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Evaluation of Optimal Conditions for Antibody Subunit Analysis on a Quadrupole-Orbitrap Mass Spectrometer

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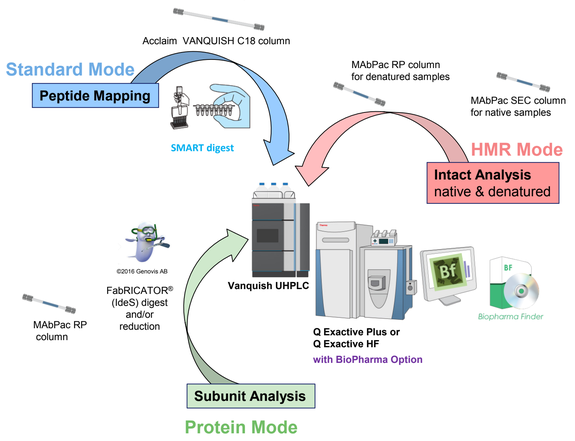
INTRODUCTION

Mass spectrometric analysis of antibodies at the protein and peptide levels is critical during development and production of biopharmaceuticals. Intact mass and peptide mapping analyses have become essential techniques in mAb analysis. The analysis of antibody subunits often provides additional and complementary information with the advantage of requiring only very little sample preparation. Reduction is an optional step in the sample preparation, and when combined with enzymatic digestion with IdeS resulting in ~23-25 kDa subunits. Subunits in that molecular weight range are most amenable to top-down analysis and can provide highest sequence coverage.

Here we have reviewed three commercially available monoclonal antibodies under different reduction conditions aiming at complete reduction of **inter-** and **intra**-chain disulfide bridges for unambiguous determination of accurate intact masses of the subunits and most efficient fragmentation and sequence coverage in top-down experiments.

For top-down analysis the presence of disulfide bridges is a generally limiting factor preventing efficient fragmentation in particular in the regions between two cysteine residues involved in a disulfide bridge. Whereas the reduction of **inter**-chain disulfide bridges is often very efficient, reduction of the **intra**-chain disulfide bridges often require more stringent conditions for efficient reduction and show higher variability amongst different antibodies.

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



MATERIALS AND METHODS

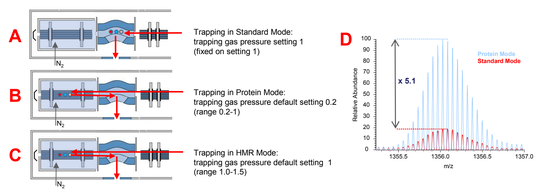
The three commercially available monoclonal antibodies Trastuzumab, Infliximab and Rituximab, obtained either as solution or dried powder and then dissolved according to the manufacturer's protocol, were used for all experiments.

Sample Preparation:
For analysis of light and heavy chain (LC, HC) samples were either reduced with a) DTT b) DTT in the presence of 4M guanidinium hydrochloride (GdHCl), c) TCEP, or d) TCEP in the presence of 4M GdHCl., each performed at room temperature or 57 °C. For LC, Fc/2, and Fd' subunit analysis antibodies were first digested with FabRICATOR® (Genovis) enzyme according to the manufacturer's protocol and then reduced using conditions a)-d) as listed above.

Chromatography:
Reversed phase chromatography of subunits was performed on a Thermo Scientific™ Vanquish™ Horizon UHPLC system using a 2.1 x 100 mm MAbPac RP column with a gradient of solvent A consisting of water 0.1% formic acid and solvent B consisting of acetonitrile/0.1% formic acid. The gradient started at 25 %B, held for 1 min and then ramped to 32 %B in 12 min, further ramped to 80 %B and held for 2 min before returning back to 25 %B.

Mass Spectrometry:
The mass spectrometer used for all experiments was the Thermo Scientific™ Q Exactive™ HF mass spectrometer equipped with the BioPharma option (Figure 1) which includes in addition to the Standard Mode the Protein Mode and High Mass Range Mode (HMR) (Figure 2), The mass spectrometer was operated with Thermo Scientific™ Xcalibur™ 4.0 software in combination with Thermo Scientific SII for Xcalibur 1.3.

Figure 2: Schematic of the differences in the trapping path in the three different operating modes available: **A)** Standard Mode, **B)** Protein Mode and **C)** HMR Mode. **D)** Illustration of improvement in signal intensity for +17 charge state of a mAb light chain comparing Protein Mode and Standard Mode.



