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High Resolution Mass Spectrometry of Antibody Drug Conjugates Using the Orbitrap Mass Analyzer

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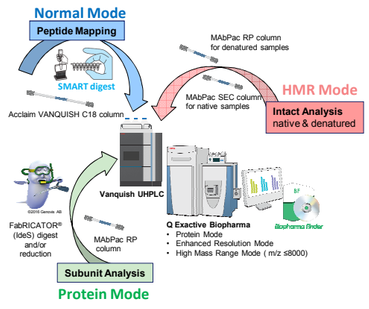
INTRODUCTION

The complexity of modern therapeutic proteins presents a great analytical challenge. Most often a whole set of analytical techniques is used to characterize these proteins. With mass spectrometry alone several complementary methods are required to analyze protein drugs on the intact protein and on the peptide levels. Native mass spectrometry on the intact protein level allows also for the analysis of molecules which rely on non-covalent interactions to preserve critical structural features, such as antibody-drug conjugates (ADC). In addition the use of 100% aqueous buffers in native MS analysis produces lower charge states detected at higher m/z values compared to analysis under denaturing condition and thus improves mass separation of heterogeneous mixtures [1]. Recent technical advance-ments on the benchtop Orbitrap mass spectrometry platform offer now complete characterization of the complex conjugates composed of small molecule drugs attached to antibodies on a single instrument platform. In this presentation relevant workflows covering chromato-graphy, mass spectrometry and data analysis for ADC characterization are laid out and data obtained from two different types of ADCs are presented.

Figure 1: Operating modes for the three major BioPharma workflows: Normal Mode, Protein Mode and HMR mode



MATERIALS AND METHODS

Samples:
Samples used in this study are Trastuzumab (Roche, UK; tradename Herceptin),Trastuzumab emtansine (Roche UK; tradename Kadcyla) and Brentuximab vedotin (Seattle Genetics, US; tradename Adcetris).
All antifbody samples were prepared for intact mass analysis by dissolving the dried substances according to the manufacturer's instructions. For denaturing LC-MS intact analysis 1 µg of protein samples were separated using a 10 min gradient of 10-90% ACN in H_2O and 0.1% formic acid (Thermo MAb-Pac RP; flow rate 250 µL/min). For native LC-MS intact analysis 10 µg of sample was desalted online using size exclusion chromatography with 50 mM NH_4OAc using isocratic elution at a flow rate of 300 µL/min and directly infused into the mass spectrometer via electrospray ionization.

Chromatography:
A Vanquish UHPLC system was used for all LC/MS experiments. For native analysis, 50 mM ammonium acetate buffer (99.99%, Sigma Aldrich) was used. On a size exclusion column. Reversed phase chromatography was performed with water/0.1% formic acid and acetonitrile/0.1% formic acid on a MAbPac RP 2.1x50 mm column.

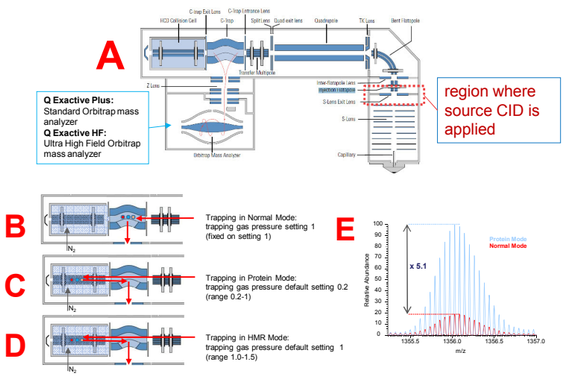
Mass Spectrometry:
Mass spectrometers used in this study were the commercially available Thermo Scientific™ Q Exactive™ Plus and Q Exactive™ HF equipped with BioPharma Option. The instruments were operated under Tune 2.8 instrument control software in HMR mode which allows for detection of a mass range up to 8,000 m/z. The S-lens RF level was allowed to be increased to a setting of 200 in HMR mode and set to that level for all experiments shown here. Resolution settings between 15,000 and 70,000 were applied.

