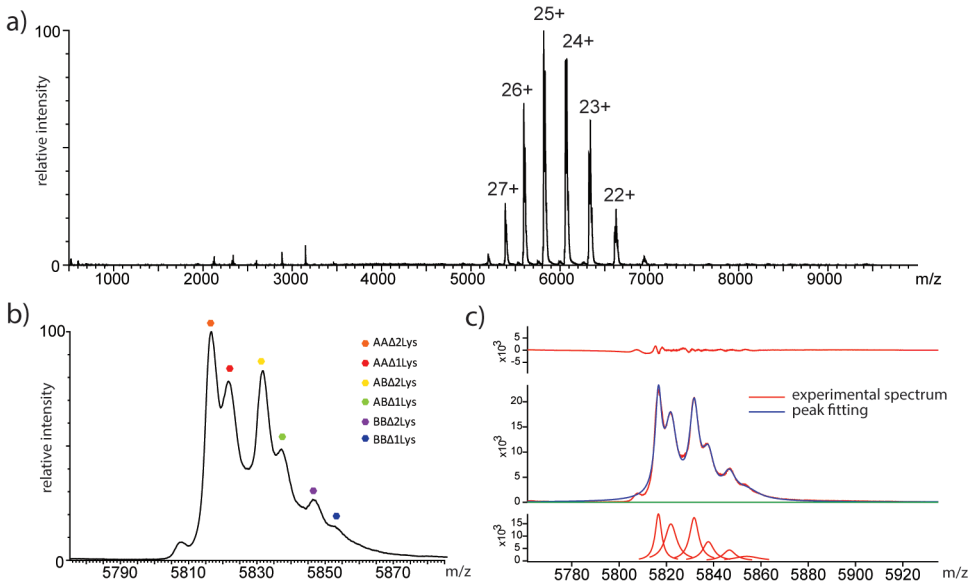


Figure S-3. Binary mixture of monoclonal antibodies expressed in CHO-K1 cells. (a) Full native ESI-MS spectrum. (b) Zoom-in of the 25+ charge state peaks. (c) Igor Pro based peak fitting of the 25+ charge state peaks: (top) residuals; (middle) composite peak fitting; (bottom) individual fitted peaks.

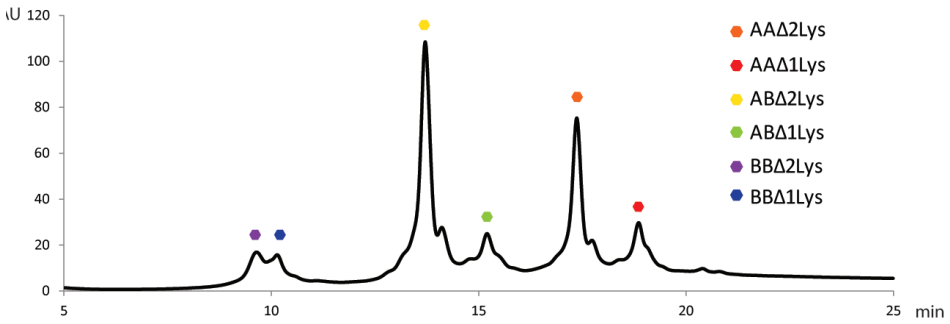
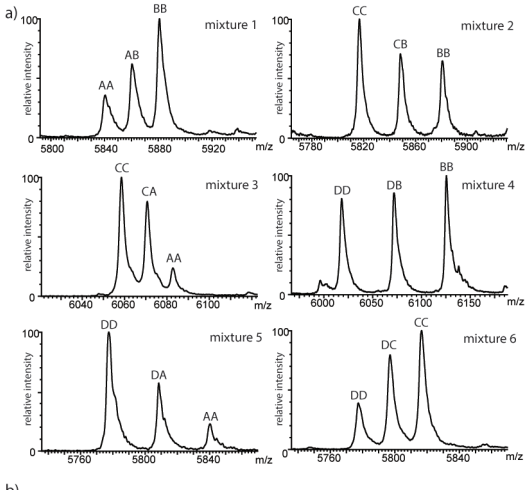


Figure S-4. CEX chromatogram of a binary mixture of monoclonal antibodies expressed in CHO-K1 cells. Peaks assignment was done according to species abundance as determined by native MS and by theoretical pI.

ID	Mass (Da)	Relative abundance	
		nMS	CEX
AAΔ2Lys	145394.8 ± 1.8	17%	23%
AAΔ1Lys	145519.4 ± 3.1	28%	12%
AA	n.d.	n.d.	n.d.
ABΔ2Lys	145769.8 ±2.5	24%	39%
ABΔ1Lys	145899.7 ±3.9	15%	12%
AB	n.d.	n.d.	n.d.
BBΔ2Lys	146138.6 ± 8.6	9%	7%
BBΔ1Lys	146283.8 ± 12.1	7%	7%
BB	n.d.	n.d.	n.d.

Table S-1. Mass determination and semi-quantitation of the components present in the binary mixture of antibodies expressed in CHO-K1 cells. Antibody species retaining both C-terminal lysines were not detected by both native MS and CEX.



b)

Mixture	Construct		Relative abundance (%)		
1	A	B	AA	AB	BB
			11	30	59
2	C	B	CC	CB	BB
			41	30	29
3	C	A	CC	CA	AA
			43	40	17
4	D	B	DD	DB	BB
			37	30	33
5	D	A	DD	DA	AA
			61	27	12
6	D	C	DD	DC	CC
			22	36	41

Figure S-5. Mixtures of monoclonal antibodies used to construct a composite mixture of ten antibodies. (a) Native ESI-MS spectra zoomed-in on the 25+ charge state. (b) Relative quantitation of the components for each mixture.

ID	Mass (Da)	Predict. %	Expt. %
DD	144582	20	21
DC	145054	6	8
DA	145363	5	2
CC	145527	21	24
DB*	145836	12	13
CA*	145856		
AA	146145	7	4
CB	146329	5	7
AB	146638	5	4
BB	147131	20	18

* overlapping components.

Table S-2. Mass determination and relative abundances (predicted vs experimental) of ten antibodies species in a composite mixture.

