Middle-Down Analysis of Unmodified and Stressed Monoclonal Antibodies Using an Orbitrap Fusion Lumos Tribrid Mass Spectrometer

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ABSTRACT

Purpose: To characterize unmodified and stressed monoclonal antibodies using a middle-down approach.

Methods: Samples were first digested with IdeS and MS1 and targeted MS2 data were acquired. Two different modes of fragmentation, electron-transfer dissociation (ETD) and ultraviolet photodissociation (UVPD), were used, and data were processed with Thermo Scientific™ BioPharma Finder™ software version 3.0.

Results: By combining different activation times and modes of fragmentation, very high sequence coverage was obtained for the NIST mAb. One targeted MS2 experiment was enough to pinpoint some sites of oxidation for a mAb after an oxidative stress. Specific insights on hotspots were also revealed.

INTRODUCTION

Peptide mapping is the gold standard for in-depth characterization of biotherapeutics, but the sample preparation, LC runtime, and data analysis can be time-consuming. Considering that subunit mass analysis is a common assay in the biopharmaceutical industry, getting sequence information using the same sample preparation and LC settings would be advantageous. Automated data processing has historically been one of the bottlenecks for middle-down experiments; however, dedicated software developed for biotherapeutics characterization is available today.

MATERIALS AND METHODS

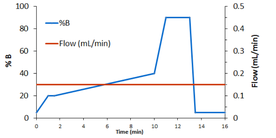
Sample Preparation

Trastuzumab and NIST mAbs were used as standard samples. The stressed samples were generated by incubating mAbs in 0.015% (v/v) of H_2O_2 for 5 or 24 hours. Stressed samples were then buffer exchanged in 0.1 M Tris HCl using a spin column. Finally, all samples were digested with IdeS protease and then reduced under denaturing conditions to generate three ~25 kDa subunits (scFc, LC, Fd).

Methods

For MS1 and MS2 experiments, data were acquired at 120K resolution. MS2 Targeted (wide isolation window or multiplexing) LC-MS experiments were performed using a Thermo Scientific™ Orbitrap Fusion™ Lumos™ Tribrid™ mass spectrometer with an ETD source and 213 nm UVPD source coupled with a Thermo Scientific™ Vanquish™ UHPLC system. A Thermo Scientific™ MAbPac™ 1 mm × 100 mm column was used for all experiments.

Figure 1. Gradient details.



time	%A : Water 0.1% Formic Acid (v/v)	%B : Acetonitrile 0.1% Formic Acid (v/v)	Flow (mL/min)
0	95	5	0.15
1	80	20	0.15
1.5	80	20	0.15
10	60	40	0.15
11	10	90	0.15
13	10	90	0.15
13.5	95	5	0.15
16	95	5	0.15

Data Analysis

LC-MS raw files were processed with BioPharma Finder 3.0 software. For MS1 experiments, the sliding window coupled to the Xtract deconvolution algorithm was used. Time-defined MS/MS spectra were automatically deconvoluted using Xtract, and fragment ion maps were directly generated within the software. The signal-to-noise ratio (SNR) threshold for fragment peak picking was set to 7 and the mass tolerance of fragment ions that can be matched to theoretical fragments was set to 10 ppm. The different proteoforms with one or two oxidations were generated automatically inside BioPharma Finder software by defining the number of oxidation and the site of modification.

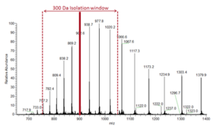
Figure 2. Data were collected using a Vanquish UHPLC system (a) coupled to an Orbitrap Fusion Lumos mass spectrometer (b) and processed with BioPharma Finder 3.0 software (c).

(a) (b) (c)

RESULTS

A) NIST mAb: Acquisition with a wide isolation window and ETD and UVPD modes of fragmentations

Figure 3. Illustration of ions that will be fragmented for the LC when a wide isolation window of 300 Da centered at 900 *m/z* is used.



A wide isolation window of 300 Da centered at 900 *m/z* was used for all three subunits. For each subunit, ETD fragmentation will provide higher sequence coverage than UVPD (Figure 4). On average, the sequence coverages using ETD were respectively 47%, 56%, and 39% for the scFc, LC, and Fd. For UVPD, on average the sequence coverages were respectively 26%, 34%, and 29% for the scFc, LC, and Fd. For UVPD or ETD, by combining the matched fragment ions from different reaction times, the sequence coverage will increase. The sequence coverage for the combined reaction times ranged from 61 to 77 for ETD and from 58 to 67 for UVPD.

Figure 4. Sequence coverage for the NIST mAb subunits for different reaction times using ETD or UVPD.

