Monoclonal Antibody and Related Product Characterization Under Native Conditions Using a Benchtop Mass Spectrometer

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Key Words
Orbitrap native MS, extended mass range MS, monoclonal antibody (mAb), monoclonal antibody-drug-conjugate (ADC), monoclonal antibody antigen complexes (mAb/Ag)

Goal
Demonstrate the characterization of mAbs, antibody-drug conjugates (ADC), mAb/antigen (mAb/Ag) complexes, and a mixture of mAbs under their native conditions by using a high-resolution, accurate-mass (HRAM) benchtop mass spectrometer with extended mass range (EMR) in combination with a chip-based electrospray ionization interface.

Introduction
Native mass spectrometry (MS) has emerged as a valuable technique for characterization of intact noncovalent protein complexes, reaching a high level of reliability within the last ten years.[1] For the analysis of intact monoclonal antibodies (mAbs), native MS yields accurate mass measurements of the molecules, glycoform identification, and assessment of higher-order structures (dimer, trimer, tetramer), thus providing a robust, fast, and reliable first-line analytical characterization tool.[2,3] This approach can now be applied to the routine characterization of heterogeneous therapeutic monoclonal antibodies. Native MS has gained interest not only for analysis of intact mAb, but also for analysis of antibody-drug conjugates (ADCs), bispecific mAbs, antibody-antigen complexes, and characterization of antibody mixtures. It benefits from simplified data interpretation due to the presence of fewer charge states compared to classical denaturing MS.

This application note describes the use of a new Orbitrap mass spectrometer with an extended mass range of up to *m/z* 20,000 and improved detection of high-mass ions for the characterization of mAbs, ADCs, mAb/Ag, and mAb mixtures under native conditions.

Figure 1. Exactive Plus EMR mass spectrometer equipped with a TriVersa NanoMate chip-based electrospray ionization interface

Experimental
Sample Preparation
The intact trastuzumab (Herceptin®, Roche), the monoclonal antibody-drug conjugate brentuximab vedotin (ADC, Adcetris®, Seattle Genetics), the mAb/antigen complexes of J10.4 mAb/JAM-A, and one mixture of eleven distinct IgG antibodies were introduced using the TriVersa NanoMate® (Advion, USA) onto the Thermo Scientific™ Exactive™ Plus EMR Orbitrap™ mass spectrometer.

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Brentuximab vedotin was deglycosylated using EndoS endoglycosidase (IgGZERO™, Genovis). Titration experiments involving J10.4 mAb and JAM-A were monitored by native MS in order to determine the binding stoichiometry. The fixed amount of J10.4 (5 µM) was incubated with increasing amounts (1:1, 1:2, 1:4, 1:8) of JAM-A up to 40 µM. The mixture of eleven distinct deglycosylated humanized IgG antibodies included two marketed therapeutic mAbs (rituximab and trastuzumab) and nine point mutation variants of the Hz6F4-2 mAb [4, 5]. They were mixed together prior to PNGase-F deglycosylation.

Finally, all the samples were buffer exchanged against 150 mM ammonium acetate (AcONH4) pH 7.5. Trastuzumab, deglycosylated Brentuximab vedotin, and the mAb/antigen complexes of J10.4 mAb/JAM-A were injected at 5 µM, and the deglycosylated IgG mixture was injected at 1 µM on the Exactive Plus EMR Orbitrap mass spectrometer.

Direct-Infusion Native MS Conditions

Chip-based infusion conditions	
Instrumentation	TriVersa NanoMate® (Advion, USA) system
Ionization voltage (kV)	1.6–1.8
Gas pressure (psi)	0.3–0.6

The ESI Chip® consists of an array of 400 nanoelectrospray emitters with 5 µm inner diameters.

