Full Characterization of Heterogeneous Antibody Samples Under Denaturing and Native Conditions on a Hybrid Quadrupole-Orbitrap Mass Spectrometer

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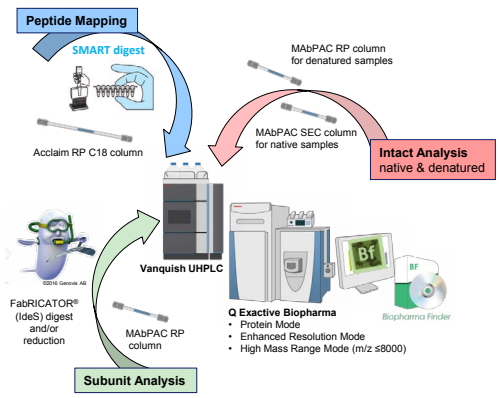
INTRODUCTION

MS analysis of antibodies at the protein and peptide levels is critical during development and production of biopharmaceuticals. The compositions of current generation therapeutic proteins are often complex due to their heterogeneity caused by various modifications which are relevant for their efficacy. Intact proteins analyzed by ESI-MS are detected in higher charge states that also provide more complexity in mass spectra. Protein analysis in native or native-like conditions with zero or minimal organic solvent and neutral or weakly acidic pH decreases charge state value resulting in mAb detection at higher m/z ranges with more spatial resolution.

Here we have analyzed the profiles of three monoclonal antibodies under denaturing and native conditions by direct infusion with offline desalting and with on-line desalting via size exclusion and reversed phase type columns. The samples were analyzed with three different workflows (Figure 1): 1) the analysis on the intact level under native and denaturing conditions; 2) the analysis of subunits following IdeS digestion and or reduction; and 3) peptide mapping following sample preparation applying the Thermo Scientific™ SMART Digest™ kit.

The mass spectrometer used for all experiments was a commercially available hybrid quadrupole-Orbitrap mass spectrometer with modified instrument control software to allow for improved high mass transmission and mass detection up to 8000 m/z. This modification is necessary for the analysis of antibody samples on the intact level under native conditions requiring the detection of masses beyond the standard mass range of up to 6000 m/z.

Figure 1. Workflow overview covered in this poster



MATERIALS AND METHODS

The three commercially available monoclonal antibodies Trastuzumab, Trastuzumab-Emtansine, Infliximab and Bevacizumab obtained in manufacturer's formulation buffer were used for all experiments.

Sample Preparation:

For native intact mass analysis using SEC-LC/MS, the antibodies were injected without any further dilution. For direct infusion analysis under denaturing conditions, samples were desalted via Bio-Rad P6 desalting columns and diluted to achieve 50% ACN/0.1% formic acid in the solvent. For subunit analysis samples were either reduced in 4M GdHCl/50mM TCEP or first digested with FabRICATOR® (Genovis) enzyme according to the manufacturer's protocol and then reduced. For peptide analysis antibodies were proteolytically digested using the SMART Digest kit following reduction with 10mM DTT. For disulfide bridge analysis, part of the sample was analyzed non-reduced.

Chromatography:

For intact mass analysis under native conditions proteins were online desalted using size exclusion chromatography (SEC) and isocratic elution with 50mM or 100mM ammonium acetate. For reversed phase chromatography of proteins, subunits and peptides under denaturing conditions a gradient of solvent A consisting of water 0.1% formic acid and solvent B consisting of acetonitrile/0.1% formic acid was used.

Mass Spectrometry:

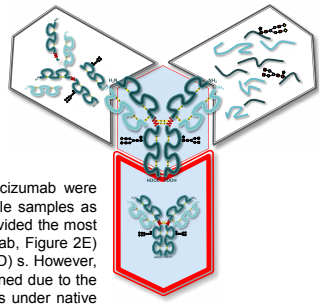
The mass spectrometers used for all experiments were commercially available Thermo Scientific™ Q Exactive™ Plus and Q Exactive™ HF mass spectrometers with modified instrument control software to allow for improved high mass transmission and mass detection up to 8000 m/z.