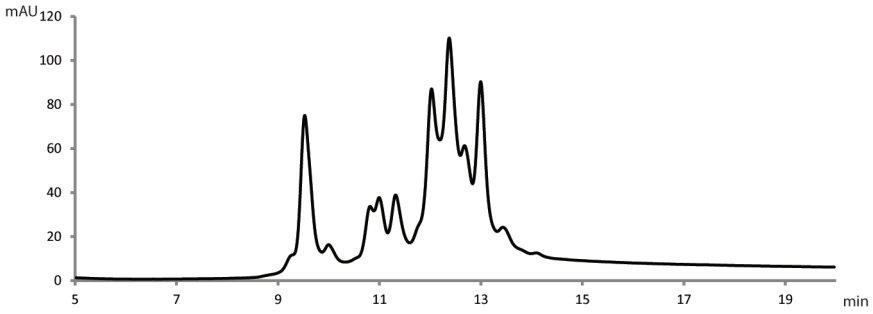


Figure S-6. CEX chromatogram of the composite mixture of ten monoclonal antibodies.

Exploring an Orbitrap Analyzer for the Characterization of Intact Antibodies by Native Mass Spectrometry

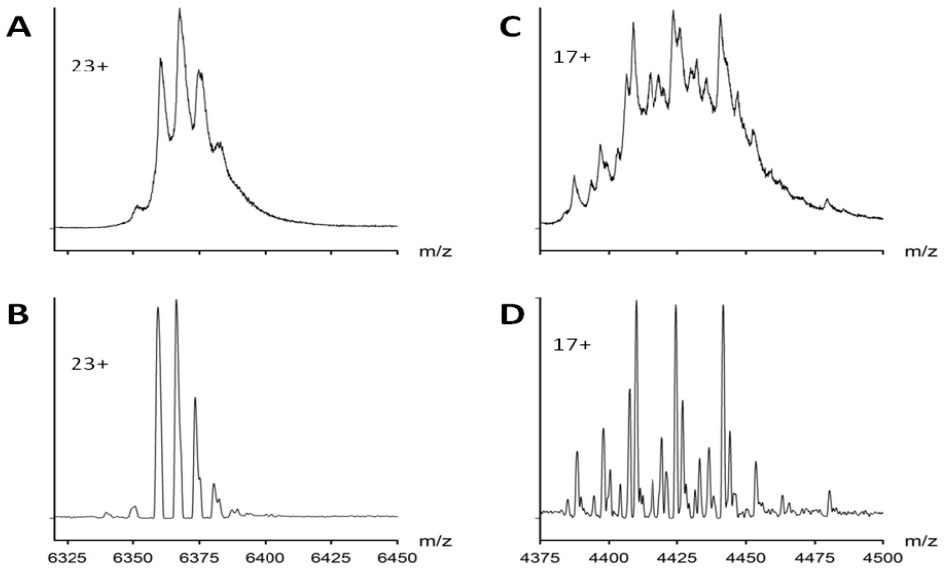


Figure S1. Comparison of experimentally observed peak widths on Q-Tof and Orbitrap analyzers, both modified to allow high m/z detection. (A,B) An IgG antibody with a simple glycosylation pattern or (C,D) a 'half'-IgG (*i.e.* one heavy chain-light chain pair) with complex glycosylation pattern were analysed with (A,C) a modified Q-Tof instrument and (B,D) the modified Exactive Plus. The 23+ charge state is shown for the intact IgG and the 17+ charge state for the half-IgG. The observed peak widths are significantly wider than the instruments' mass resolution, indicating that other peak broadening processes, such as incomplete desolvation and unresolved molecular heterogeneity, are affecting the observed peak shapes.

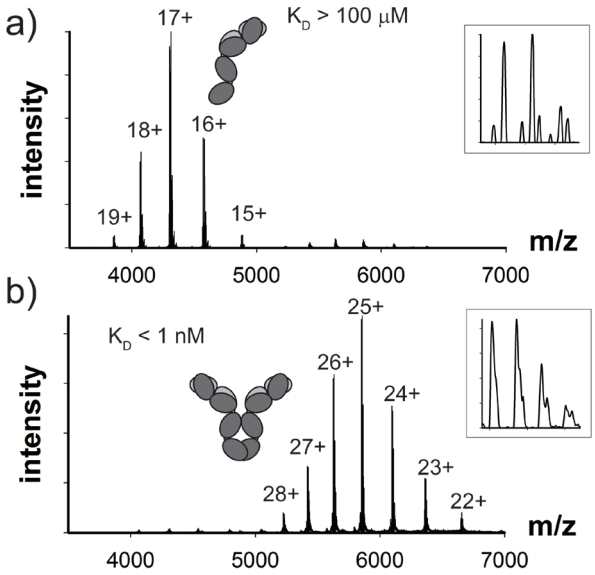


Figure S2. Analysis of non-covalently interactions between antibody half-bodies by Orbitrap native MS. Full native mass spectra of two variants of hingeless-IgG4: (a) F405Q, which preferentially populates a 'monomer' state (K_D > 100 μM), and (b) R409K, which forms a non-covalent 'dimer' structure (K_D < 1 nM). The antibody species observed are indicated by the cartoon structures. Insets: zoom-in of one charge state peak showing resolution of different glycoforms.

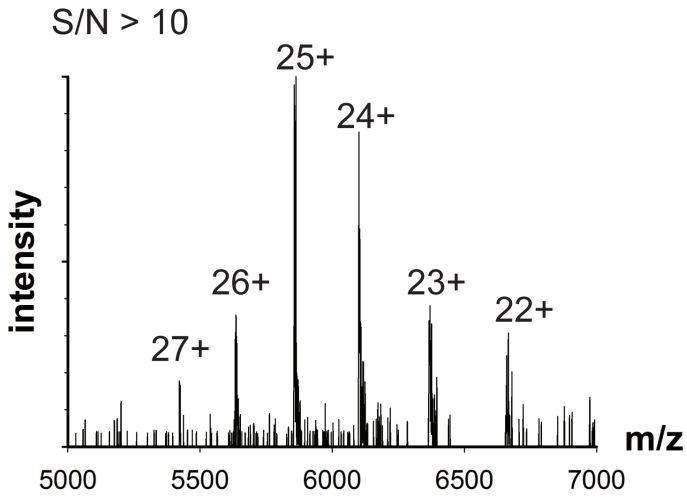


Figure S3. Sensitivity of the Orbitrap native MS for analysing mAbs. Native mass spectrum of a deglycosylated IgG at a concentration of 5 nM, with data being acquired for 15 seconds, equating to the consumption of approximately 20 attomoles of IgG material.