MS conditions	
Instrumentation	Exactive Plus EMR Orbitrap MS system (Figure 1)
EMR mode	ON
Mass range *(m/z)*	350–20,000
Resolution	17,500 to 140,000, depending on spectral complexity
Target value	3×10^6
Microscans	10
Max injection time (ms)	300
Insource CID energy (eV)	60 to 150, manually tuned for optimized desolvation
S-lens level (%)	100 to 200, manually tuned for optimized transmission and avoiding in-source fragmentation
Injection flatapole DC (V)	8
Inter flatapole lens (V)	7
Bent flatapole DC (V)	6
C-Trap entrance lens tune offset (V) EMR	0
Trapping gas pressure setting factor	4
Spectra average	Enabled (10 to 50 scans are averaged to achieve S/N ratio of >100)

Data Processing

Software	Thermo Scientific™ Protein Deconvolution software version 2.0 SP2 and version 3.0
Deconvolution parameters	
Number of iterations	4
Noise compensation	On
Minimum adjacent charges	1 to 3

Results and Discussion

High-Resolution Native MS Analysis of Intact Monoclonal Antibody Trastuzumab

Trastuzumab (Herceptin®) is a humanized IgG1 mAb, approved for HER2-overexpressing breast cancer treatment since 1998. Several mechanisms of action are thought to contribute to trigger the tumor-inhibitory effect of this protein therapeutic. Among them, trastuzumab can mediate the effector functions of immune cells through its constant region (Fc) by binding to the Fc gamma receptor III (FcγRIII) and triggering antibody-dependent, cell-mediated cytotoxicity (ADCC).

Based on the published amino acid sequence of both the light and heavy chain of trastuzumab, the calculated mass of this protein is $C_{6560}H_{10132}O_{2090}N_{1728}S_{44}$ = 148,057 Da. This calculation includes 16 disulfide bridges (–32 Da), two main glycoforms (G0F; +1445 Da), and near 99% cleavage of two heavy chain C-terminal lysines (–128 × 2 Da). Partial cyclization of one or two N-terminal glutamic acids (–18 Da) may also occur as well as methionine oxidations (+16 Da). Three Asn deamidation/Asp isomerization hot spots have also been described in the CDRs and shown to negatively impact HER2 antigen binding when degraded (Figure 2A).

Trastuzumab was analyzed on the Exactive Plus EMR MS with resolution set at both 17,500 and 35,000. The deconvoluted mass spectrum calculated using Protein Deconvolution software version 2.0 SP2 represents the classical glycosylation pattern of a mAb with baseline-resolved glycan peaks. Figure 2B shows the complete mass spectrum at resolution of 35,000 and a zoom of the corresponding 23^+ charge state of trastuzumab acquired with the resolution set at both 17,500 and 35,000 in native conditions. Compared to the raw spectrum acquired at 17,500 resolution, an interference peak can be resolved by using a resolution of 35,000 or higher. The high resolution can resolve the analyte from the interferences, therefore, ensuring the low ppm mass accuracy. Molecular weights of each trastuzumab glycoform were measured with good mass accuracy in the low ppm range, as shown in Figure 2C. The mass differences between species are +146 Da and +162 Da, corresponding to a fucose or to the addition of multiple hexose units, respectively.

