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Assessing Oxidation in IgG1 Monoclonal Antibodies and Correlating at both Intact Protein and Peptide Levels

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ABSTRACT

Purpose: Evaluate the performance of the new Thermo Scientific™ Orbitrap Exploris™ 240 mass spectrometer equipped with BioPharma option for the oxidation assessment of monoclonal antibodies (mAbs).

Methods: (1) Intact mass analysis under denaturing conditions using Reversed-phase Liquid Chromatography mass (RP LC-MS), (2) Subunit analysis using RP LC-MS and (3) Peptide mapping using RP LC-MS/MS.

Results: The Orbitrap Exploris 240 MS delivers confident tracking of post-translational modifications (PTMs) in mAbs at intact, subunit, and peptide levels with operational simplicity, simplified spectral interpretation, and exceptional mass accuracy.

INTRODUCTION

Oxidation is a common PTM, with methionine residues particularly susceptible. During the production of biotherapeutics, the levels of oxidation must be assessed as it is known to have an impact on product safety and efficacy[1]. Here, we investigate the susceptibility of methionine residues by subjecting the IgG1 mAbs ipilimumab and golimumab to oxidative stress by incubation with hydrogen peroxide (H_2O_2), to determine the sites of potential Critical Quality Attributes (CQAs). Samples were assessed at the intact protein, subunit and peptide levels to pinpoint the locations of oxidation hotspots within the primary sequence, and to provide comprehensive orthogonal characterization.

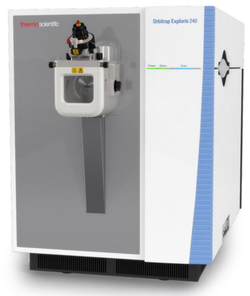
MATERIALS AND METHODS

Ipilimumab (Yervoy) and golimumab (Simponi) were exposed to varying levels of H_2O_2 (50, 100 and 500 ppm) for 24 hours to induce oxidation. All analyses were performed using a Thermo Scientific™ Vanquish™ Duo UHPLC system coupled to an Orbitrap Exploris 240 Mass Spectrometer. Data were processed using Thermo Scientific™ BioPharma Finder™ 4.0 software.

Table 1 - MS conditions

MS Conditions	Intact Denaturing	Subunit Analysis	Peptide Mapping
Method Type	Full MS	Full MS	Full MS-ddTop5 HCD
Scan range (*m/z*)	1500-5000	600-3000	200-2000
Resolution (at *m/z* 200)	30,000	120,000	120,000/15,000
RF Lens (%)	125	80	0
AGC target value (full MS/MS2)	300	300	300/200
Max inject time (ms)	200	200	100/ 200
Microscans (Full MS/MS2)	10	5	1/1
Source Settings			
Spray voltage (+ve acquisition mode)	3700	3800	3800
Ion transfer tube temperature (°C)	320	320	320
Sheath gas (a.u.)	25	25	25
Aux gas (a.u.)	10	10	10
Sweep gas (a.u.)	0	0	1
Vaporizer temp (°C)	150	150	150
Application mode	Intact Protein	Intact Protein	Peptide
Pressure mode	Standard	Low Pressure	Standard

Figure 1 - Orbitrap Exploris 240 Mass Spectrometer



The Exploris 240 MS has application-specific MS tune and acquisition settings which are templated and provided within the software. Settings are directly transferable from instrument to instrument to enable easy method transfer and operational simplicity.

Table 2A and B - Chromatographic conditions

2A. Intact Denatured + Subunit (IdeS with Reduction)

Column:	MAbPac RP 2.1 x 50 mm, 4 µm
Column Temp:	80°C
Flow Rate:	300 µL/min
Solvent A:	Water/0.1% formic acid
Solvent B:	ACN/0.1% formic acid

Intact		*Subunit*	
Time [min]	***%B***	***Time [min]***	***%B***
0.0	25	0.0	25
4.5	50	1.0	25
5.0	80	16.0	45
6.0	80	16.1	80
6.2	25	19.0	80
10.0	25	19.1	25
		26.0	25