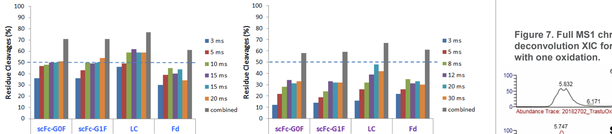
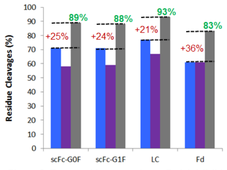
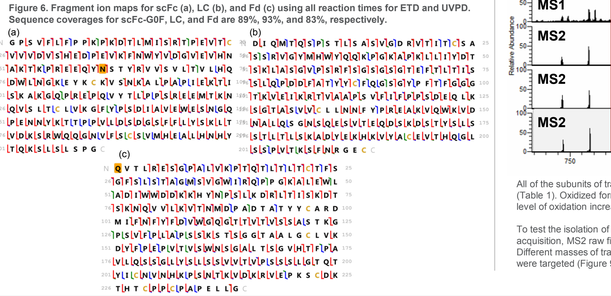


Figure 5. Comparison of the sequence coverage when all of the different reactions for ETD or/and UVPD are combined.



The highest sequence coverages, between 83% and 93%, are only observed when the ETD and UVPD runs are combined (Figure 5). For any sub-unit, adding the matched fragments ions from UVPD at different reaction times will increase the sequence coverage by at least 21%. UVPD is a complementary fragmentation technique to ETD.

Figure 6. Fragment ion maps for scFc (a), LC (b), and Fd (c) using all reaction times for ETD and UVPD. Sequence coverages for scFc-G0F, LC, and Fd are 89%, 93%, and 83%, respectively.



B) Oxidative stress of trastuzumab

1) MS1 experiment

Table 1. Results for duplicate raw files for each condition (unmodified or 0 h and after an oxidative stress of 5 h or 24 h)

Protein Name	Modification	Monoisotopic Mass (mean)	Theoretical Mass (Da)	Matched Mass Error (ppm) (mean)	Relative Abundance	Number of Files Observed	Apex RT (mean)
LC		23428.614	23428.524	3.9	100.00	6	6.695
Fd		25367.619	25367.517	4.0	65.50	6	7.543
scFc	1xA2G0F	25220.550	25220.463	3.4	21.00	6	5.825
scFc	1xA2G1F	25382.588	25382.516	2.8	17.33	6	5.807
scFc	1xA2G1F,1xOxidation (MW)	25398.589	25398.511	3.1	8.67	4	5.706
scFc	1xA2G0F,1xOxidation (MW)	25236.540	25236.458	3.2	6.28	6	5.722
Fd	1xOxidation (MW)	25383.612	25383.512	3.9	6.17	4	7.411
scFc	1xA2G1F,2xOxidation (MW)	25414.570	25414.506	2.5	2.30	2	5.616
scFc	1xA2G2F	25544.618	25544.569	1.9	1.75	6	5.811
scFc	1xA2G0F,2xOxidation (MW)	25252.521	25252.453	2.7	1.62	4	5.68
scFc	1xA2G0	25074.471	25074.405	2.6	1.45	6	5.853

Figure 7. Full MS1 chromatogram and deconvolution XIC for scFc G0F and Fd both with one oxidation.

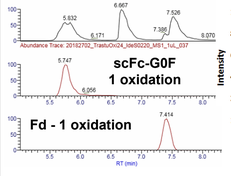
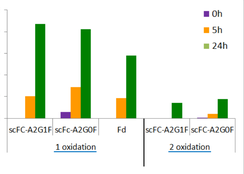
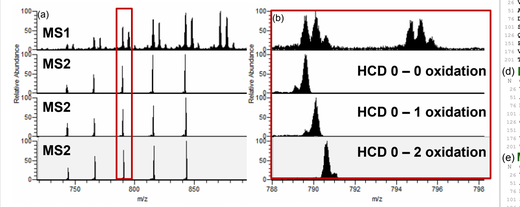


Figure 8. Intensity of oxidized subunits of trastuzumab unmodified or after 5 hours or 24 hours of an oxidative stress with H_2O_2.



2) Visualization of the multiplexing capability to target specific level of oxidation (non modified, 1 oxidation or 2 oxidation).

Figure 9. Difference between an MS1 acquisition or acquisition by multiplexing five charge states (MSX5) for the different level of oxidation on scFc (a). Zoom in the mass range between 788 and 798 *m/z* (b).



All of the subunits of trastuzumab were identified and matched to the theoretical mass at less than 4 ppm (Table 1). Oxidized forms of scFc and Fd eluted before the non-modified subunit (Figure 7). As expected, the level of oxidation increased for longer times of incubation in H_2O_2 (Figure 8).

To test the isolation of the unmodified and oxidized subunits of trastuzumab for the multiplexing mode of acquisition, MS2 raw files were acquired using the HCD mode of fragmentation but with no collision energy. Different masses of trastuzumab scFc-G0F corresponding to the unmodified or with one or two oxidations were targeted (Figure 9a), and each subunit can be separated from each other (Figure 9b).

3) MS2 experiments: ETD (8 ms activation time) on scFc-G0F by multiplexing five charge states

Figure 10. scFc of trastuzumab unmodified. Data searched with G0F on asparagine at position 61 and no oxidation (a) or one oxidation at position 16 (b) (modified peptides are highlighted in green).