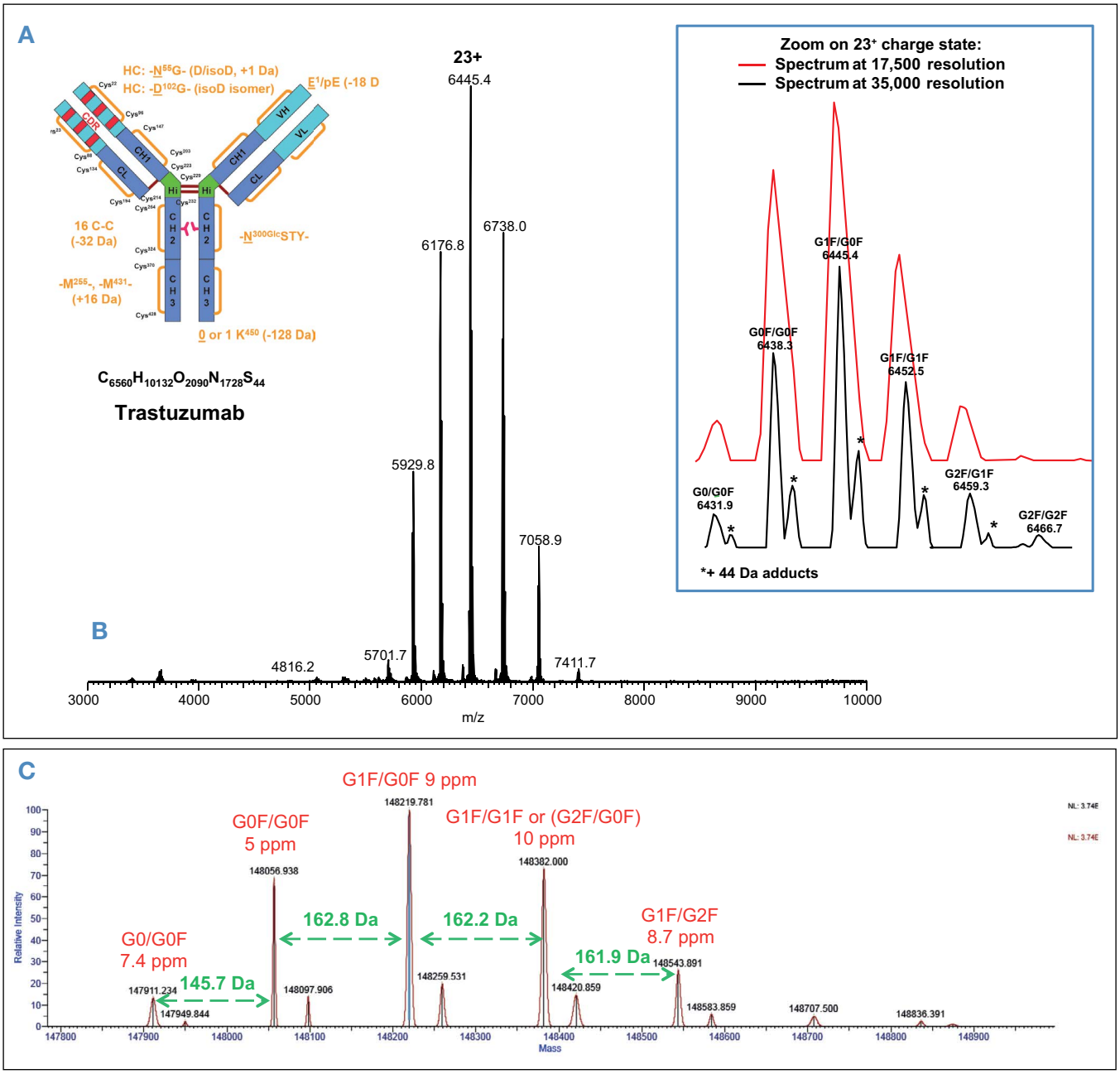

Figure 2. Orbitrap native MS detection of intact monoclonal antibody trastuzumab. A. Intact mAb trastuzumab. B. High-resolution, native MS showing complete mass spectrum and zoom of corresponding 23^{+} charge state. C. Deconvoluted spectrum showing molecular weights of each trastuzumab glycoform with low ppm mass accuracy.

Orbitrap Native MS Analysis of a Monoclonal Antibody-Drug-Conjugate (ADC) Brentuximab Vedotin

Antibody-drug conjugates (ADCs) are an increasingly important modality for treating several types of cancer. The impact of ADCs in this field is due to the exquisite specificity of antibodies that deliver the conjugated cytotoxic agent to targeted tumor cells preferentially, thus reducing the systemic toxicity associated with traditional chemotherapeutic treatments. ADCs are differentiable on the basis of the drug, linker, and also the amino acid residue of attachment on the antibody. Recently, two ADCs were approved by the FDA (Adcetris®, brentuximab vedotin, and Kadcyla®, trastuzumab emtansine) and 35 more are being investigated in clinical trials.