2B. Peptide Mapping

Column:	Acclaim VANQUISH C18 2.1 x 250 mm, 2.2 µm
Column Temp:	25°C
Flow Rate:	300 µL/min
Solvent A:	Water/0.1% formic acid
Solvent B:	ACN/0.1% formic acid

Time [min]	***%B***
0.0	2
45.0	40
46.0	80
50.0	80
50.5	2
65.0	2

RESULTS

Intact Protein (mAb) Level

Following incubation with H_2O_2, ipilimumab and golimumab samples were analyzed at the intact level by RP-LC-MS over a 5 minute linear gradient

Figure 2 - Intact Level Oxidative Stress (ipilimumab)

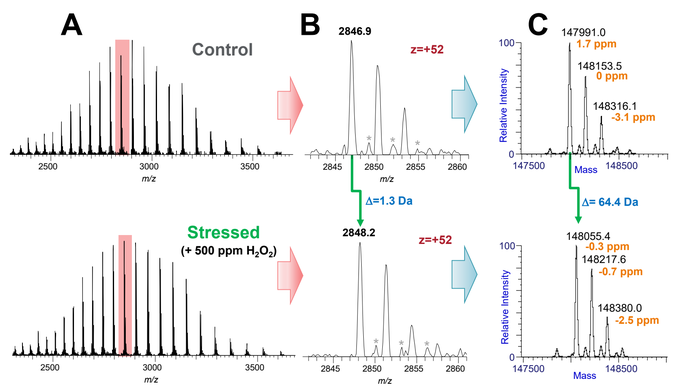


Figure 2 from left to right shows A) the full charge envelope of the intact mAb ipilimumab control and stressed samples (500 ppm treatment), B) a zoom of the +52 charge state representing a baseline resolved glycoform pattern. The grey asterisk indicate adducts that were found to be solvent related. C) The spectra on the right were obtained upon deconvolution using the ReSpect algorithm in BioPharma Finder software. Files were deconvoluted with default settings as available in the software. Data were acquired with a resolution setting of 30,000 (at *m/z* 200) and provided average masses with mass accuracy between 0.0 and 3.1 ppm for the three most abundant glycoforms of ipilimumab. A mass shift of ~64 Da was observed for the stressed ipilimumab sample which potentially indicates a potential increase in four oxidized methionine residues at the intact mAb level.

Subunit Level

Control and stressed samples (+ 50 and 500 ppm H_2O_2) of ipilimumab and golimumab were digested using IdeS protease (FabRICATOR, Genovis), then denatured and reduced by the addition of Guanidine HCl & TCEP. RP LC-MS was used to separate the 3 subunits (Fc/2, LC, and Fd') over 16 min linear gradient.

Figure 3 - Subunit Level Oxidation (ipilimumab and golimumab)

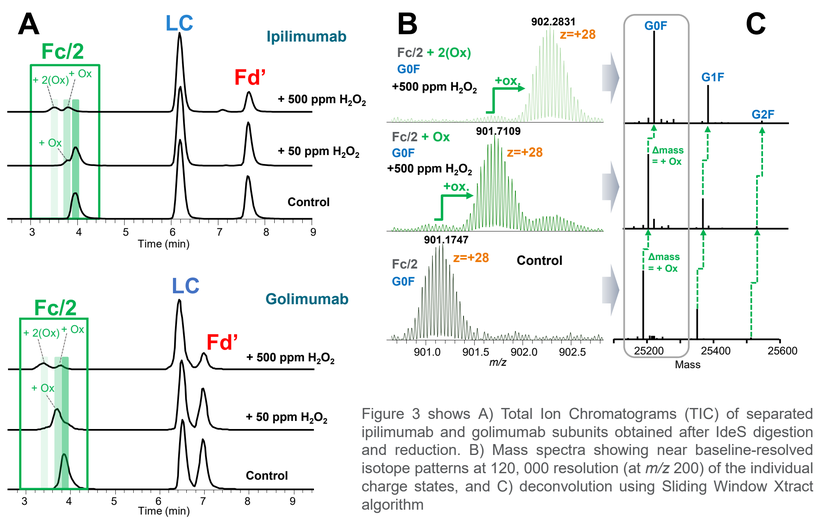


Figure 3 shows A) Total Ion Chromatograms (TIC) of separated ipilimumab and golimumab subunits obtained after IdeS digestion and reduction. B) Mass spectra showing near baseline-resolved isotope patterns at 120, 000 resolution (at *m/z* 200) of the individual charge states, and C) deconvolution using Sliding Window Xtract algorithm

Peptide Level

Control and stressed samples of ipilimumab were digested using the magnetic bulk resin option of Thermo Scientific™ SMART Digest™ Trypsin kits (P/N: 60109-101-MB) on the Thermo Scientific™ KingFisher™ Duo Prime Purification System (Catalog #: 5400100). The resulting peptides were separated by reverse phased chromatography.