Data Analysis:
Data analysis was performed with Thermo Scientific™ BioPharma Finder™ 2.0 software using the ReSpect™ algorithm and Sliding Window feature. DAR ratios were calculated automatically based on deconvolution species that were identified using the publicly-available FASTA sequences for Brentuximab vedotin and Trastuzumab emtansine, a mass tolerance of 50 ppm, and a static modification of Gln>Pyro-Glu for the heavy chain.

RESULTS

There are many factors that play a key role in the analysis of proteins, some of which relate to sample preparation (buffers, solvents, additives) while others relate to the mass spectrometer's source conditions as well as the physical environment inside the instrument [2,3]. The Q Exactive Plus and Q Exactive HF mass spectrometers (Figure 2A) have previously been introduced with the Protein Mode option, which was one of many advancements for intact protein analysis on the Orbitrap platform. For these two instruments an automated HCD gas control was introduced by using an electronically controlled valve for nitrogen gas in the HCD cell for easier optimization of experimental conditions required for different types of analyses wished to run on a single platform.
In Normal Mode pressure settings are factory-optimized, suitable for most analyses and ions are cooled in the C-trap (Fig. 2B). In Protein Mode the default trapping gas pressure setting is 0.2 and that corresponds to a ΔHV which is 5x lower than in Normal Mode. Additionally, ions are transferred and cooled in the HCD cell and thus have a longer flight path (Fig. 2C). The new High Mass Range (HMR) Mode that is especially required for the analysis of proteins under native conditions when samples are kept in aqueous buffers with no organic solvents involved at near neutral pH. For HMR mode the default trapping gas pressure setting is the same as in Normal Mode and can even be slightly increased for even improved trapping of certain species such as protein complexes and heterogeneous large proteins (e.g. antibody drug conjugates). The trapping path in HMR mode is the same as in Protein Mode with ion cooling taking place in the HCD cell. And also, mass detection is enabled ranging up to m/z 8000 compared to m/z 6000 in the two other modes.

Figure 2: A) Schematic of the Q Exactive Plus/HF mass spectrometers and differences in the trapping path in the three different operating modes available: B) Normal Mode, C) Protein Mode and D) HMR Mode. E) Illustration of improvement in signal intensity for +17 charge state of a mAb light chain comparing Protein Mode and Normal Mode.



Native MS intact protein analysis allows direct observation of molecules which rely on non-covalent interactions to preserve critical structural features, such as maintaining interchain associations which hold together cysteine-linked ADCs. The use of 100% aqueous physiological pH buffers in native MS analysis produces a fewer number and lower charge states (increased m/z) compared to denaturing conditions (Figure 3) and improves mass separation which is beneficial in particular for heterogeneous mixtures. We demonstrate this phenomenon using the cysteine-linked ADC *Brentuximab vedotin* (Figures *4 and 5)* and the Asparagine-linked ADC Trastuzumab emtansine (Figure 6). Based on mass spectra acquired under native condition DAR values for both ADCs were determined that are well in agreement with previously reported numbers [4,5].

Figure 3. Trastuzumab analyzed under native (A) and denaturing (B) conditions resulting in highly similar deconvolution results (C).

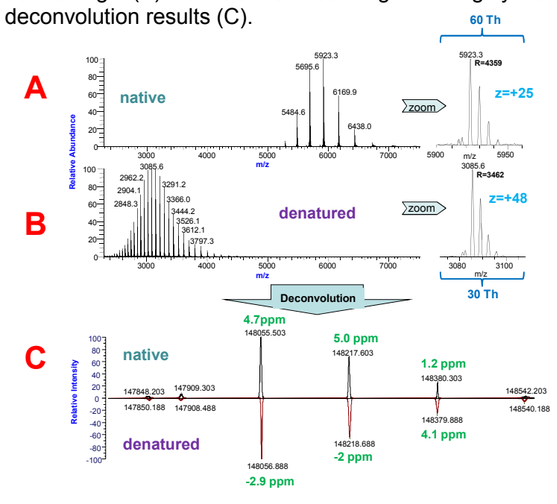


Figure 4: LC-MS analysis of intact Brentuximab vedotin under denaturing conditions: (A) Reversed phase chromatogram and the resulting averaged MS spectrum provides a complex mixture of charge state envelopes as well as vcMMAE-specific reporter fragment ion at m/z 718. **(B)** Data analysis with ReSpect deconvolution and Sliding Window integration show roughly six covalently-structured forms of unraveled cysteine-linked ADC.