The brentuximab vedotin mass spectrum was recorded at a resolution of 35,000 and in-source CID voltage was set to 75 eV. Figure 3A shows the native deconvoluted mass spectrum of the deglycosylated ADC. Populations with zero (grey), two (black), four (blue), six (red), and eight (green) molecules loaded onto the antibody (payloads) were detected with a mass difference between peaks corresponding to the addition of two payloads (+2,634 Da). The drug loading clearly increases in steps of two, which corresponds to binding of one payload to the two accessible cysteine amino acids after disulfide bridge reduction. For each set of peaks, the drug-to-antibody ratio (DAR) can be determined. Relative ratios of each detected compound were determined using MS peak intensities and served to estimate the mean DAR (4.2), which is in agreement with hydrophobic chromatography data (data not shown). Figure 3B shows the corresponding raw mass spectrum with the entire charge state distribution of brentuximab vedotin under native conditions.

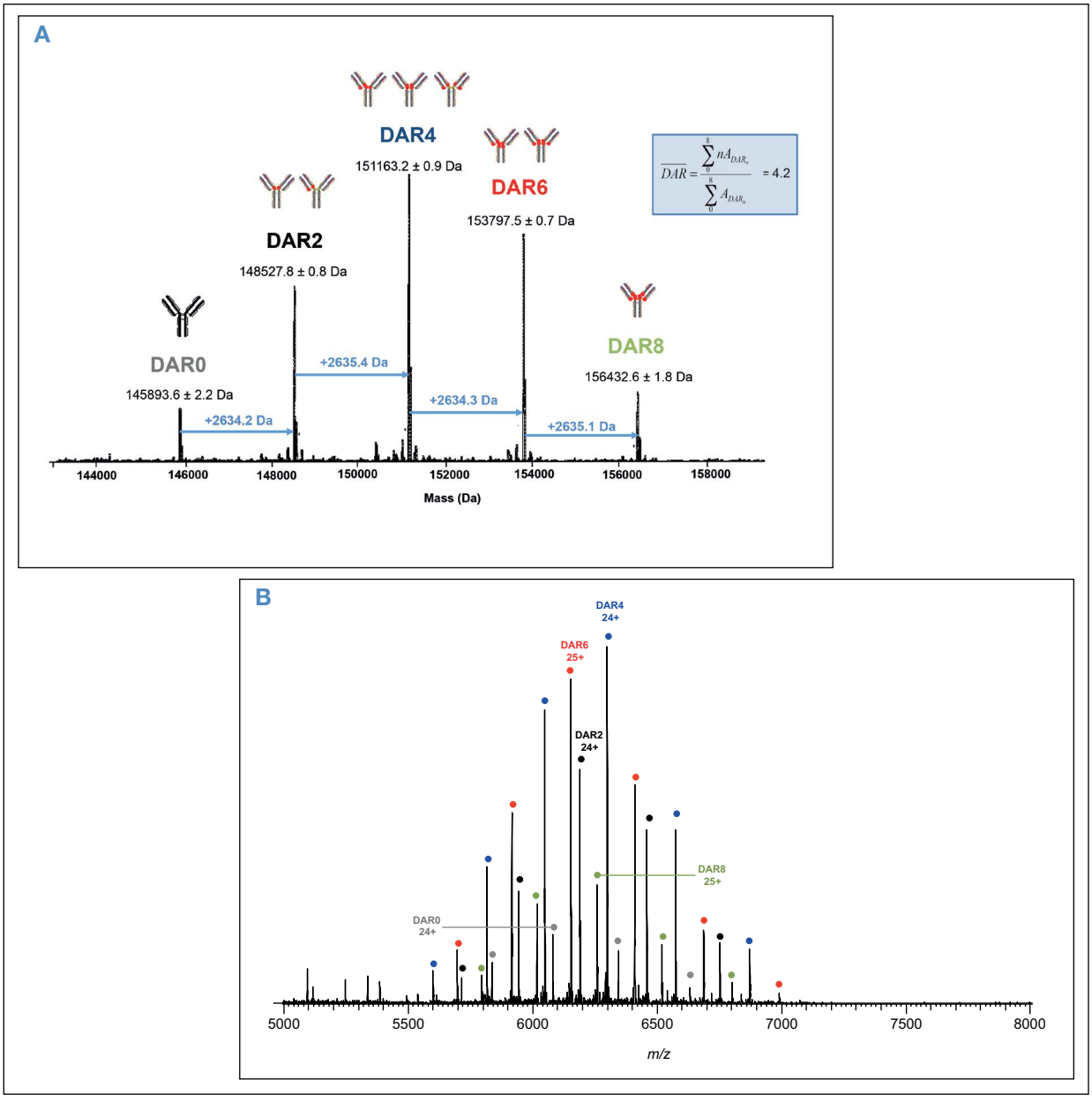

Figure 3. Orbitrap Native MS analysis of a monoclonal Antibody-Drug-Conjugate (ADC). A. Native deconvoluted mass spectrum showing the determination of drug-to-antibody ratio (DAR). B. Raw mass spectrum with the entire charge state distribution of ADC under native conditions.

Orbitrap Native MS Analysis of Immune mAb/Antigen Complexes

Native MS can also be used to analyze mAb/antigen (mAb/Ag) complexes, providing additional information including mAb/antigen binding stoichiometries, specificities and affinities.