Data Analysis:
Raw data files were analyzed with Thermo Scientific™ BioPharma Finder™ 2.0 software for deconvolution. For top-down analysis ProSight Lite was used after deconvolution with Xtract.

RESULTS

The levels of **inter-** and **intra**chain disulfide bond reduction for subunits (Figure 3) from the different antibodies Trastuzumab, Rituximab and Infliximab were evaluated using DTT and TCEP at room temperature, at 57 °C and with and without denaturation.

The level of reduction can be derived by reviewing the shape of the charge envelope on the Full MS level. As Figure 4B and 5C-E demonstrate spectra obtained from subunits containing intact **intra**chain disulfide bridges show charge envelopes with bimodal distributions whereas in reduced condition the envelope has a rather Gaussian shape representing rather high charge states. The shift towards higher charge states is also supported by the Protein Mode providing a longer flight path an reduced pressure for ion trapping.

All data in this study were acquired in Protein Mode, thus differences in charge state distributions are exclusively due to different structural shapes of the subunits resulting from disrupted (reduced that is) or intact disulfide bridges.

Figure 3. A) Schematic representing the generation of mAb subunits via straight reduction or FabRICATOR (IdeS) digest followed by reduction. **B)** Generic structure of an IgG1 antibody highlighting the **inter-** and **intra**chain disulfide bridges.

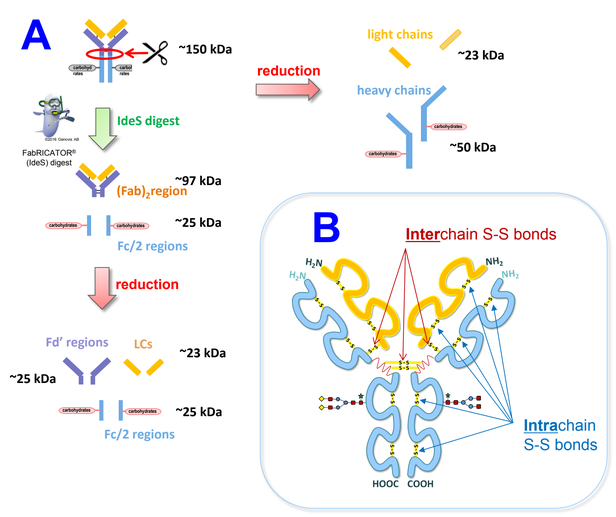
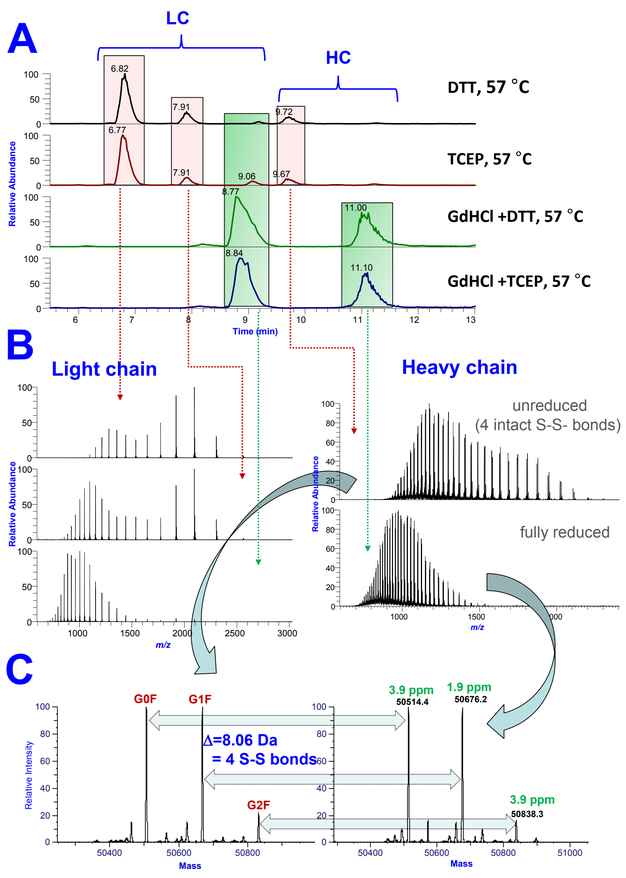


Figure 4. A) Total ion chromatograms of **Rituximab** subunit LC-MS analysis after reduction with DTT, TCEP, DTT with GdHCl and TCEP with GdHCl. **B)** Full MS spectra of the Light and heavy chains. Early eluting peaks represent species with intact **intra**chain disulfide bridges. Whereas the alter eluting peaks represent semi- or fully reduced subunits which can be deducted based on the mass obtained after deconvolution **(C)**.