Data Analysis:

Intact protein, IdeS digest and peptide mapping raw data files were analyzed with Thermo Scientific™ BioPharma Finder™ 1.0 SP1 software. Data for the disulfide bridge mapping was performed with BioPharma Finder 2.0 beta-software. For top-down analysis data ProSight Lite was used after Xtract spectral deconvolution.

RESULTS – Intact mAb Analysis

- **Native vs. denatured**
- **Antibody Drug Conjugate (ADC)**



The three antibodies Trastuzumab, Infliximab and Bevacizumab were analyzed under native and denaturing conditions as single samples as well as in a mixture (Figure 2A,C). The mixed sample provided the most complex pattern (also due to Lys-heterogeneity of Infliximab, Figure 2E) which can be well resolved in both conditions (Figure 2 B,D) s. However, under native conditions a higher spatial resolution is obtained due to the detection at higher m/z. The benefit of analyzing samples under native conditions becomes even more apparent for the antibody drug conjugate Trastuzumab-Emtansine (Figure 3 A,B) resulting in higher spatial resolution and improved, baseline resolved peaks even of overlapping peak patterns.

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Figure 2. Intact mAb analysis under native and denaturing conditions

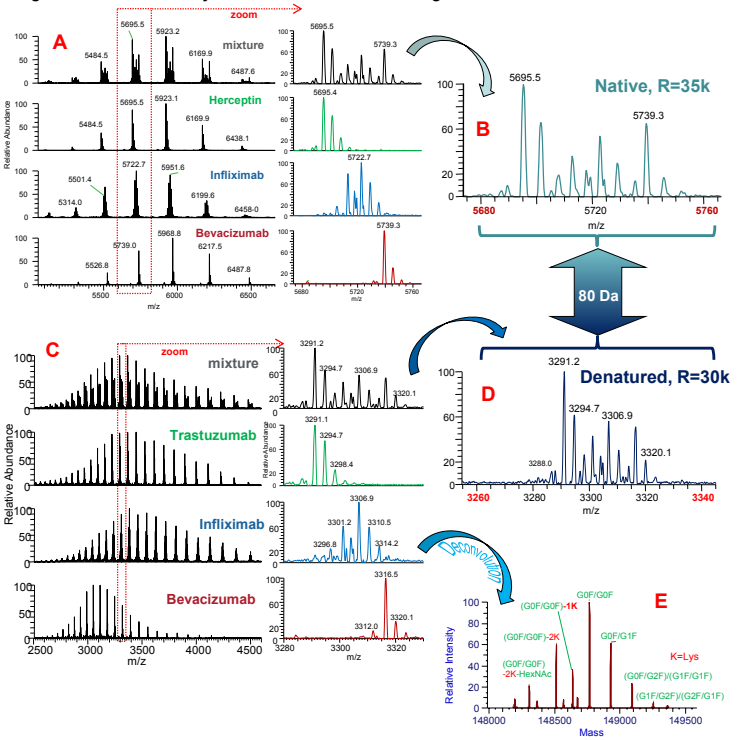


Figure 3. Trastuzumab-Emtansine ADC under native (A) and denaturing conditions (B)