[4] These properties are essential for originator and biosimilar candidates comparison studies. ESI-MS presents the advantage to allow the direct observation of noncovalent immune complexes without any chemical modification. J10.4 is a commercial mouse monoclonal IgG1 raised against recombinant JAM fusion protein of human origin that is recommended for detection of JAM-A by western blotting and immunopurification techniques. JAM-A, used here as antigen, is a single transmembrane protein belonging to the immunoglobulin superfamily. JAM-A localizes in tight junctions in normal epithelial and endothelial cells where homophilic JAM-A interactions have been shown to be important for regulation of epithelial barrier function.[4,5] This newly identified target is over-expressed in many tumor tissues and therefore is of prime interest as a target in oncology. Two JAM-A molecules are expected to bind to one J10.4 mAb.

The native mass spectrum of mAb/antigen complexes was recorded at a resolution of 35,000 with the in-source CID voltage set to 150 eV. As shown in Figure 4A, when an 4-fold excess of JAM-A (20 µM) is added to J10.4 mAb (5 µM), three species are detected: the intact free mAb (MW 150237.1 ± 1.1 Da, black), 1:1 (MW 174304.4 ± 2.0 Da, blue) and 1:2 (MW 198369.6 ± 2.3 Da, red) mAb:JAM-A complexes. Native MS thus confirmed that two JAM-A molecules can bind to J10.4 mAb. MWs correspond to the main G0F/G0F glycoforms. Relative abundances were estimated from MS peak intensities and proportions of mAb:Ag complexes at 1:1 and 1:2 stoichiometries were observed to be 37% and 30%, respectively, while free mAb represents 33%. Figure 4B shows the corresponding mass spectrum with the entire charge state distribution in native conditions.