A more precise measure of the number and status of the disulfide bonds is the intact accurate mass of the subunits obtained after deconvolution of the mass spectra.

For all antibodies investigated it was found that TCEP is slightly stronger in reducing capabilities. Both DTT and TCEP without further additives are reducing **inter**chain disulfide bonds very efficiently but to only a very small extent also the **intra**chain disulfide bonds (Figures 4 and 5).

Achieving complete reduction of all disulfide bonds requires denaturation. We found 4M GdHCl as a final concentration efficient to support complete reduction. Additionally, the temperature applied for reduction is also playing a role as room temperature is not sufficient for complete reduction of the heavy chain and Fc/2 subunits (Figure 5A). Top down analysis strongly benefit from complete reduction of the proteins (Figure 6). Doublet peaks in a chromatogram are not necessarily an indication of incomplete reduction but can also result from different isoforms or as in the case of Infliximab due to different levels of C-terminal Lys-clipping at the Fc/2 subunit (Figure 7), both of which are fully reduced.

Figure 5: **A)** Total ion chromatograms of **Trastuzumab** subunits after FabRICATOR digest followed by reduction using DTT or TCEP at room temperature or at 57 °C, and/or in the presence of 4M GdHCl. **B)** Enlarged TIC of the chromatogram obtained from the sample reduced with TCEP at 57 °C highlighting the three subunits in non-reduced, semi-reduced and fully reduced states. The further the retention times shift the fewer intact **intra**molecular disulfide bonds are present which can also be deducted based on the shapes of the charge envelopes represented in the Full MS spectra (**D-E**) and is confirmed by the masses obtained after deconvolution. **F)** Comparison of simulated and measured isotope patterns of one charge state each for the light chain unreduced, (left) with one disulfide bridge reduced and one still intact (center), and fully reduced (right).