Figure 4 - Peptide Level Oxidation (ipilimumab)

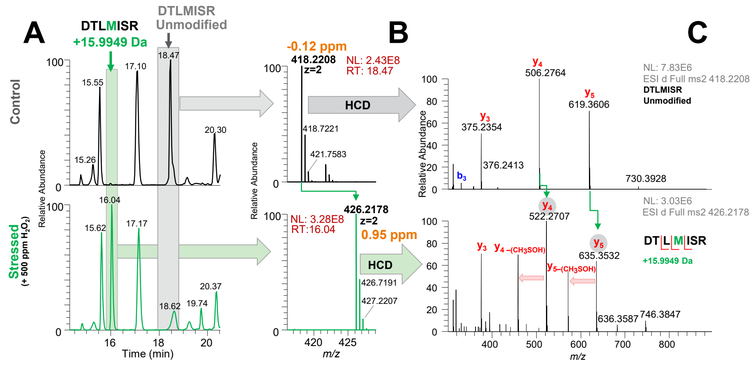


Figure 4 shows A) Peptide mapping TIC zoom of the digested ipilimumab control sample vs. stressed. The figure highlights the tryptic 'DTLMISR' peptide of the heavy chain. A mass shift that is extremely close to theoretical +15.9949 Da, indicate with a high degree of confidence that methionine oxidation has occurred in the sample subjected to oxidative stress with 500 ppm H_2O_2. The modified variant is favoring earlier chromatographic elution at RT 16.04 min compared to the unmodified peptide eluting at RT 18.47 min. B) Zoom into the full MS spectra of DTLMISR displays the isotope patterns and relative abundances of the doubly charged peptides with <1 ppm mass accuracy, selected for HCD fragmentation. C) MS/MS fragment ion spectra (zoom into mass range 300-900 *m/z*). Identification is further supported by confident assignment of a series of y-ions. Mass shifts of approximately +15.9949 Da were observed for the y4 and y5 fragment ions. Moreover, both the y4 and y5 fragment ions show the diagnostic loss of methane sulfenic acid (CH_3SOH) in the oxidized peptide[2].

Figure 5 - Ipilimumab and golimumab % abundance methionine oxidation

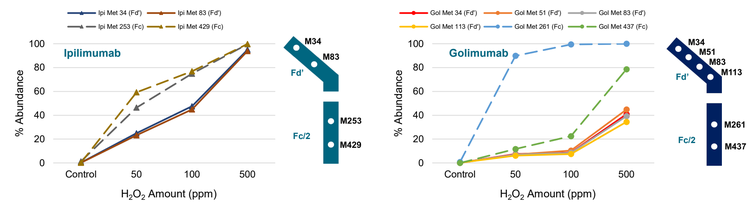


Figure 5 shows the % abundance of oxidation at methionine residues that were observed for both ipilimumab and golimumab from the acquired peptide mapping data. The results aid in pinpointing methionine hotspots in the Fc region of both mAbs and provide complementary and orthogonal data supporting the increase in mass observed at both the intact protein and subunit levels. The susceptibility of methionine residues in the Fc region of both mAbs is likely the results of side chains which are surface exposed and in contact with the solvent[3].

CONCLUSIONS

In this work we demonstrated outstanding performance of the new Orbitrap Exploris 240 mass spectrometer for confident tracking of PTMs in mAbs at intact, subunit, and peptide level with operational simplicity and exceptional mass accuracy.

REFERENCES

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2. Guan, Z *et al*, J Am Soc Mass Spectrom 2003, 14, 605–613
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TRADEMARKS/LICENSING

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