Protein Mass Spectrometry

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

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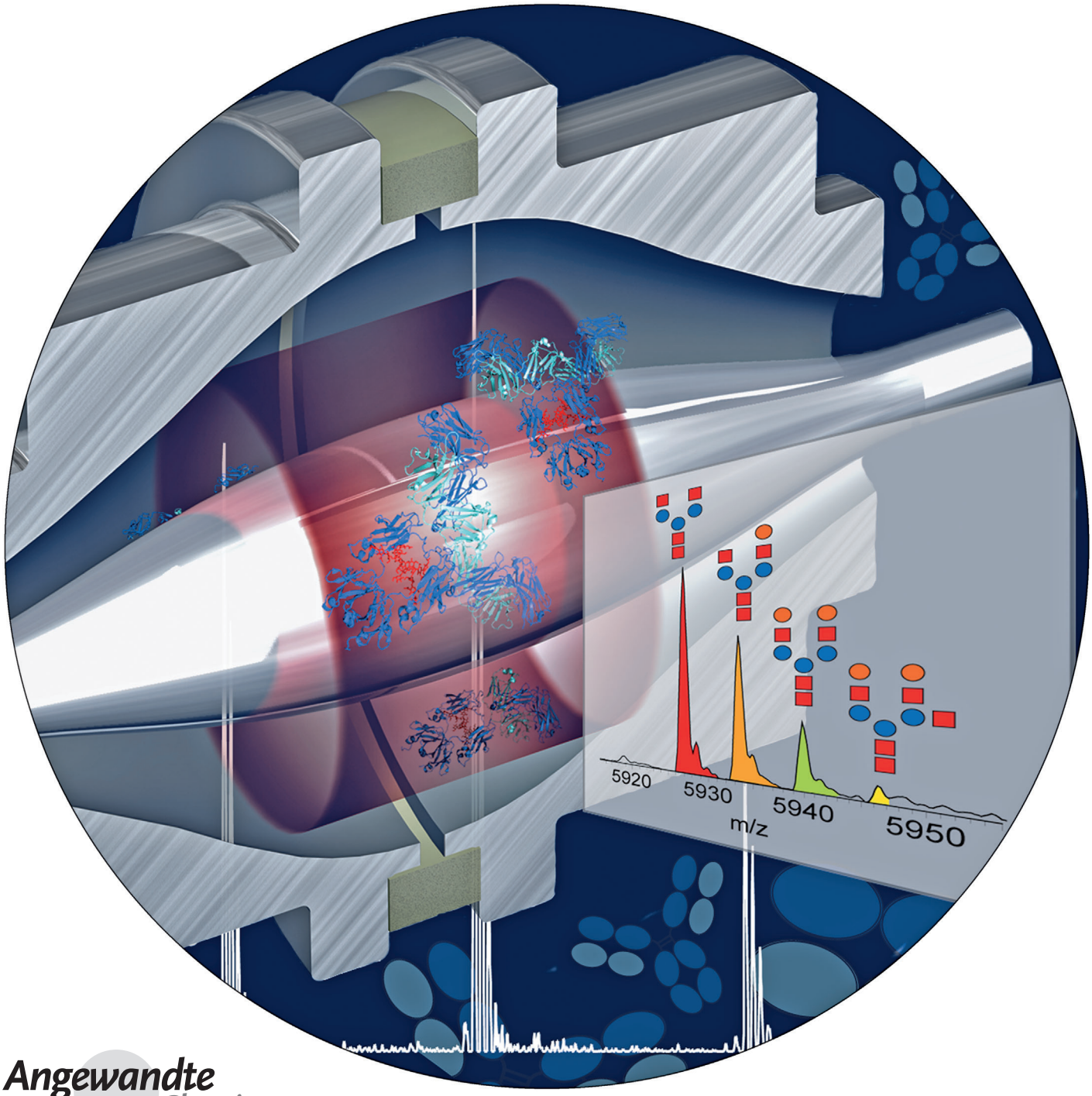

Monoclonal antibodies (mAbs) represent a major class of therapeutic biomolecules being developed by the biopharmaceutical industry, their popularity largely being a result of their high specificity towards targets. Therapeutic mAbs are being developed for various diseases, with a strong focus on cancer and immunological disorders.[1] All antibody drugs currently approved for clinical use are based on the immunoglobulin G (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 a range of analytical tools are necessary to characterize them fully.

