Comprehensive Characterization of Intact Monoclonal Antibody Using High Resolution Benchtop Quadrupole-Orbitrap LC-MS/MS

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Overview

Purpose: A LC/MS-based workflow solution was developed for robust, accurate and comprehensive intact monoclonal antibody (mAb) characterization.

Methods: Thermo Scientific Q Exactive quadrupole-Orbitrap mass spectrometers were used for intact mass measurement and top-down sequencing. Full MS spectra of intact or reduced mAb were analyzed using Thermo Scientific Protein Deconvolution 2.0 software that utilizes the ReSpect™ algorithm for molecular mass determination. The top-down msx HCD spectra were analyzed using Thermo Scientific ProSight PC 2.0.

Results: A mass error of less than 10 ppm was routinely achieved for intact mAb mass measurement. Low mass modifications, such as oxidation, can be confidently identified on substructure level such as intact Fab, or light chain. Using an on-line high resolution top-down MSMS approach, over 30% of the fragmentation site was covered for intact light chain as well as for Fab heavy chain. Sequence coverage from top-down approach also confirmed disulfide linkage on partially reduced samples.

Introduction

Monoclonal antibodies (mAbs) are increasingly developed and utilized for the diagnostic and therapeutic treatment of diseases including cancer. mAb can exhibit heterogeneity and thorough analytical characterization is required to obtain the approval to use mAb as a therapeutic product. Among the analytical tools used for the analysis of therapeutic mAb, mass spectrometry has become important in providing valuable information on various protein properties, such as intact mass, amino acid sequence, post-translational modification including glycosylation form distribution, minor impurities due to sample processing and handling and high order structure, etc. Characterization at intact protein level is usually the first step. In this study, a high resolution LC-MS based workflow solution was developed for robust, accurate and comprehensive mAb characterization at intact protein level. The fast chromatography, the superior resolution and mass accuracy provided by the Q Exactive Orbitrap™ MS, and accurate data analysis of this workflow provides high-confident screening tool to accelerate biopharmaceutical product development cycles.

Methods

Samples: Four intact mAbs were used in this study. To reduce intact mAb, the sample was incubated for one hour at 60 °C or 37 °C in 6 M guanidine-HCl containing 5 mM DTT for complete or partial reduction, respectively. Fab was generated using papain in 1mM EDTA, 10 mM Cys, 50 mM sodium phosphate buffer, pH 7,0. Before digestion, the enzyme suspension (10mg/ml) was activated for 15 min at 37C in the same buffer at an enzyme: buffer ratio of 1:9. The digestion was performed at 37C overnight using an enzyme: antibody ratio of 1:99 w/w.

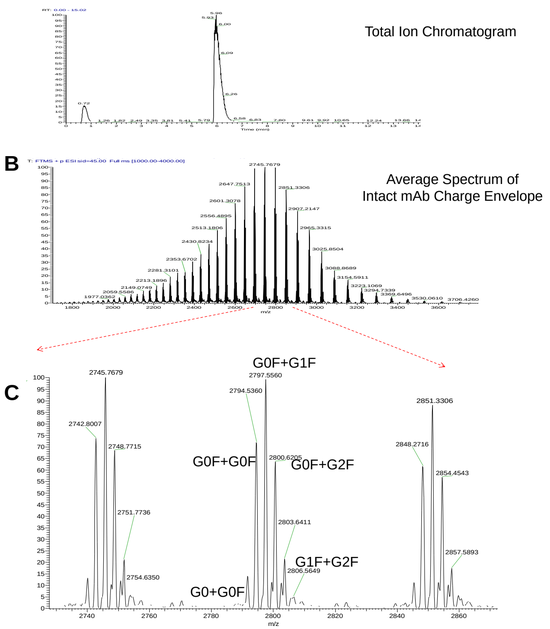
HPLC: Thermo Scientific ProSwift RP-10R monolithic column (1 x 50mm) was used for desalting and separation of light and Fab heavy chain. LC solvents are 0.1% formic acid in H_2O (Solvent A) and 0.1% formic acid in acetonitrile (Solvent B). Column was heated to 80°C during analysis. Flow rate was 60 µL/min. After injection of 1 µg mAb, a 15 min gradient was used to elute mAbs from the column (0.0min, 20%B; 1.0min, 35%B; 3.0min, 55%B; 4.0min, 98%B; 7.0min, 98% B; 7.1min, 20%B; 15.0min, 20%B).