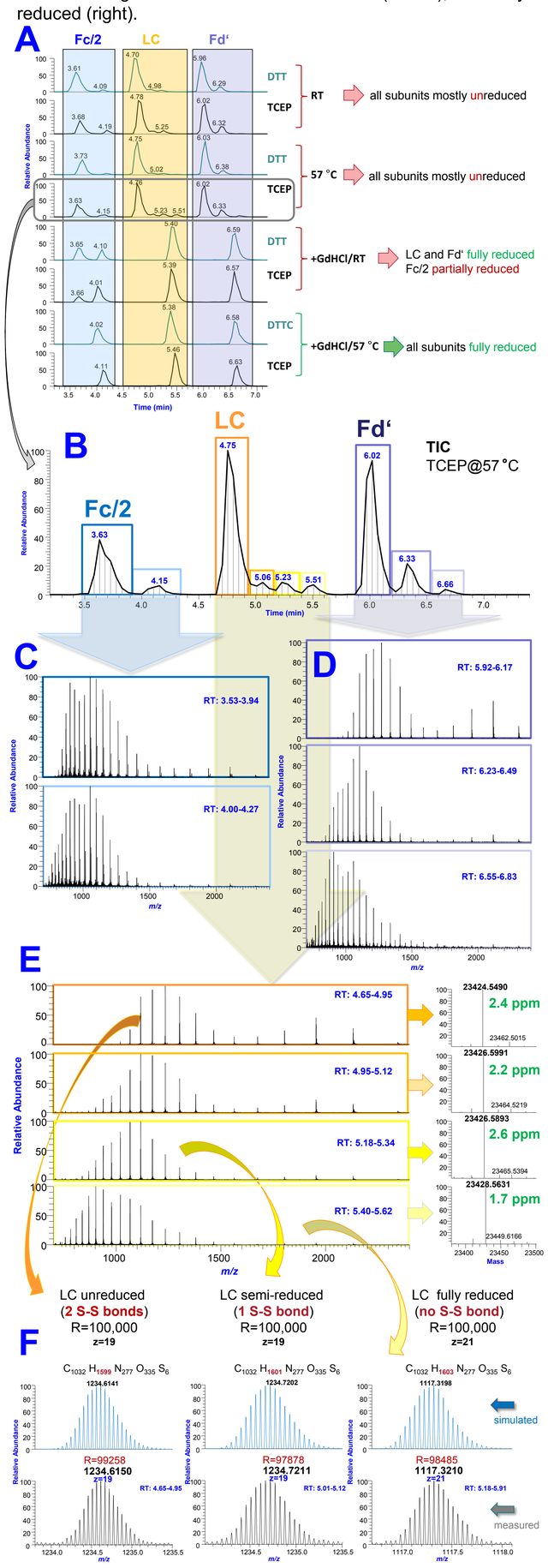


Figure 6: **A)** Full MS spectrum of a light chain with intact **intra**chain disulfide bridges (top) and fully reduced (bottom). **B)** Top down spectra of the most abundant charge state of the unreduced light chain (top) and the fully reduced light chain (bottom).

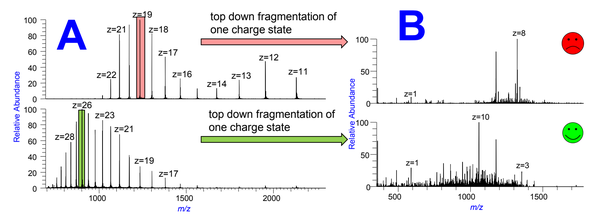
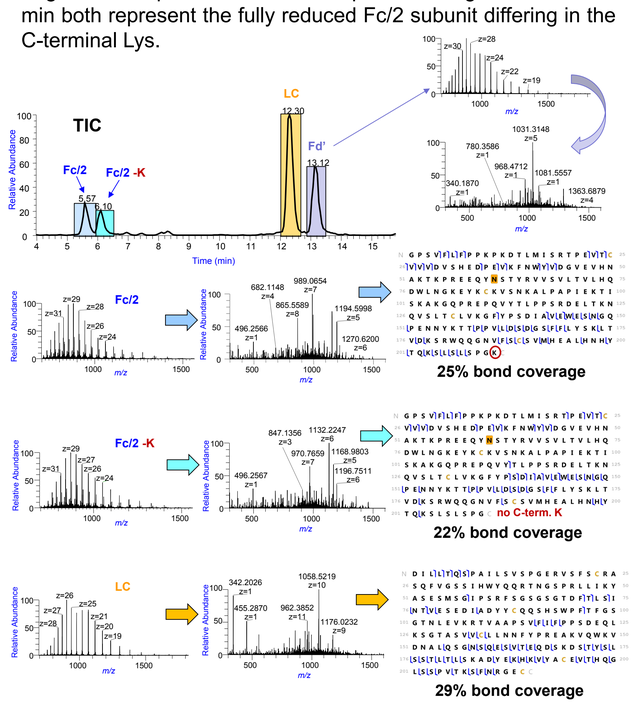


Figure 7: TIC of FabRICATOR digest followed by reduction with TCEP at 57 °C in the presence of 4M GdHCl of **Infliximab** and full MS spectra of the four abundant peaks in the chromatogram and their top down spectra acquired with a resolution setting of 120,000, a wide isolation window of 200 Th around a center mass of 950 m/z, stepped collision energies with NCE 10,13, and 16% and sequence coverages obtained based on top down fragment ion spectra. The two first peaks eluting at 5.6 and 6.1 min both represent the fully reduced Fc/2 subunit differing in the C-terminal Lys.



CONCLUSIONS

- The Q Exactive BioPharma offers three modes (Standard Mode, Protein Mode, and High Mass Range Mode) that predominantly relate to the ion trapping path and pressure regimes inside the mass spectrometer to cover all major workflows for the characterization of biopharmaceuticals.
- The high resolution settings of 120,000 and 240,000 are essential for the intact mass and top down analyses of mAb subunits to obtain accurate monoisotopic masses and for amino acid sequence confirmation.
- For complete reduction of mAb subunits it is not sufficient to reduce with either DTT or TCEP without any denaturing agent such as GdHCl.
- 4M GdHCl in the presence of DTT or TCEP at elevated temperatures of 57 °C were found appropriate conditions to achieve complete reduction of all **inter-** and **intra**chain disulfide bridges.
- Complete reduction using either DTT or TCEP, elevated temperature and 4M GdHCl is essential for achieving an unambiguous intact mass for antibody subunits and significantly improves the quality of top down spectra in regards to the number of fragments detected supporting increased sequence confirmation.
- Doublet peaks in a chromatogram can either represent subunits with various numbers of intact disulfide bonds or may also represent variants such as Fc/2 regions with and without Lys-clipping.

TRADEMARKS/LICENSING

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