In this context, mass spectrometry (MS) is gaining importance as a technique for the analysis of mAbs.[2] MS is versatile and can address numerous structural issues, including characterization of the glycan profile, mapping of disulfide bonds, determination of post-translational modification, and mapping of epitopes.[3] 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 under denaturing conditions is now becoming routine,[2b,c] MS under nondenaturing conditions, that is, native MS, also represents a viable alternative for the accurate measurement of the mass of intact antibodies. Native MS, as defined previously,[4] is used to probe the folded tertiary and quaternary structures of proteins and protein complexes. It may provide information inaccessible by other approaches, thus 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,[5] to evaluation of structural features, dynamics, and interaction strengths,[6] to qualitative and semiquantitative analysis of mixtures of mAbs.[7] Analysis of native intact antibodies could benefit from increases in sensitivity, mass accuracy, resolving power, and robustness. Towards that goal, we recently pioneered native MS by using a modified orbitrap mass analyzer, which resulted in significant improvements in performance compared to the TOF instruments typically used for such measurements (see Figure S1 in the Supporting Information).[2b,8] We initially proved the power of this new technique for the analysis of very large protein complexes, such as *E. coli* GroEL (800 kDa) and the 20S proteasome (730 kDa) from *S. cerevisiae*.[9] Here, we further demonstrate the great versatility of this new orbitrap platform for the characterization of mAbs, thus enabling important needs of the biopharmaceutical industry to be met.

One of the challenges of characterizing IgGs is the presence of an N-linked glycosylation site in each heavy chain. The nature of this glycan chain has been found to influence both the binding of the antibody to the corresponding Fc receptor and its half-life in serum.[10] The glycan chain 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 (ca. 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 ESIMS spectra, the spectrum is spread over only a few charge states, primarily 23+ to 27+. Different glycoforms of the antibody are clearly baseline-resolved, thus allowing accurate assignments of the glycan identity ($\Delta Mw = 162$ Da, which corresponds to different numbers of hexose (galactose) units present, Figure 2b). Mass differences down to 25 Da can be sufficiently resolved at the resolution obtained in this spectrum, thus allowing the identification of different modifications such as glycosylation, C-terminal lysine cleavage, or primary sequence mutations.

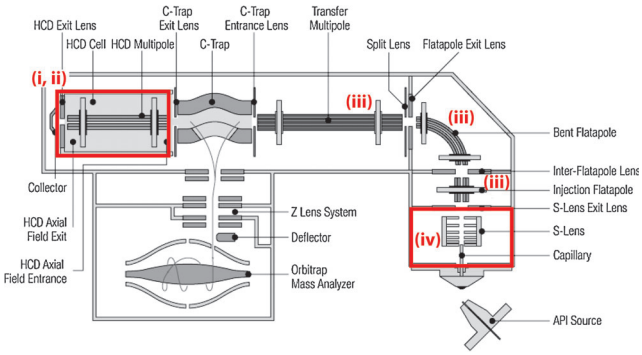

Figure 1. Schematic representation of the modified Exactive Plus instrument (ThermoFisher Scientific, Germany) as described in detail in Ref. [9]. Modifications included: i) storing ions in the HCD cell, rather than trapping in the C trap, thus allowing more efficient trapping and increased desolvation, ii) manual regulation of the Xe pressure in the HCD cell, iii) altering the voltage offset on the flatapoles and transfer multipole, 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 values up to 30000 to be measured.

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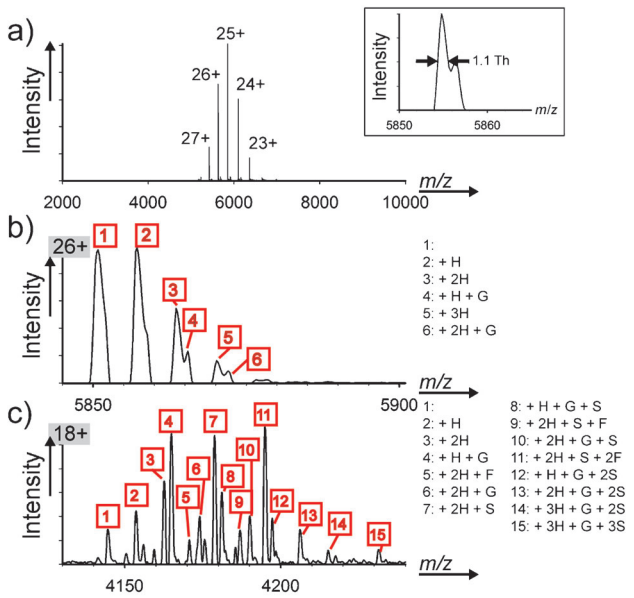