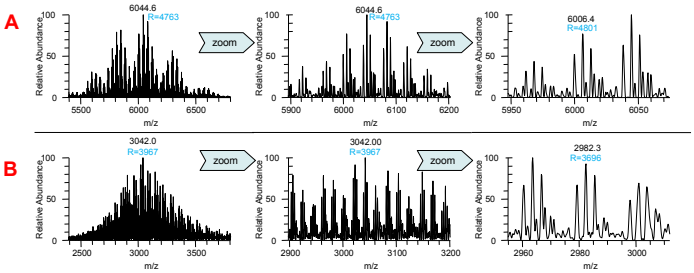


Figure 3: Intact (A) and (B) top down subunit analysis of reduced Trastuzumab

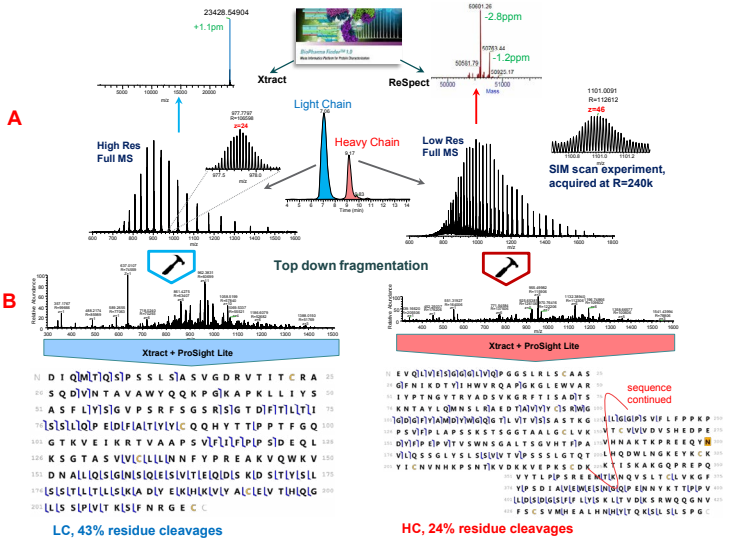
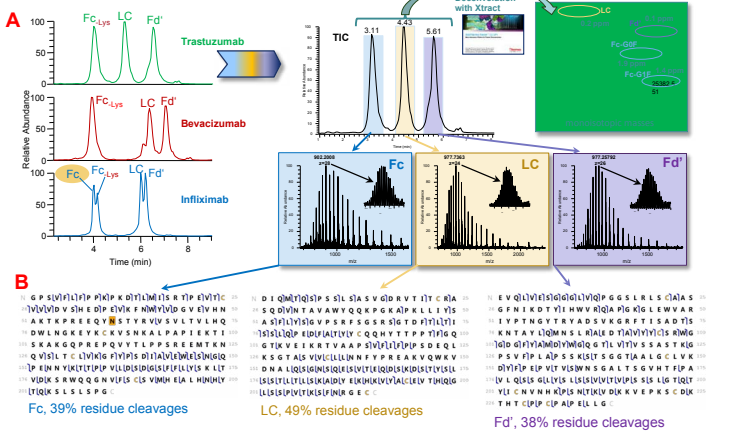
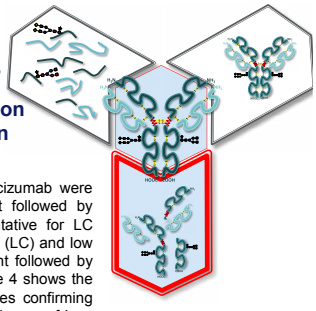


Figure 4. Intact (A) and (B) Top Down Subunit Analysis after FabRICATOR digest and reduction



RESULTS – mAb Subunit Analysis

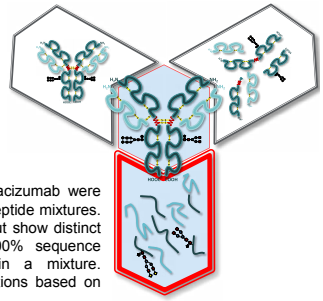
- **Reduced mAb /middle down fragmentation**
- **IdeS digest + middle down fragmentation**



The three antibodies Trastuzumab, Infliximab and Bevacizumab were analyzed after reduction and after FabRICATOR digest followed by reduction. Figure 3 shows Trastuzumab as a representative for LC separation of light and heavy, Full scan acquisition at high (LC) and low (HC) resolution settings to achieve intact molecular weight followed by top down analysis to obtain sequence confirmation. Figure 4 shows the LC chromatograms obtained for the 3 individual antibodies confirming heterogeneity of the FC region of Infliximab due to a low degree of Lys-truncation. Very accurate intact masses for the Fc, LC and Fd' subunits are obtained and top town analysis thereof provides very good sequence coverage.