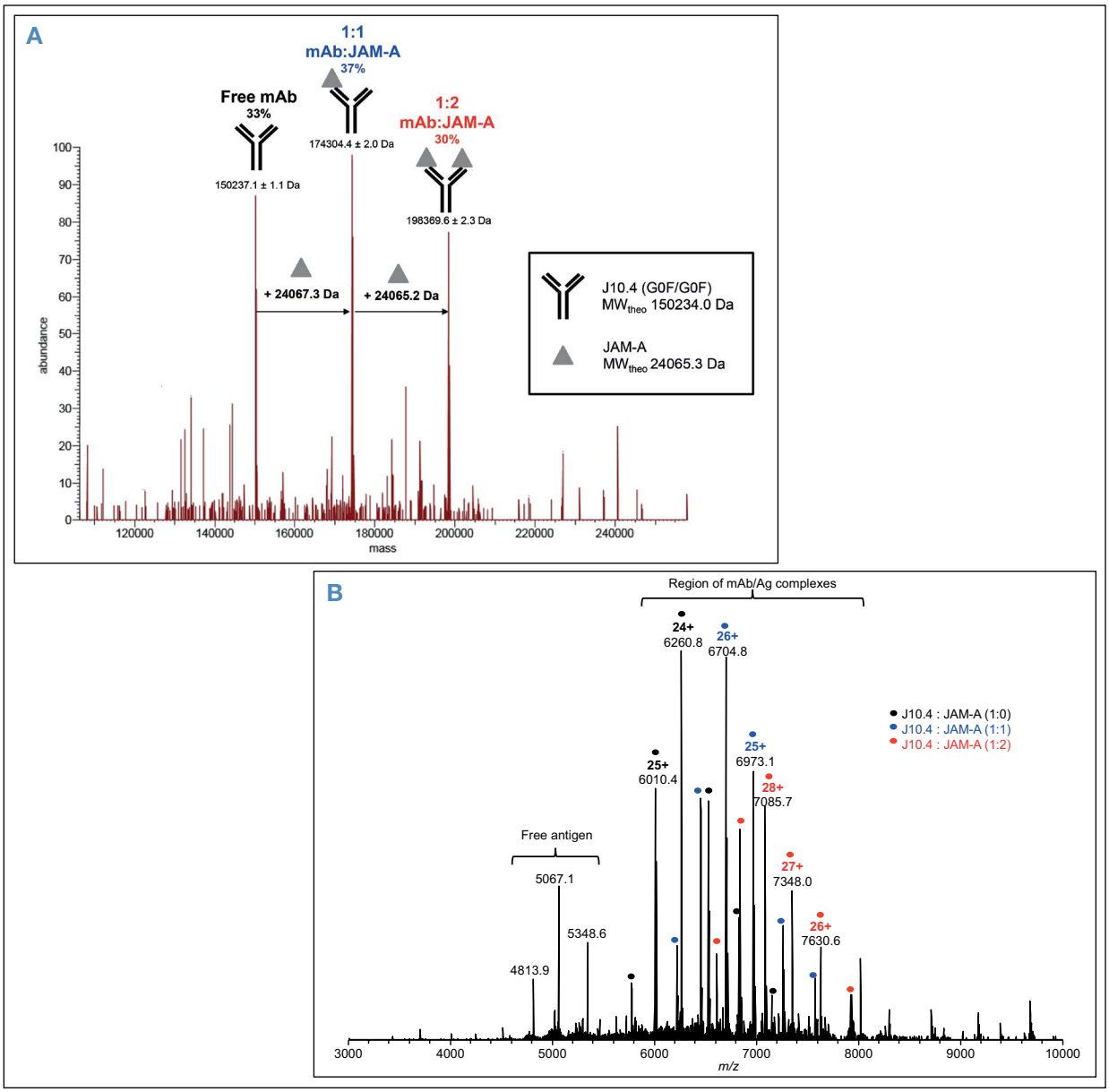

Figure 4. Orbitrap native MS detection of immune mAb/antigen complexes. A. Deconvoluted mass spectrum showing mAb/antigen binding stoichiometries. B. Charge state distribution in native conditions.