Figure 2. Antibody glycoform profiling by orbitrap native MS. Native mass spectra of IgG with increasingly complex glycosylation profiles. a) Full native mass spectrum of a deglycosylated IgG, showing that all ion signals accumulate in 5 main charge states ($23 \le z \le 27$). The accurate mass determined from this spectrum (146.352.4 Da) was within $\delta = 2$ 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 spectra 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 values between signals, 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.

To illustrate this, we resolved and assigned glycoforms for an antibody-based construct with a very heterogeneous glycan pattern (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 resolution up to 12000 at m/z 6000 could be achieved in combination with a high mass accuracy (< 1.5 Da/δ = 10 ppm, that is, 0.001 %), thus allowing for confident assignment of modifications to the antibody. Even higher resolution could be achieved for denatured antibodies at lower m/z values by using a FTICR instrument, albeit at the expense of sensitivity and detection speed.[11]

Besides the structural heterogeneity caused by glycosylation and other modifications, further complexity may arise because of the pharmaceutical industry moving towards artificial constructs of increased intricacy, for example, coexpressed mixtures, antibody–drug conjugates, and antibody–DNA conjugates for diagnostic purposes. Mixtures of monoclonal antibodies are increasingly being applied in the pharmaceutical industry as potential new therapeutic entities with synergic effects for minimal extra costs, and require dedicated novel analytical tools.[7,12] To explore the capabilities of the orbitrap analyzer for this purpose, a complex mixture of ten monoclonal antibodies was analyzed after deglycosylation (Figure 3a). Nine distinct signals could be observed for each of the charge states (see Figure 3a,b); these signals 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 that the width of the isotopic distribution for an antibody is approximately 25 Da).[2b] The experimental resolving power of the orbitrap also allows mixtures of glycosylated mAbs to be analyzed (Figure 3c,d). Each of four different IgG species is seen to result in five well-resolved signals, attributed to five different glycan combinations. It is also clear from the spectrum 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.

Similar data as described above can be achieved on Q-TOF platforms under "native" conditions, albeit at a lower resolving power (see Figure S1 in the Supporting Information).[7,8] 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, thereby reducing the likelihood of overlapping ion signals from other protein species present. Moreover, by using native MS it is also

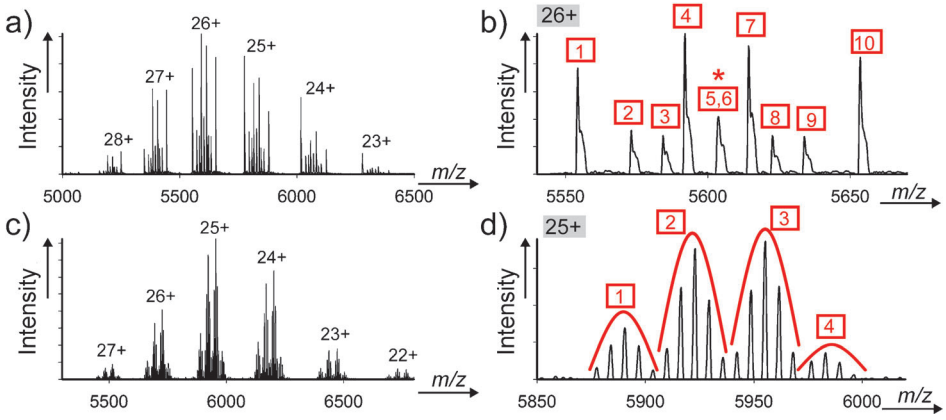

Figure 3. Composite mixtures of antibodies analyzed by orbitrap native MS. a) Full native mass spectra of a mixture of ten distinct deglycosylated IgG antibodies and b) enlargement of the 26+ charge state. The well-resolved ion signals and accurate masses measured enable the unambiguous assignment and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

possible to measure noncovalent interactions, such as protein–protein and protein–ligand interactions, simultaneously. In the context of mAbs, this includes monitoring antibody–antigen binding,[5,13] noncovalently assembled antibody structures,[6] and oligomerization or aggregation.[14] To demonstrate that such analyses are now also possible on the orbitrap platform we next analyzed noncovalent antibody species and antibody–antigen interactions.