In-Depth Qualitative and Quantitative Analysis of Composite Glycosylation Profiles and Other Micro-Heterogeneity on Intact Monoclonal Antibodies by High-Resolution Native Mass Spectrometry Using a Modified Orbitrap

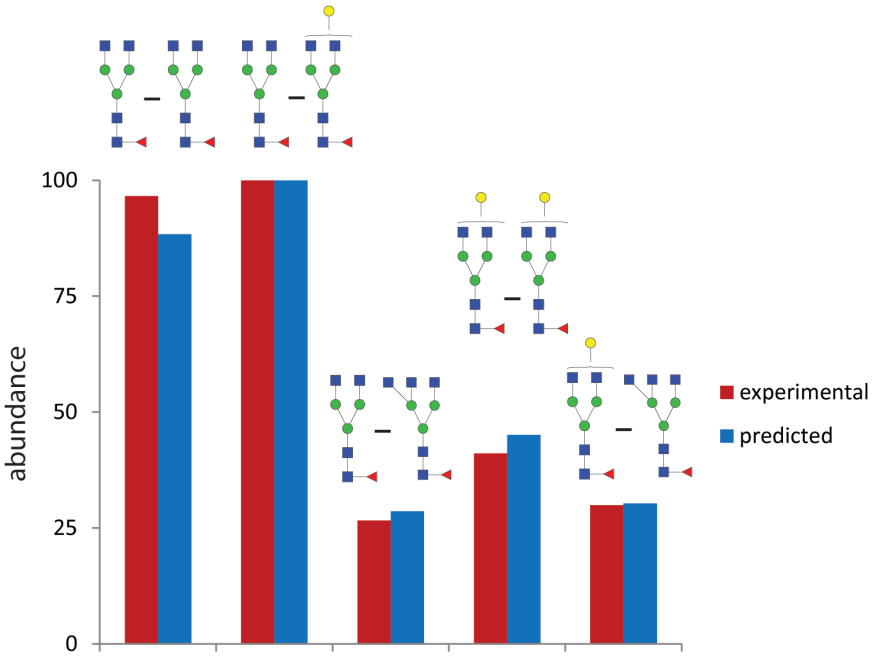


Figure S1. Comparison between experimental and predicted intensities of the identified glycoforms of the ΔhingeIgG4WT full-antibody. These predictions are based on the glycosylation pattern observed on the half-antibody assuming a statistical distribution on the full-antibody.

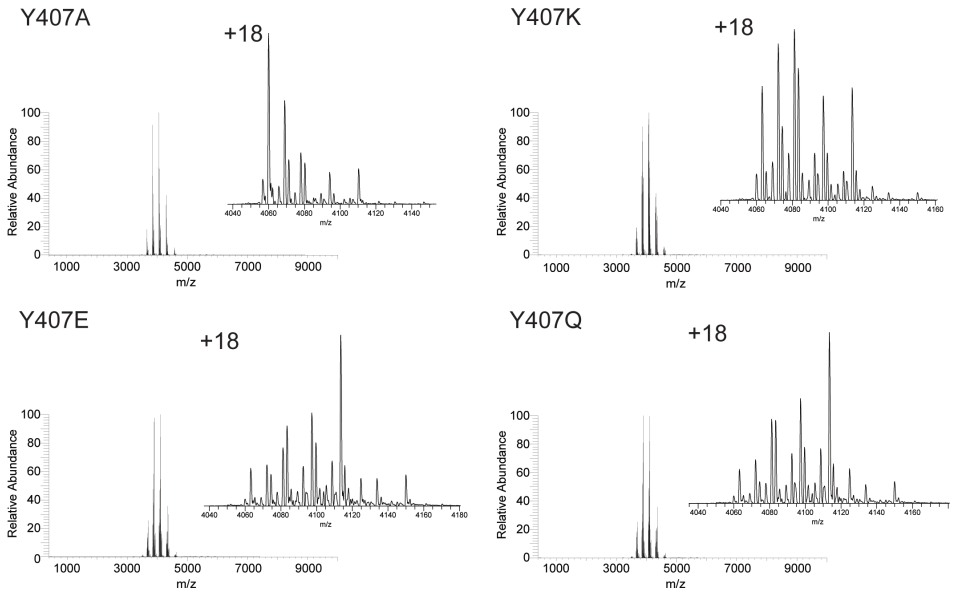


Figure S2. Full native Orbitrap mass spectra of the ΔhingeIgG4 mutants. The in-sets zoom-in on a single charge state highlighting the variance in micro-heterogeneity caused by the differential glycosylation.

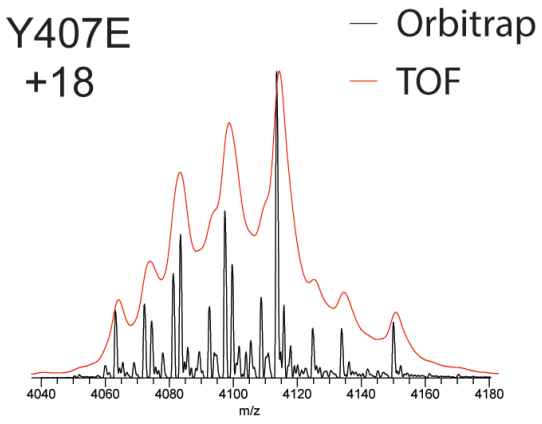


Figure S3. Comparison of the experimental peak-widths obtained using a TOF and a Orbitrap analyzer. To illustrate the improved resolving power obtained using the Orbitrap platform the IgG4Y407E mutant was analyzed on the Orbitrap and on a TOF instrument (LCT, Waters). The overlay of the zoom-in's on the 18+ charge state highlights the improved resolving power of the Orbitrap instrument that allows the separation of peaks otherwise buried under the high abundant ones. As argued previously (*e.g.* reference[24]), the narrower peak-width observed on the Orbitrap platform are largely due to the improved desolvation in the Orbitrap instrument, and less due to the instrument mass resolution, which on modern TOF instruments should potentially also allow narrower peak widths than experimentally observed.