Mass Spectrometry: Q Exactive Orbitrap instruments were used for this study. Intact and reduced mAbs were analyzed by ESI-MS for intact molecular mass. Top-down MSMS was performed using high energy collision dissociation with a unique spectrum multiplexing feature (msx HCD). In this data acquisition mode, fragment ions produced from several individual HCD events, each on a precursor of a different charge state of the reduced mAb, were detected together in the Orbitrap mass analyzer. The spray voltage was 4kV. Sheath gas flow rate was set at 10. Auxiliary gas flow rate was set at 5. Capillary temperature was 275°C. S-lens level was set at 55. In-source CID was set at 45 eV. For full MS, resolution was 17,500 for intact mAb and intact Fab average mass measurement, or 140,000 for light chain and Fab heavy chain monoisotopic mass measurement. Resolution was set at 140,000 for top-down MSMS. The AGC target was set at 3E6 for full scan and 2E5 for MSMS. Maximum IT was set at 250 ms.

Data Processing: Full MS spectra were analyzed using Protein Deconvolution 2.0™ that utilizes the ReSpect algorithm for molecular mass determination. Mass spectra for deconvolution were produced by averaging spectra across the most abundant portion of the elution profile for the mAb. A minimum of at least 8 consecutive charge states from the input m/z spectrum were used to produce a deconvoluted peak. To identify glycoforms, the masses were compared to the expected masses with the various combinations of commonly found glycoforms. The top-down msx HCD spectra were analyzed using ProSightPC™ software under the single protein mode with a fragment ion tolerance of 5 ppm.

Results

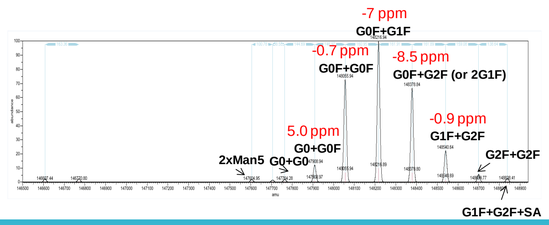
Figure 1: Intact mAb analysis using LC MS



One micrograms of mAb were desalted and eluted from a Dionex ProSwift RP-10R™ monolithic column using a 15min gradient and analyzed using ESI-MS on the Q-Exactive mass spectrometer. As shown in Figure 1., the mAb was eluted over one minute as shown in (A). The average spectrum over the elution time shows a nicely distributed complete charge envelope of the mAb (B). A zoom-in view of each charge state reveals five major glycosylation forms that are baseline separated (C).

After each of the mAb datasets were analyzed using the Protein Deconvolution software, the masses were compared to the masses expected for the known amino acid sequence with the various combinations of glycoforms commonly found on mAbs. One such result is shown below in Figure 2.

Figure 2: Deconvoluted spectrum for a mAb with known composition and mass errors of average molecular mass



To measure the mass accuracy and reproducibility of mAb samples on the Q Exactive mass spectrometer in conjunction with Protein Deconvolution, the mAb sample was analyzed several times using two different instruments over three different days. The results for ppm mass accuracy are shown in Table 1 and the results for relative abundance of the various glycoforms are shown in Table 2.

Table 1: ppm mass deviations from expected target masses for the 5 most abundant glycoforms

RAW file	Q Exactive	G0+G0F	G0F+G0F	G0F+G1F	G0F+G2F	G1F+G2F
1	1	-10.5	0.7	-10.5	-13.8	-18.0
2	1	-11.6	-1.1	-8.8	-11.2	-12.0
3	1	5.1	-5.0	-2.6	5.1	5.6
4	2	-14.3	3.0	-6.9	-5.4	-5.9
5	2	-8.6	-2.2	-12.2	-12.5	-12.9
6	2	-14.3	-6.6	-12.3	-14.8	-10.1

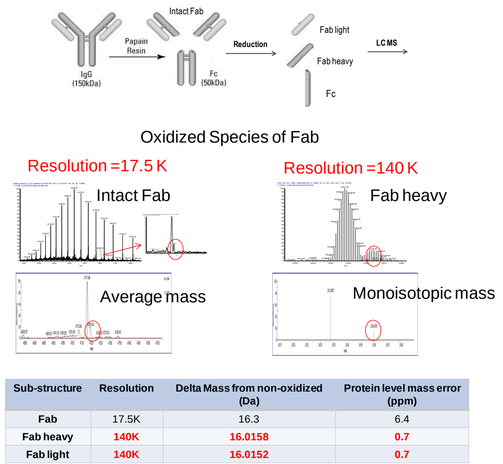
The average ppm error for all 34 measurements of four different mAbs on multiple instruments was **6.9 ppm** with a standard deviation of **6.4 ppm** (not all the data are shown here). This indicates that the Q Exactive is a very powerful platform for confirmation of protein primary structure.