Native MS Analysis of a Mixture of Eleven N-deglycosylated Humanized Antibodies

Analysis of mAb mixtures is of utmost interest for high-throughput screening purposes and for therapeutic use to block simultaneously multiple epitopes. Indeed cocktails of mAbs with additive or synergic effects are increasingly foreseen as potential new therapeutic entities.

Figure 5A presents a convoluted mass spectrum of a mixture of eleven distinct deglycosylated humanized IgG antibodies. This mix includes two marketed therapeutic mAbs (rituximab and trastuzumab) and nine point mutation variants of the Hz6F4-2 mAb.[4,5] Figure 5B shows a full native mass spectrum of the mAb mix with an in-source CID energy set to 100 eV.

The well-resolved ion signals at a detection resolution of 140,000 and accurately measured masses enable the unambiguous assignment of ten out of the eleven compounds. Trastuzumab and Hz6F4-2v6 could not be differentiated due to very close molecular weights (2 Da). Peaks corresponding to Hz6F4-2 and Hz6F4-2v3, which differ by only 21 Da in mass, are clearly distinguished on the mass spectrum. However, they are not baseline resolved, and when combined with the low signal-to-noise (S/N) ratio (S/N < 20), that causes a relatively low mass accuracy for Hz6F4-2. However, with a good signal-to-noise ratio (S/N > 50), even without baseline-resolved peaks, for example, peaks of Hz6F4-2v9 and 6F4-2v10, the mass accuracies are achieved in the low ppm range for both species. The measured and theoretical masses for the mixture of eleven N-deglycoslated humanized antibodies are listed in Table 1.