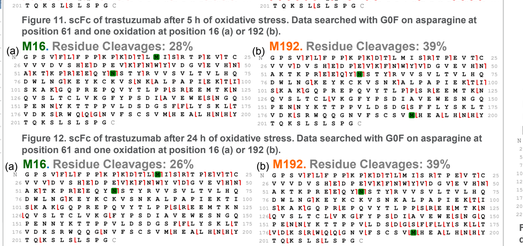


Figure 11. scFc of trastuzumab after 5 h of oxidative stress. Data searched with G0F on asparagine at position 61 and one oxidation at position 16 (a) or 192 (b).

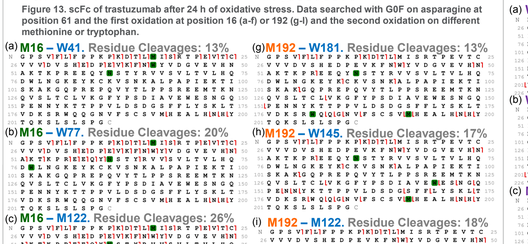


Figure 12. scFc of trastuzumab after 24 h of oxidative stress. Data searched with G0F on asparagine at position 61 and one oxidation at position 16 (a) or 192 (b).

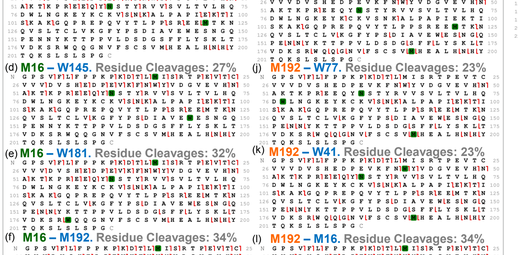


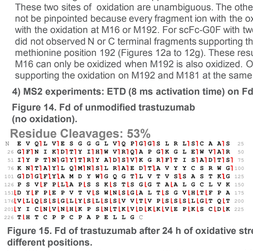
Figure 13. scFc of trastuzumab after 24 h of oxidative stress. Data searched with G0F on asparagine at position 61 and the first oxidation at position 16 (a-f) or 192 (g-l) and the second oxidation on different methionine or tryptophan.

For a rapid identification of the oxidation sites, only one raw file per level of oxidation was acquired and the only mode of fragmentation used was ETD with an activation time of 8 ms. For the unmodified scFc, masses corresponding to only G0F as a modification were targeted. By multiplexing five charge states, a sequence coverage of 57% was observed (Figure 10a). The same deconvolution masses were searched against scFc-G0F with one oxidation at position 16, and no N terminal fragments were matched after the methionine at position 16 assuring that random matching or false positive is at a low level or not occurring.

For scFc-G0F with one modification, the highest sequence coverage for 5 h or 24 h was identified with the oxidation on the methionine at position 192 and the sequence coverage was 39% (Figures 11b and 12b) . Numerous fragment ions also support an oxidation on methionine at position 16 (Figures 11a and 12a). These two sites of oxidation are unambiguous. The other sites of oxidation for scFc with one oxidation can not be pinpointed because every fragment ion with the oxidation at a different position could be explained with the oxidation at M16 or M192. For scFc-G0F with two oxidations, the oxidation on M16 was fixed and we did not observed N or C terminal fragments supporting the second oxidation until the oxidation was on methionine position 192 (Figures 12a to 12g). These results suggest that for scFc-G0F with two oxidations, M16 can only be oxidized when M192 is also oxidized. On the other hand, three fragment ions are also supporting the oxidation on M192 and M181 at the same time (Figure 13j).

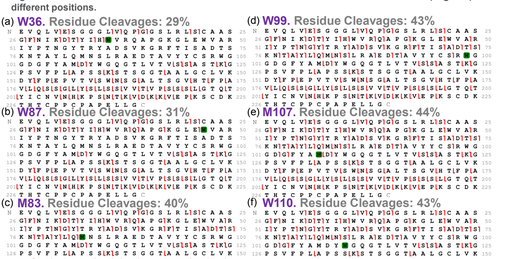
4) MS2 experiments: ETD (8 ms activation time) on Fd by multiplexing 5 charge states

Figure 14. Fd of unmodified trastuzumab (no oxidation).



For the unmodified Fd, a sequence coverage of 53% was observed (Figure 14). For Fd with one oxidation, N and C terminal fragments supporting the oxidation on methionine at position 107 were observed and the sequence coverage was 44% (Figure 15e). All of the sites identified by middle-down were also confirmed by peptide mapping.

Figure 15. Fd of trastuzumab after 24 h of oxidative stress. Data searched with one oxidation (in green) at different positions.



CONCLUSIONS

- High sequence coverage of mAb subunits can be obtained by combining different modes of fragmentation (ETD and UVPD) and reaction times.
- Multiplexing different charge states represented different levels of oxidation of a stressed mAb can be a strategy to quickly identify sites of oxidation and can provide unique information.
- The identification of the oxidation site through a middle-down experiment can be unambiguous, but when subunits are oxidized at numerous sites, the analysis can be challenging.

TRADEMARKS/LICENSING

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