RESULTS – Peptide Mapping

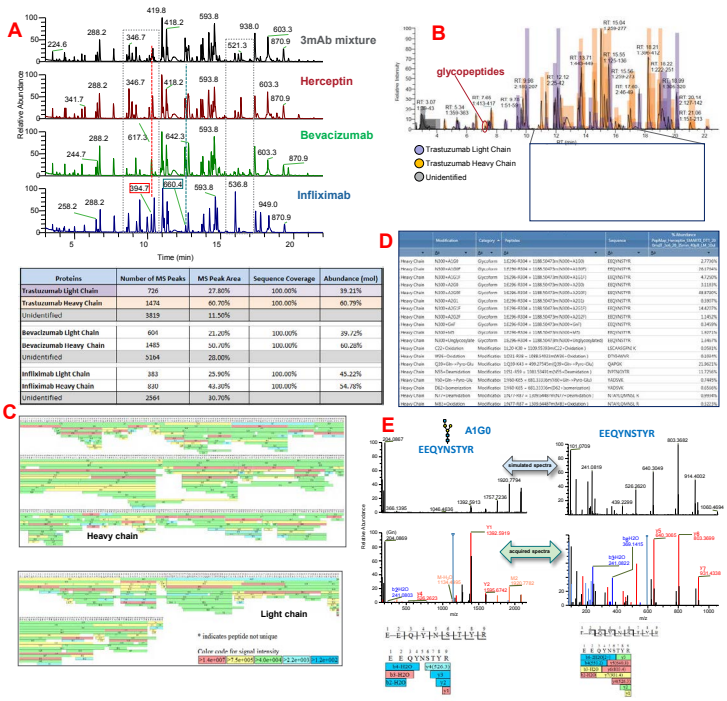
- **Sequence coverage**
- **Modifications**
- **Disulfide bridge mapping**



The three antibodies Trastuzumab, Infliximab and Bevacizumab were analyzed after performing a SMART digest resulting in peptide mixtures. Obtained Base Peak Chromatograms are very similar but show distinct differences. All antibodies can be identified with 100% sequence coverage when analyzed separately as well as in a mixture. Glycopeptides are confidently identified in many variations based on MS/MS spectra as shown in one example.

Figure 7 highlights as well on the peptide level the low degree of Lys-truncation of Infliximab compared to Trastuzumab and Bevacizumab, confirming the results obtained on the intact and subunit levels.

Figure 6. A) Base peak chromatograms obtained for peptide mapping (B) of individual and mixed mAbs with sequence coverage (B) , identified glycoforms (D) and (E) example spectra for a peptide in a glycosylated and unglycosylated forms



Proteins	Number of MS Peaks	MS Peak Area	Sequence Coverage	Abundance (mol)
Trastuzumab Light Chain	726	27.80%	100.00%	39.21%
Trastuzumab Heavy Chain	1474	60.70%	100.00%	60.79%
Unidentified	3819	11.50%		
Bevacizumab Light Chain	604	21.20%	100.00%	39.72%
Bevacizumab Heavy Chain	1485	50.70%	100.00%	60.28%
Unidentified	3164	28.00%		
Infliximab Light Chain	383	25.90%	100.00%	45.22%
Infliximab Heavy Chain	820	48.80%	100.00%	54.78%
Unidentified	2564	30.70%		

Figure 7. XICs (A) and (B) Infliximab Full MS spectra of +1/+2 charge states for Lys-truncated vs. full length C-terminal peptides for the 3 mAbs