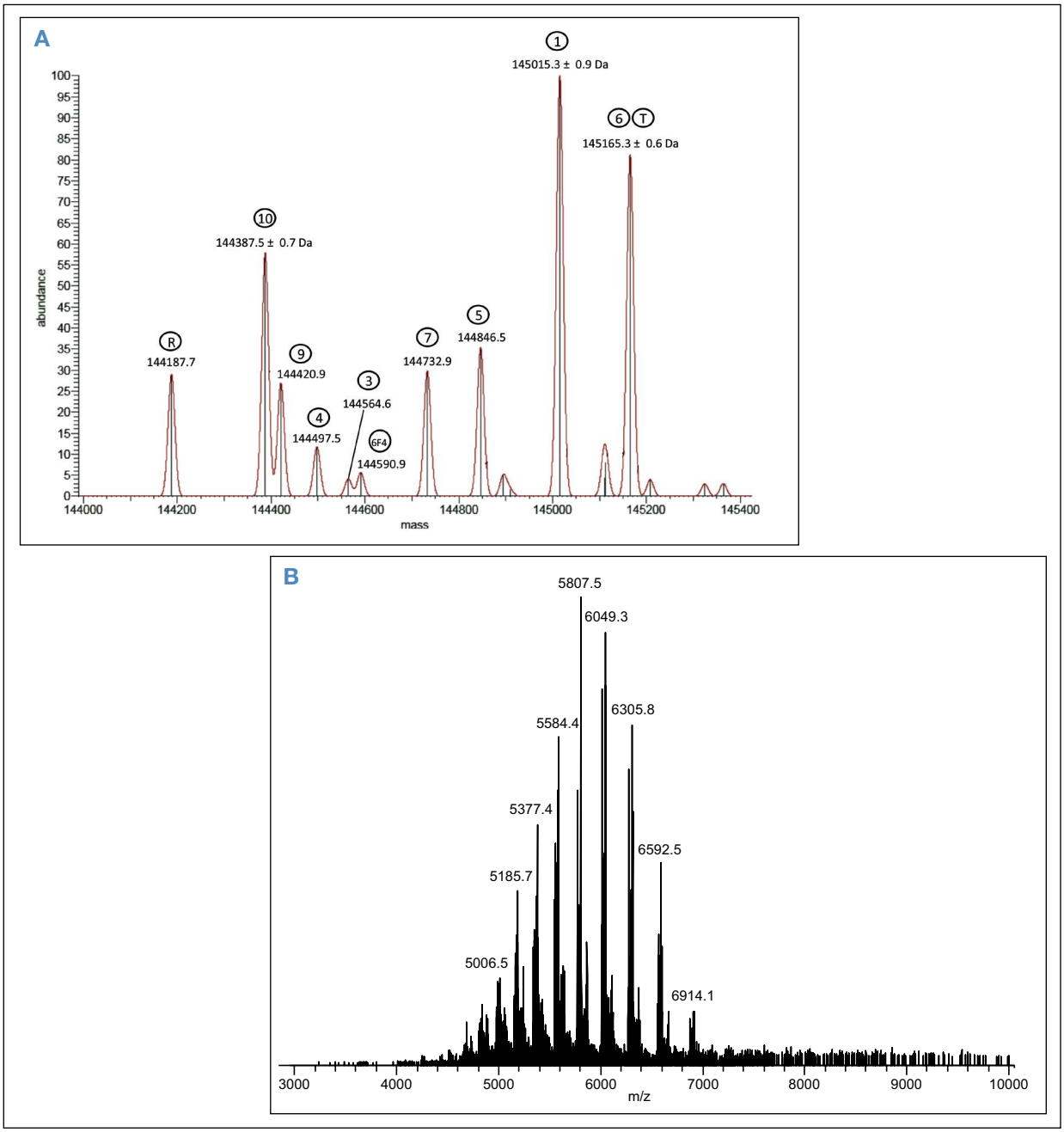

Figure 5. Native MS analysis of a mixture of eleven N-deglycosylated humanized antibodies. A. Deconvoluted mass spectrum. B. Charge state distribution in native conditions.

Table 1. Measured and theoretical masses for the mixture of eleven N-deglycosylated humanized antibodies at an Orbitrap detection resolution of 140,000

	Species	Theoretical Masses (Da)	Measured Masses (Da)	Mass Accuracy (ppm)
R	Rituximab	144186.3	144187.7	9.7
10	6F4-2 v10	144388.3	144387.5	5.5
9	6F4-2 v9	144420.5	144420.9	2.8
4	6F4-2 v 4	144498.4	144497.5	6.2
3	6F4-2 v3	144564.4	144564.6	1.4
6F4	6F4-2	144585.5	144590.9	37.3
7	6F4-2 v7	144732.5	144732.9	2.8
5	6F4-2 v5	144846.9	144846.5	2.8
1	6F4-2 v1	145015.3	145015.3	0
6	6F4-2 v6	145163.3	N.D	N.D
T	Trastuzumab	145165.5	145165.3	1.4

Conclusion

In the analysis (0.3–5 min) using the Exactive Plus EMR MS, molecular weight measurements of mAb and related products in the low ppm mass deviation range allowed the identification of all species simultaneously present in solution. The number of DAR and relative abundance of mAb/Ag complexes was also assessed with the peaks intensities serving for relative quantification of the detected species.

- The high resolving power of the Orbitrap mass analyzer can baseline resolve a native mAb's glycan peaks, as well as the interference peaks, ensuring an excellent mass accuracy in the low ppm range.
- The Exactive Plus EMR MS is able to sensitively characterize ADC complexes with mass differences between peaks corresponding to different additional number of payloads/drugs. For each set of peaks, the drug-to-antibody ratio (DAR) can be determined as well as the relative ratio of each detected compound in order to assess the mean DAR value.
- Native Orbitrap MS can reveal the number of antigens bound to mAbs. Relative abundances of mAb/Ag complexes at different stoichiometries can be achieved from MS peak intesities.
- The Exactive Plus EMR MS enables the high throughput screening of mAb mixtures, ensuring a excellent mass accuracy for each individual mAb.

Acknowledgements

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