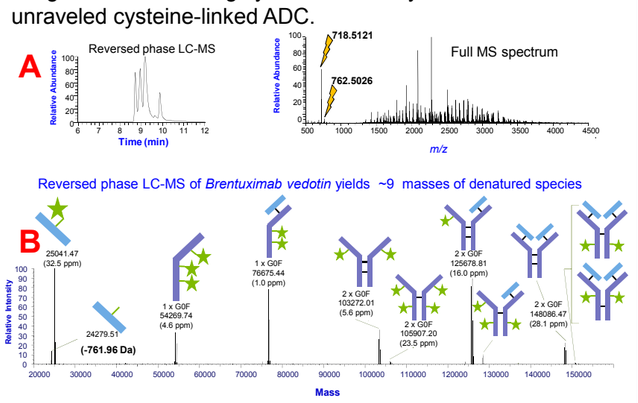
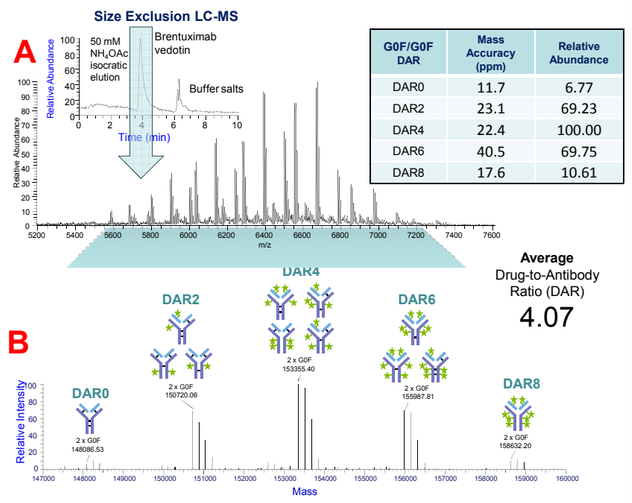
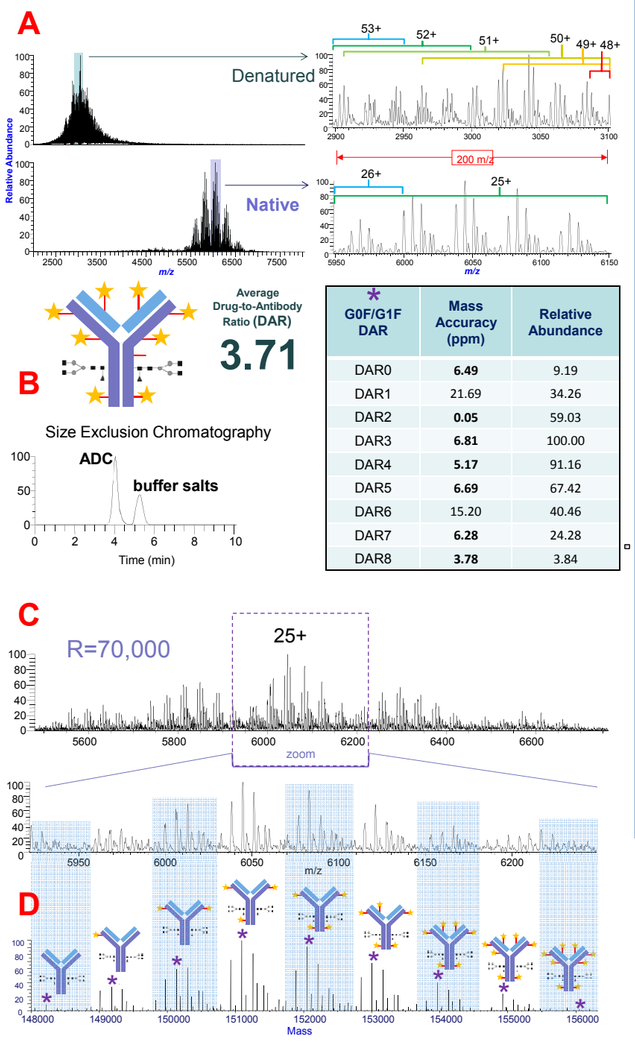


Figure 5: LC-MS analysis of intact Brentuximab vedotin under native conditions:
A) Size exclusion chromatogram and mass spectrum obtained from averaging 2 min chromatographic time which includes all DAR forms (DAR 0-8). **B)** ReSpect deconvolution result using the Sliding Window feature. A pattern of lower abundance species were detected corresponding to a low abundance loss of 762 Da from each glycoform at each DAR. Based on the individual deconvolved abundances of the G0F/G0F glycoform, an average DAR value of 4.07 was calculated which is consistent with previous reports [4].