Table 2. Relative abundance for the 5 most abundant glycoforms

RAW file	Q Exactive	G0+G0F	G0F+G0F	G0F+G1F	G0F+G2F	G1F+G2F
1	1	12.9	74.1	100.0	67.0	23.4
2	1	12.0	72.8	100.0	66.2	22.0
3	1	12.2	75.0	100.0	67.0	23.6
4	2	12.7	75.7	100.0	63.6	21.6
5	2	13.2	75.4	100.0	64.8	21.0
6	2	12.9	76.6	100.0	64.7	21.6
CV		**3.4%**	**1.6%**	**N.A.**	**3.9%**	**4.4%**

For the top 5 glycoforms, the relative intensity reproducibility is within a few percent.

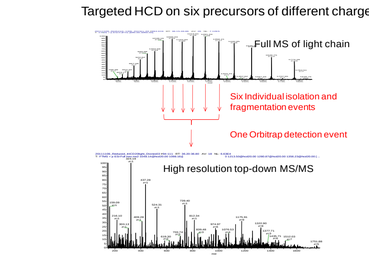
Figure 3: Identification of oxidation on intact Fab, light and Fab heavy chain



Sub-structure	Resolution	Delta Mass from non-oxidized (Da)	Protein level mass error (ppm)
Fab	17.5K	16.3	6.4
Fab heavy	**140K**	**16.0158**	**0.7**
Fab light	**140K**	**16.0152**	**0.7**

Further characterization at substructure level is shown in Figure 3 to Figure 5. Fab was generated using papain which cleaves this molecule at hinge. Fab was then reduced to generate light chain and Fab heavy chain (Figure 3, top). LC-MS of intact Fab, light chain and Fab heavy chain identified oxidation species as shown above (Figure 3, middle). The mass errors of the identification at protein level were 6.4 ppm at resolution 17,500 for Fab and less than 1 ppm at resolution 140,000 for light chain and Fab heavy chain.(Figure 3 bottom)

Figure 4: Top-down sequencing of light and Fab heavy chain using LC-MS/MS



Light chain

30% backbone fragments

P Score = 4.9E-19

Fab heavy chain

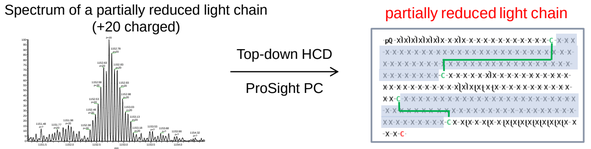
34% backbone fragments

P Score = 2.2E-34

Besides molecular mass, amino acid sequence can be obtained at intact protein level using a top-down LC-MS/MS approach. High resolution top-down HCD was performed using a multiplexing mode where multiple precursors, which were the same protein molecule carrying different number of charges, were isolated, fragmented separately and the resulting fragment ions were then detected all together in a single Orbitrap detection event (Figure 4 top). More than 30% of fragments from backbone cleavage were detected for both light chain and Fab heavy chain (Figure 4 bottom) with excellent P-score from ProSight PC software.

Top-down sequencing was also performed on a partially-reduced light chain which is 4.02 Da less in molecular mass than the fully reduced species. ProSight PC analysis of the HCD spectrum matched two disulfide linkages which is typical of this type of IgG molecule (Figure 5).

Figure 5: Top-down sequencing maps disulfide linkage on partially-reduced light chain



Conclusion

In this study, a workflow was developed that combines high resolution Orbitrap MS, fast chromatography, high throughput msx HCD and accurate data analysis to characterize intact mAb. The precise mass measurement and extensive, high confident amino acid sequence obtained from this workflow provides the following information for intact mAb and its substructure:

- Accurate measurement of intact molecular mass
- Reproducible quantification of glycoform relative abundances
- Confident amino acid sequence information and protein structural information