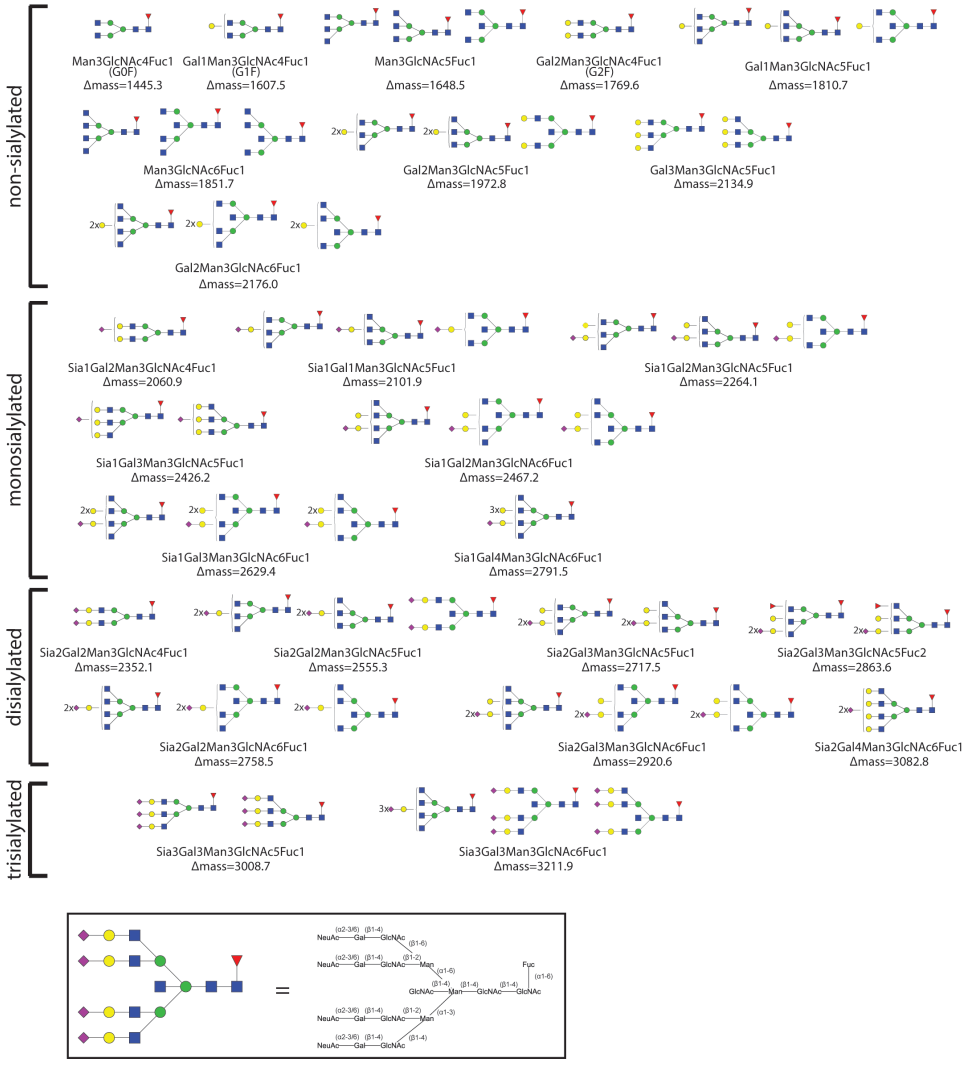


Figure S4. Overview of assigned N-glycan structures, based on $(Neu5Ac_a)(Gal_b)Man_cGlcNAc_dFuc_e$ compositions, for IgG4 WT and mutant antibodies. For the translation of the composition of the molecular masses in terms of numbers of Sia, Hex, HexNAc, and dHex, obtained from the MS data, into N-glycan structures (Sia = Neu5Ac / N-acetylneuraminic acid; Hex = Gal + Man / Galactose + Mannose; HexNAc = GlcNAc / N-acetylglucosamine; dHex = Fuc / Fucose) use is made of the general knowledge about the N-glycan biosynthetic pathways in human cells and known N-glycosylation patterns of human mAbs, including intersected GlcNAc. When more than one structural isoform is possible, multiple structures are given. In total 25 glycan compositions could be assigned. The in-set shows the relationship between the structure of a tetra-sialylated tetra-antennary N-glycan and the used symbolic notation for such a structure, clarifying also the symbolic notation of

diantennary and triantennary structures. For the triantennary structures both the 2,4-disubstituted Man(α1-3) and the 2,6-disubstituted Man(α1-6) possibilities are postulated. However, it has been shown for HEK-293F cells that both (α2-3)- and (α2-6)-linked Neu5Ac can occur.

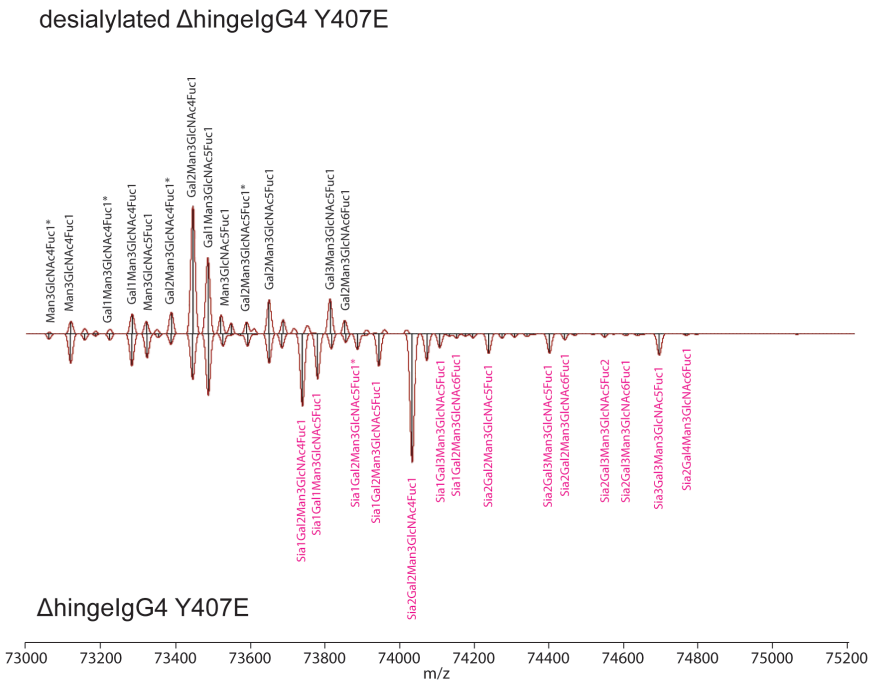


Figure S5. Deconvoluted native mass spectrum of the ΔhingeIgG4Y407E mutant before and after desialylation. All peaks corresponding to sialylated glycoforms (assigned in pink) have disappeared after the treatment with a sialidase enzyme.