IgG4 molecules can exist as noncovalently 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 the heavy chain association can be further altered by single-point mutations.[6] Thus, 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 (see Figure S2a in the Supporting Information), and a dimeric species with a molecular weight of 150 kDa for the latter construct (see Figure S2b in the Supporting Information). These represent the "half" and "intact" structures, as expected, and prove that the noncovalent association of the heavy chains is completely retained during orbitrap analysis.

Antigen-binding studies are clearly also of pivotal importance for the development of therapeutic antibodies. The three-dimensional structures of proteins, and as a consequence their binding properties, can be at least partially preserved by using native MS.[13] 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, which result 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, thus providing information about each individual binding state, as opposed to the average amount of binding determined by other techniques.

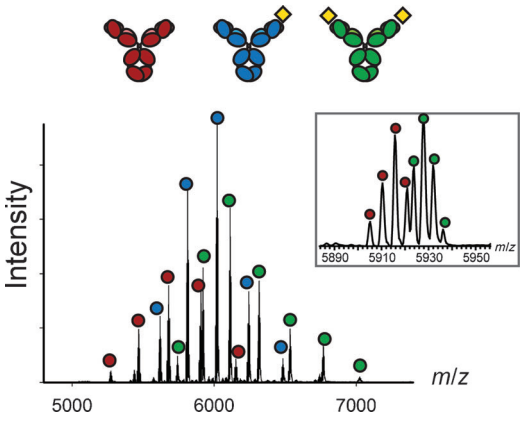

Figure 4. Monitoring antibody–antigen binding by orbitrap native MS. Native mass spectrum of an anti-IL6 antibody incubated with the IL6 antigen. Three well-resolved charge-state envelopes are observed, which correspond to unbound mAb (red circles, 147.639.2 Da), mAb–IL6 (blue circles, 168.547.5 Da), and mAb–$(IL6)_2$ (green circles, 189.452.8 Da), as indicated by the schematic structures. Inset: enlargement of the m/z 5890–5950 region, showing resolution of glycoforms of species with similar m/z values. Masses quoted are for the most abundant glycoforms.

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 as an example a raw spectrum of a 5 nM solution, acquired over 15 seconds, and with a clear charge-state envelope and signal-to-noise ratio greater than 10:1. Estimating the flow-rate from the nano-electrospray capillaries as 1 μL 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 values that can be achieved 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, noncovalent interactions, and antibody–antigen binding. The method introduced here allows analysis of very complex mixtures of compounds and 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 could be exploited to enhance significantly the analysis of therapeutic antibodies within the pharmaceutical industry.

Experimental Section

Antibody samples, produced in HEK293T cells, were kindly provided by Merus and Genmab. All purified proteins were exchanged into ammonium acetate (100 mM or 150 mM, pH 7 or 7.5) using either 10 or 5 kDa molecular-weight cut off (MWCO) spin-filter columns (Vivaspin 500; Sartorius Stedim Biotech GmbH, Goettingen, Germany). The 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 one unit of N-glycosidase F (PNGaseF; Roche Diagnostics, Mannheim, Germany) at 37°C overnight prior to buffer exchange. An antibody/antigen ratio of 1:4 was used for the antibody–antigen binding experiment. Serial dilutions of deglycosylated IgG1 down to 1 nM were made for sensitivity tests.

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 Plus instrument[7] over the m/z range 400–30000 Th. Ions were stored in the HCD cell before return to the C trap for increased desolvation and higher sensitivity. The pressure of the 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. The resolution settings were adjusted as required, between 9000 and 70000 at *m/z* 200. An in-source dissociation energy was applied (100–200 V) to aid in desolvation. Data were calibrated using clusters of ammonium hexafluorophosphate or caesium iodide.

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