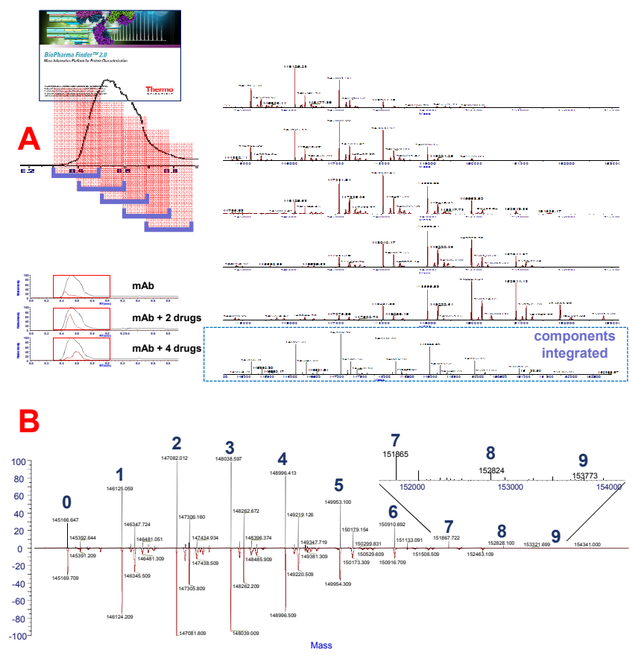
G0F/G0F DAR	Mass Accuracy (ppm)	Relative Abundance
DAR0	11.7	6.77
DAR2	23.1	69.23
DAR4	22.4	100.00
DAR6	40.5	69.75
DAR8	17.6	10.61

Figure 6: **A**) Mass spectra obtained for Trastuzumab emtansine under denaturing and native conditions. **B**) Calculation of DAR ratio based on **C**) the deconvoluted spectra obtained using the Sliding Window feature in BioPharma Finder software.



G0F/G1F DAR	Mass Accuracy (ppm)	Relative Abundance
DAR0	**6.49**	9.19
DAR1	21.69	34.26
DAR2	**0.05**	59.03
DAR3	**6.81**	100.00
DAR4	**5.17**	91.16
DAR5	**6.69**	67.42
DAR6	15.20	40.46
DAR7	**6.28**	24.28
DAR8	**3.78**	3.84

Figure 7: A) The Sliding Window feature in BioPharma Finder provides a composite spectrum based on stepwise deconvolution results across the full RT range. **B)** Low abundant species are represented more accurately and are less prone to be underrepresented which is in particular useful for low abundant, low and high drug loads of ADCs.



CONCLUSIONS

- The BioPharma option extends the capabilities of the Q Exactive Plus and Q Exactive HF mass spectro-meters allowing for mass detection up to m/z 8000 in the new High Mass Range (HMR) Mode
- This new operating mode extends the instrument's capabilities to cover all three major workflows for BioPharma characterization and allows for intact mass analysis of larger proteins such as antibodies and antibody drug conjugates under native conditions.
- Native LC/MS intact mass analysis of Brentuximab vedotin resulted in detection of intact ADC forms, DAR0-8. ReSpect deconvolution and Sliding Window integration showed an average DAR of 4.07, consistent with previous studies.
- Native LC/MS intact mass analysis of Trastuzumab emtansine resulted in detection of intact ADC forms, DAR0-8. ReSpect deconvolution and Sliding Window integration showed an average DAR of 3.71, consistent with previous studies.
- The sliding window feature in BioPharma Finder software is essential for accurate determination of DAR ratios.

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TRADEMARKS/LICENSING

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