glycoform	abundance	theoretical mass (Da)	experimental mass (Da)	Δ_{exp-th} mass (Da)	fucose	hexNAc	hexose	sialic acid (Neu5Ac)	glycan exp. mass (Da)	glycan th. mass (Da)	glycan Δ_{exp-th} mass (Da)	Δ mass (ppm)
ΔhingeIgG4 (half-antibody)												
deglycosylated	-	71706,8	71709,7	2,9	-	-	-	-	-	-	-	-
Man3GlcNAc4Fuc1	54,0%	73152,2	73155,3	3,1	1	4	3	0	1445,6	1445,3	-0,3	-3,5
Gal1Man3GlcNAc4Fuc1	30,0%	73314,3	73317,1	2,8	1	4	4	0	1607,4	1607,5	0,1	1,4
Man3GlcNAc5Fuc1	8,0%	73355,3	73356,7	1,4	1	5	3	0	1647,0	1648,5	1,5	20,6
Gal2Man3GlcNAc4Fuc1	4,4%	73476,4	73478,4	2,0	1	4	5	0	1768,8	1769,6	0,8	11,6
Gal1Man3GlcNAc5Fuc1	3,6%	73517,5	73519,0	1,5	1	5	4	0	1809,3	1810,7	1,4	18,9
ΔhingeIgG4 (full-antibody)												
deglycosylated	-	143413,7	143419,8	6,1	-	-	-	-	-	-	-	-
Man3GlcNAc4Fuc1/Man3GlcNAc4Fuc1	32,8%	146304,3	146310,5	6,2	2	8	6	0	2890,7	2890,6	-0,1	-0,4
Man3GlcNAc4Fuc1/Gal1Man3GlcNAc4Fuc1	34,0%	146466,4	146472,0	5,6	2	8	7	0	3052,3	3052,8	0,5	3,4
Man3GlcNAc4Fuc1/Man3GlcNAc5Fuc1	9,1%	146507,5	146515,7	8,2	2	9	6	0	3095,9	3093,8	-2,1	-14,3
Gal1Man3GlcNAc4Fuc1/Gal1Man3GlcNAc4Fuc1	14,0%	146628,6	146636,1	7,5	2	8	8	0	3216,3	3214,9	-1,4	-9,7
Man3GlcNAc5Fuc1/Gal1Man3GlcNAc4Fuc1	10,2%	146669,6	146675,8	6,1	2	9	7	0	3256,0	3256,0	0,0	-0,3
ΔhingeIgG4 Y407A												
*deglycosylated**	-	71557,6	71561,7	4,0	-	-	-	-	-	-	-	-
deglycosylated	-	71614,6	71617,3	2,6	-	-	-	-	-	-	-	-
*Man3GlcNAc4Fuc1**	4,5%	73002,9	73005,0	2,1	1	4	3	0	1443,3	1445,3	2,0	27,3
Man3GlcNAc4Fuc1	29,3%	73060,0	73062,9	3,0	1	4	3	0	1445,6	1445,3	-0,3	-4,5
*Gal1Man3GlcNAc4Fuc1**	3,2%	73165,1	73165,9	0,8	1	4	4	0	1604,2	1607,5	3,2	44,1
Gal1Man3GlcNAc4Fuc1	18,1%	73222,1	73224,9	2,8	1	4	4	0	1607,6	1607,5	-0,2	-2,1
Man3GlcNAc5Fuc1	7,3%	73263,1	73265,5	2,3	1	5	3	0	1648,2	1648,5	0,3	4,5
*Gal2Man3GlcNAc4Fuc1**	2,0%	73327,2	73329,0	1,8	1	4	5	0	1767,4	1769,6	2,2	30,7
Gal2Man3GlcNAc4Fuc1	8,9%	73384,2	73386,7	2,4	1	4	5	0	1769,4	1769,6	0,2	2,6
Gal1Man3GlcNAc5Fuc1	6,9%	73425,3	73427,5	2,2	1	5	4	0	1810,2	1810,7	0,4	5,6
*Gal2Man3GlcNAc5Fuc1**	0,9%	73530,4	73534,0	3,6	1	5	5	0	1972,4	1972,8	0,4	5,7
Gal2Man3GlcNAc5Fuc1	1,8%	73587,4	73589,6	2,1	1	5	5	0	1972,3	1972,8	0,5	6,9
*Sia1Gal2Man3GlcNAc4Fuc1**	1,0%	73618,5	73622,7	4,2	1	4	5	1	2061,0	2060,9	-0,2	-2,3
Sia1Gal2Man3GlcNAc4Fuc1	5,4%	73675,5	73678,4	2,9	1	4	5	1	2061,1	2060,9	-0,2	-3,0
Sia1Gal1Man3GlcNAc5Fuc1	1,6%	73716,5	73717,7	1,2	1	5	4	1	2100,4	2101,9	1,5	20,0
Sia1Gal2Man3GlcNAc5Fuc1	0,9%	73878,7	73880,4	1,7	1	5	5	1	2263,1	2264,1	0,9	12,5
*Sia2Gal2Man3GlcNAc4Fuc1**	0,9%	73909,7	73912,3	2,6	1	4	5	2	2350,7	2352,1	1,4	19,5
Sia2Gal2Man3GlcNAc4Fuc1	5,9%	73966,8	73969,6	2,8	1	4	5	2	2352,3	2352,1	-0,2	-2,7
Sia2Gal2Man3GlcNAc5Fuc1	0,5%	74169,9	74172,9	3,0	1	5	5	2	2555,6	2555,3	-0,3	-4,2
Sia2Gal3Man3GlcNAc5Fuc1	0,4%	74332,1	74334,6	2,6	1	5	6	2	2717,4	2717,5	0,1	1,0
Sia3Gal3Man3GlcNAc5Fuc1	0,4%	74623,3	74626,2	2,9	1	5	6	3	3008,9	3008,7	-0,2	-2,9
ΔhingeIgG4 Y407K												
*deglycosylated**	-	71614,7	71617,8	3,1	-	-	-	-	-	-	-	-
deglycosylated	-	71671,7	71675,5	3,8	-	-	-	-	-	-	-	-
*Man3GlcNAc4Fuc1**	2,2%	73060,0	73062,5	2,5	1	4	3	0	1444,8	1445,3	0,5	7,5
Man3GlcNAc4Fuc1	8,9%	73117,0	73120,1	3,1	1	4	3	0	1444,6	1445,3	0,7	9,3
*Gal1Man3GlcNAc4Fuc1**	3,2%	73222,2	73224,1	1,9	1	4	4	0	1606,3	1607,5	1,1	15,7
Gal1Man3GlcNAc4Fuc1	12,5%	73279,2	73282,2	3,0	1	4	4	0	1606,7	1607,5	0,7	9,8
Man3GlcNAc5Fuc1	5,9%	73320,2	73321,2	1,0	1	5	3	0	1645,8	1648,5	2,8	37,6
*Gal2Man3GlcNAc4Fuc1**	3,9%	73384,3	73386,5	2,2	1	4	5	0	1768,8	1769,6	0,8	11,3
Gal2Man3GlcNAc4Fuc1	13,8%	73441,3	73443,9	2,6	1	4	5	0	1768,5	1769,6	1,1	15,6