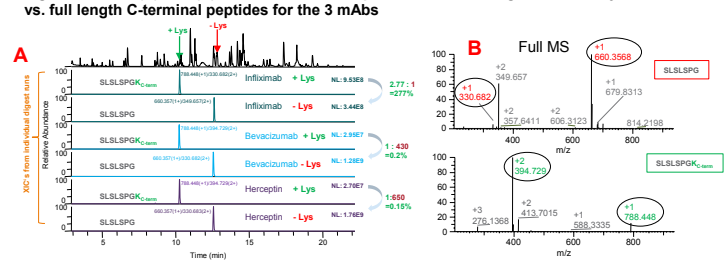
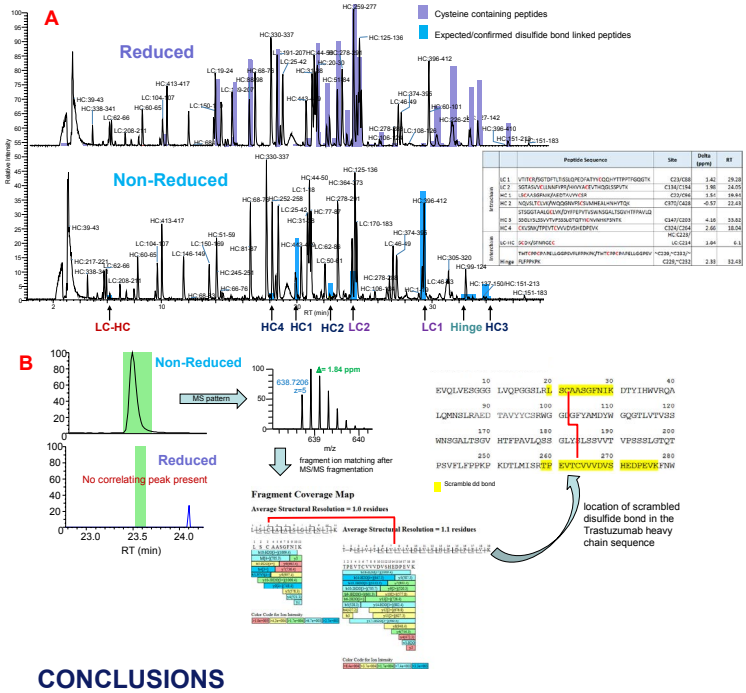


Figure 8 highlights results from a disulfide bond study on Trastuzumab comparing a reduced vs. non-reduced sample. Differences in the base peak chromatograms are obvious by visual inspection and using BioPharma Finder software they are identified as either free Cys-containing peptides (reduced sample) or as part of disulfide linked peptides (non-reduced sample) and provided as shaded peaks in the chromatogram. Also, a number of scrambled, unexpected disulfide bonds are identified and represented by one example showing a peak unique to the reduced sample, the isotope profile detected with superb mass accuracy and identification of scrambled peptide supported by MS/MS.

Figure 8. (A) Disulfide mapping analysis results confirming the eight expected disulfide bonds (table) and (B) an example of an unexpected scrambled disulfide bond



CONCLUSIONS

- Here we cover the three major workflows for characterization of biopharmaceuticals on one single instrument LC-MS platform using single and mixed antibody samples: 1) intact mass analysis, 2) subunit analysis and 3) peptide mapping - aiming at confirming antibody sequences and disulfide bonds, elucidating modifications and probing for scrambled disulfide bonds.
- The new High Mass Range mode now also allows for analysis of antibodies and antibody drug conjugates under native conditions requiring a higher mass range up to m/z 8000.
- Excellent mass accuracy, resolution and sequence coverages are obtained for results at all stages of the workflows: intact molecular masses, masses of subunits, top-down fragments of subunits as well as peptides providing high confidence results.
- For Infliximab unusually low Lys-truncation was observed and confirmed at all molecular levels: in the fully intact mAb, in the Fc subunit and at the peptide level.

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