Gal1Man3GlcNAc5Fuc1	10,5%	73482,4	73484,0	1,6	1	5	4	0	1808,5	1810,7	2,1	29,0
Man3GlcNAc6Fuc1	2,2%	73523,4	73522,9	-0,5	1	6	3	0	1847,4	1851,7	4,3	58,3
*Gal2Man3GlcNAc5Fuc1**	1,7%	73587,5	73588,7	1,2	1	5	5	0	1970,9	1972,8	1,9	25,5
Gal2Man3GlcNAc5Fuc1	3,7%	73644,5	73646,4	1,9	1	5	5	0	1970,9	1972,8	1,9	25,2
*Sia1Gal2Man3GlcNAc4Fuc1**	2,5%	73675,6	73681,1	5,5	1	4	5	1	2063,3	2060,9	-2,4	-32,9
Sia1Gal2Man3GlcNAc4Fuc1	8,3%	73732,6	73735,4	2,8	1	4	5	1	2059,9	2060,9	1,0	13,0
Sia1Gal1Man3GlcNAc5Fuc1	3,5%	73773,6	73774,5	0,9	1	5	4	1	2099,0	2101,9	2,9	39,1
Gal2Man3GlcNAc6Fuc1	0,4%	73847,7	73847,4	-0,3	1	6	5	0	2172,0	2176,0	4,0	54,5
*Sia1Gal2Man3GlcNAc5Fuc1**	1,4%	73878,8	73881,4	2,7	1	5	5	1	2263,7	2264,1	0,4	5,2
Sia1Gal2Man3GlcNAc5Fuc1	2,1%	73935,8	73937,5	1,7	1	5	5	1	2262,0	2264,1	2,1	27,9
*Sia2Gal2Man3GlcNAc4Fuc1**	1,8%	73966,8	73971,3	4,5	1	4	5	2	2353,5	2352,1	-1,4	-19,3
Sia2Gal2Man3GlcNAc4Fuc1	8,6%	74023,8	74026,5	2,7	1	4	5	2	2351,0	2352,1	1,1	14,8
Sia1Gal3Man3GlcNAc5Fuc1	0,8%	74097,9	74100,9	3,0	1	5	6	1	2425,4	2426,2	0,8	10,7
Sia2Gal2Man3GlcNAc5Fuc1	1,1%	74227,0	74229,7	2,6	1	5	5	2	2554,2	2555,3	1,1	14,9
Sia2Gal3Man3GlcNAc5Fuc1	0,6%	74389,2	74391,4	2,3	1	5	6	2	2716,0	2717,5	1,5	20,1
Sia3Gal3Man3GlcNAc5Fuc1	0,6%	74680,4	74683,3	2,9	1	5	6	3	3007,8	3008,7	0,9	11,7
ΔhingeIgG4 Y407E												
*deglycosylated**	-	71615,7	71617,6	1,9	-	-	-	-	-	-	-	-
deglycosylated	-	71672,7	71676,4	3,7	-	-	-	-	-	-	-	-
*Man3GlcNAc4Fuc1**	0,8%	73061,0	73062,9	2,0	1	4	3	0	1445,3	1445,3	0,0	-0,2
Man3GlcNAc4Fuc1	4,2%	73118,0	73120,6	2,6	1	4	3	0	1444,2	1445,3	1,1	15,7
*Gal1Man3GlcNAc4Fuc1**	1,0%	73223,1	73224,6	1,5	1	4	4	0	1607,0	1607,5	0,4	5,6
Gal1Man3GlcNAc4Fuc1	4,6%	73280,1	73282,9	2,8	1	4	4	0	1606,5	1607,5	0,9	12,9
Man3GlcNAc5Fuc1	3,5%	73321,2	73323,4	2,2	1	5	3	0	1647,0	1648,5	1,5	20,7
*Gal2Man3GlcNAc4Fuc1**	1,6%	73385,3	73387,0	1,7	1	4	5	0	1769,4	1769,6	0,2	2,9
Gal2Man3GlcNAc4Fuc1	6,6%	73442,3	73444,7	2,5	1	4	5	0	1768,3	1769,6	1,3	17,2
Gal1Man3GlcNAc5Fuc1	8,8%	73483,3	73485,8	2,5	1	5	4	0	1809,4	1810,7	1,2	16,4
Man3GlcNAc6Fuc1	1,8%	73524,4	73525,2	0,8	1	6	3	0	1848,8	1851,7	2,9	39,8
*Gal2Man3GlcNAc5Fuc1**	1,8%	73588,4	73590,0	1,6	1	5	5	0	1972,4	1972,8	0,4	5,1
Gal2Man3GlcNAc5Fuc1	4,3%	73645,5	73647,6	2,2	1	5	5	0	1971,3	1972,8	1,5	20,9
*Sia1Gal2Man3GlcNAc4Fuc1**	2,1%	73676,5	73681,7	5,1	1	4	5	1	2064,1	2060,9	-3,2	-43,5
Sia1Gal2Man3GlcNAc4Fuc1	10,3%	73733,5	73736,2	2,6	1	4	5	1	2059,8	2060,9	1,1	14,7
Sia1Gal1Man3GlcNAc5Fuc1	6,5%	73774,6	73776,5	1,9	1	5	4	1	2100,1	2101,9	1,8	24,1
Gal3Man3GlcNAc5Fuc1	2,2%	73807,6	73812,7	5,1	1	5	6	0	2136,3	2134,9	-1,4	-18,4
Gal2Man3GlcNAc6Fuc1	1,3%	73848,7	73851,1	2,5	1	6	5	0	2174,7	2176,0	1,2	16,9
*Sia1Gal2Man3GlcNAc5Fuc1**	2,3%	73879,7	73882,2	2,5	1	5	5	1	2264,6	2264,1	-0,6	-8,0
Sia1Gal2Man3GlcNAc5Fuc1	4,7%	73936,7	73939,0	2,3	1	5	5	1	2262,6	2264,1	1,4	19,6
Sia2Gal2Man3GlcNAc4Fuc1	18,3%	74024,8	74027,5	2,7	1	4	5	2	2351,1	2352,1	1,0	13,2
Sia1Gal3Man3GlcNAc5Fuc1	2,1%	74098,9	74100,8	1,9	1	5	6	1	2424,4	2426,2	1,8	24,0
Sia1Gal2Man3GlcNAc6Fuc1	0,7%	74139,9	74145,0	5,1	1	6	5	1	2468,6	2467,2	-1,4	-18,5
Sia2Gal2Man3GlcNAc5Fuc1	2,9%	74228,0	74230,8	2,9	1	5	5	2	2554,4	2555,3	0,9	11,6
Sia2Gal3Man3GlcNAc5Fuc1	2,8%	74390,1	74392,3	2,2	1	5	6	2	2716,0	2717,5	1,5	20,1
Sia2Gal2Man3GlcNAc6Fuc1	0,9%	74431,2	74433,0	1,8	1	6	5	2	2756,6	2758,5	1,9	25,5
Sia2Gal3Man3GlcNAc5Fuc2	0,5%	74536,3	74538,9	2,6	2	5	6	2	2862,5	2863,6	1,1	14,7
Sia3Gal3Man3GlcNAc5Fuc1	3,2%	74681,4	74684,2	2,8	1	5	6	3	3007,8	3008,7	0,9	12,1
Sia2Gal4Man3GlcNAc6Fuc1	0,3%	74755,5	74755,1	-0,3	1	6	7	2	3078,7	3082,8	4,0	54,0

glycoform	*abundance*	*theoretical mass (Da)*	*experimental mass (Da)*	*Δ_{exp-th} mass (Da)*	*fucose*	*hexNAc*	*hexose*	*sialic acid (Neu5Ac)*	*glycan exp. mass (Da)*	*glycan th. mass (Da)*	*glycan Δ_{exp-th} mass (Da)*	*Δ mass (ppm)*
ΔhingeIgG4 Y407Q												
*deglycosylated**	-	71614,7	71617,7	3,0	-	-	-	-	-	-	-	-
deglycosylated	-	71671,7	71674,5	2,8	-	-	-	-	-	-	-	-
*Man3GlcNAc4Fuc1**	0,8%	73060,0	73061,9	2,0	1	4	3	0	1444,3	1445,3	1,1	14,5
Man3GlcNAc4Fuc1	3,4%	73117,0	73119,8	2,8	1	4	3	0	1445,3	1445,3	0,0	0,1
*Gal1Man3GlcNAc4Fuc1**	1,1%	73222,1	73224,1	1,9	1	4	4	0	1606,4	1607,5	1,1	14,7
Gal1Man3GlcNAc4Fuc1	4,4%	73279,1	73281,7	2,5	1	4	4	0	1607,2	1607,5	0,3	3,9
Man3GlcNAc5Fuc1	2,4%	73320,2	73321,7	1,5	1	5	3	0	1647,2	1648,5	1,3	17,6
*Gal2Man3GlcNAc4Fuc1**	2,2%	73384,3	73385,9	1,7	1	4	5	0	1768,3	1769,6	1,3	18,4
Gal2Man3GlcNAc4Fuc1	8,8%	73441,3	73443,9	2,6	1	4	5	0	1769,4	1769,6	0,2	2,7
Gal1Man3GlcNAc5Fuc1	8,5%	73482,3	73484,4	2,1	1	5	4	0	1809,9	1810,7	0,7	9,9
Man3GlcNAc6Fuc1	1,6%	73523,4	73522,0	-1,3	1	6	3	0	1847,5	1851,7	4,2	56,9
*Gal2Man3GlcNAc5Fuc1**	2,0%	73587,5	73588,9	1,5	1	5	5	0	1971,2	1972,8	1,5	21,0
Gal2Man3GlcNAc5Fuc1	4,9%	73644,5	73647,0	2,5	1	5	5	0	1972,4	1972,8	0,4	4,8
*Sia1Gal2Man3GlcNAc4Fuc1**	2,7%	73675,5	73679,8	4,3	1	4	5	1	2062,1	2060,9	-1,2	-16,7
Sia1Gal2Man3GlcNAc4Fuc1	10,8%	73732,5	73735,6	3,1	1	4	5	1	2061,1	2060,9	-0,2	-3,3
Sia1Gal1Man3GlcNAc5Fuc1	5,4%	73773,6	73774,5	0,9	1	5	4	1	2100,0	2101,9	1,9	26,3
Gal3Man3GlcNAc5Fuc1	2,0%	73806,6	73810,6	4,0	1	5	6	0	2136,1	2134,9	-1,1	-15,2
Gal2Man3GlcNAc6Fuc1	0,9%	73847,7	73848,7	1,1	1	6	5	0	2174,2	2176,0	1,8	24,2
*Sia1Gal2Man3GlcNAc5Fuc1**	2,2%	73878,7	73881,4	2,7	1	5	5	1	2263,7	2264,1	0,3	4,4
Sia1Gal2Man3GlcNAc5Fuc1	5,3%	73935,7	73938,0	2,3	1	5	5	1	2263,5	2264,1	0,6	7,9
*Sia2Gal2Man3GlcNAc4Fuc1**	2,3%	73966,8	73972,0	5,2	1	4	5	2	2354,3	2352,1	-2,2	-29,1
Sia2Gal2Man3GlcNAc4Fuc1	16,7%	74023,8	74026,3	2,5	1	4	5	2	2351,8	2352,1	0,3	4,2
Sia1Gal3Man3GlcNAc5Fuc1	1,7%	74097,9	74099,4	1,5	1	5	6	1	2424,8	2426,2	1,4	18,4
Sia2Gal2Man3GlcNAc5Fuc1	3,5%	74227,0	74229,5	2,5	1	5	5	2	2555,0	2555,3	0,3	4,5
Sia1Gal3Man3GlcNAc6Fuc1	0,5%	74301,1	74300,8	-0,2	1	6	6	1	2626,3	2629,4	3,1	41,2
Sia2Gal3Man3GlcNAc5Fuc1	1,9%	74389,1	74391,6	2,5	1	5	6	2	2717,1	2717,5	0,4	4,9
Sia2Gal2Man3GlcNAc6Fuc1	0,6%	74430,2	74431,2	1,0	1	6	5	2	2756,7	2758,5	1,8	24,4
Sia1Gal4Man3GlcNAc6Fuc1	0,4%	74463,2	74461,5	-1,7	1	6	7	1	2787,0	2791,5	4,5	60,9
Sia2Gal3Man3GlcNAc5Fuc2	0,4%	74535,3	74537,6	2,3	2	5	6	2	2863,1	2863,6	0,5	7,0
Sia2Gal3Man3GlcNAc6Fuc1	0,3%	74592,3	74593,8	1,4	1	6	6	2	2919,2	2920,6	1,4	18,9
Sia3Gal3Man3GlcNAc5Fuc1	2,1%	74680,4	74682,9	2,5	1	5	6	3	3008,4	3008,7	0,3	3,9
Sia2Gal4Man3GlcNAc6Fuc1	0,2%	74754,5	74754,2	-0,2	1	6	7	2	3079,7	3082,8	3,1	41,1
Sia3Gal3Man3GlcNAc6Fuc1	0,2%	74883,6	74885,7	2,1	1	6	6	3	3211,2	3211,9	0,7	9,5

Table S1. Comparison between experimentally determined mass of ΔhingeIgG4 WT and mutant samples and their theoretical mass calculated from the protein sequence and theoretical mass of the glycans. The deviation between the experimental and theoretical mass is consistent among all half-antibodies, while it doubles for the full-antibody and is most likely due to residual binding of a few small molecules or cations (see Figure 4).

Appendix

Curriculum Vitae

List of Publications

Acknowledgements

Curriculum Vitae

Sara was born on 15th March 1985 in Termoli, Italy. In 2004 she moved to Bologna where she studied pharmaceutical chemistry and technology. During this period, in 2009, she visited the Biomolecular Mass Spectrometry & Proteomics group in Utrecht for an internship where she focused on quantitative proteomics of S*accaromices Cerevisiae*. After having obtained her master's degree from the University of Bologna in 2010, she decided to continue her studies as PhD candidate in the Biomolecular Mass Spectrometry & Proteomics group under the supervision of Prof. dr. Albert J.R. Heck. In Utrecht her research was mainly focused on developing new tools for the analysis of antibodies using native mass spectrometry. The results of her PhD research are described in this thesis.

List of publications

Kleppe, R.*, **Rosati, S.***, Jorge-Finnigan, A., Ghorbani, S., Alvira, S., Haavik, J., Valpuesta, J. M., Heck, A. J. R. & Martinez, A. Phosphorylation dependence and stoichiometry of the complex formed by tyrosine hydroxylase and 14-3-3 proteins. Mol Cell Proteomics, (2014).

Diebolder, C. A., Beurskens, F. J., de Jong, R. N., Koning, R. I., Strumane, K., Lindorfer, M. A., Voorhorst, M., Ugurlar, D., **Rosati, S.**, Heck, A. J., van de Winkel, J. G., Wilson, I. A., Koster, A. J., Taylor, R. P., Saphire, E. O., Burton, D. R., Schuurman, J., Gros, P. & Parren, P. W. Complement is activated by IgG hexamers assembled at the cell surface. Science 343, 1260-1263 (2014).

Rosati, S.*, Yang, Y.*, Barendregt, A. & Heck, A. J. Detailed mass analysis of structural heterogeneity in monoclonal antibodies using native mass spectrometry. Nat Protoc 9, 967-976 (2014).

Thompson, N. J.*, **Rosati, S.*** & Heck, A. J. Performing native mass spectrometry analysis on therapeutic antibodies. Methods 65, 11-17 (2014).

Rosati, S., van den Bremer, E. T., Schuurman, J., Parren, P. W., Kamerling, J. P. & Heck, A. J. In-depth qualitative and quantitative analysis of composite glycosylation profiles and other micro-heterogeneity on intact monoclonal antibodies by high-resolution native mass spectrometry using a modified Orbitrap. MAbs 5, 917-924 (2013).

Rosati, S., Thompson, N. J. & Heck, A. J. R. Tackling the increasing complexity of therapeutic monoclonal antibodies with mass spectrometry. Trac-Trend Anal Chem 48, 72-80 (2013).

Thompson, N. J., **Rosati, S.**, Rose, R. J. & Heck, A. J. R. The impact of mass spectrometry on the study of intact antibodies: from post-translational modifications to structural analysis. Chem Commun (Camb) 49, 538-548 (2013).

Rosati, S.*, Rose, R. J.*, Thompson, N. J., van Duijn, E., Damoc, E., Denisov, E., Makarov, A. & Heck, A. J. R. Exploring an Orbitrap Analyzer for the Characterization of Intact Antibodies by Native Mass Spectrometry. Angew Chem Int Edit 51, 12992-12996 (2012).

Nenci, S., Piano, V., **Rosati, S.**, Aliverti, A., Pandini, V., Fraaije, M. W., Heck, A. J. R., Edmondson, D. E. & Mattevi, A. Precursor of ether phospholipids is synthesized

by a flavoenzyme through covalent catalysis. Proc Natl Acad Sci U S A 109, 18791-18796 (2012).

Rosati, S., Thompson, N. J., Barendregt, A., Hendriks, L. J. A., Bakker, A. B. H., de Kruif, J., Throsby, M., van Duijn, E. & Heck, A. J. R. Qualitative and Semiquantitative Analysis of Composite Mixtures of Antibodies by Native Mass Spectrometry. Anal Chem 84, 7227-7232 (2012).

Helbig, A. O., **Rosati, S.**, Pijnappel, P. W. W. M., van Breukelen, B., Timmers, M. H. T. H., Mohammed, S., Slijper, M. & Heck, A. J. R. Perturbation of the yeast N-acetyltransferase NatB induces elevation of protein phosphorylation levels. BMC Genomics 11:685 (2010).

*These authors contributed equally.

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