Isotopically Resolved Subunit Analysis for Tracking Protein Therapeutic Stress Studies

High-resolution accurate mass enables isotopic resolution of protein subunits.

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The number and complexity of biotherapeutics are increasing as the pharmaceutical industry seeks to address novel disease areas and develop novel therapies with greater efficacy. In addition, there is a continued drive towards streamlining development efforts to focus on a smaller number of lead candidates in development by assessing efficacy and developability of new molecular entities (NMEs) before molecules transition from discovery to development efforts.

An emerging approach for these studies is the use of assays which require limited sample preparation but offer data which enable rapid assessment of key quality attributes with greater sample throughput. Specifically, protein subunit analysis performed on reduced complexes or those subjected to limited proteolysis have emerged as strong candidates. Of benefit are limited sample preparation burden and the ability to generate isotopically resolved mass spectrometric data which enable high throughput and accurate assessment of targeted post-translational modifications.

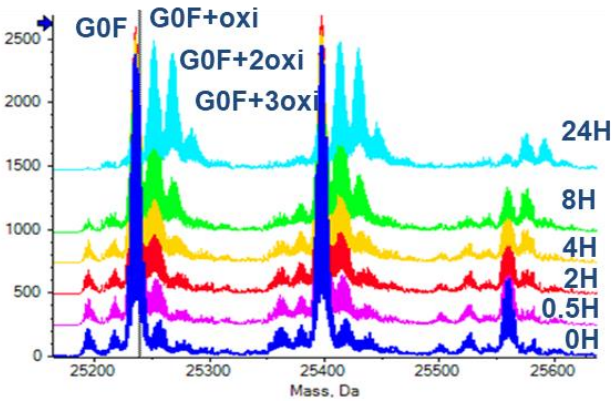

Figure 1. Reconstructed spectrum of Isotopically resolved FC/2 subunit over the timecourse of oxidative stress

Presented here is the use of the SCIEX X500B QTOF system and the ExionLC™ System operated by SCIEX OS 1.4 for the rapid analysis of protein subunits generated using the IdeS protease. This report will focus on the analysis of samples that were subjected to oxidative stress over a time course of 24 hours. The extent of oxidation on each subunit was tracked using isotopically resolved data for each protein subunit to enable accurate tracking of oxidation on each protein fragment.

Key Feature of SCIEX X500B QTOF System operated by SCIEX OS 1.4 Software

- Easy to use solution for core biopharmaceutical workflows including peptide mapping, intact and protein subunit mass analysis, and ADC DAR determination
- Isotopic resolution of proteins and subunits with high mass accuracy
- Reproducible data enabling accurate assessment of post-translational modifications

Methods

Sample Preparation

Oxidation of NIST Sample:

A total amount of 990 µg NIST sample was treated with 0.03% H_2O_2 at room temperature. At each time point (0.5h, 1h, 2h, 4h, 8h, 24h), the treated sample was vortexed and an aliquot of 150 µg NIST sample was taken out and quenched with an equal volume of 250 mM Methionine, followed by a buffer exchange with 12.5 mM L-histidine (pH 6.0), using Amicon centrifugal filter (Millipore, 10K, R8EA69651). Samples were subsequently stored at 4 °C before reduction and digestion.

IDES Digestion of Oxidative NISTmAb Samples:

The oxidative NIST samples (50 µg each) were diluted to 1mg/mL with DI water and incubated with 50 unit of IDES enzyme at 37 °C for 2 hr. Following digestion, samples were mixed with TCEP (20 mg/ml) at a 1:1 volume. Mixtures were incubated at 60°C for 15 minutes, cooled to room temperature and diluted with 0.1% formic acid to a final concentration of 0.1 mg/ml.

Chromatography

Separation was accomplished using an ExionLC™ system fitted with an Agilent PLRP-S column (2.1mm X 50mm, 300Å, 5µm) at 80°C.

Table 1. LC Gradient Conditions for IdeS Digest Analysis

%A	%B	Flow Rate (ml/min)
75	*25*	*0.250*
75	*25*	*0.250*
10	*90*	*0.250*
10	*90*	*0.250*
75	*25*	*0.250*
75	*25*	*0.250*

Mass Spectrometry

A SCIEX X500B Mass Spectrometer with a Turbo V source with a TwinSpray probe was used for data acquisition. MS instrument conditions are listed in Table 2.

Table 3. MS Parameters

Parameter	Setting
Scan Mode	*Positive*
GS1	*50*
GS2	*50*
Curtain Gas	*35*
Temperature	*400°C*
Ion Spray Voltage	*5000 V*
Time Bins to Sum	*6*
Accumulation Time (ms)	*0.5 sec*
TOF Start Mas (Da)	*400*
TOF Stop Mas (Da)	*3000*
Declustering Potential	*150.*
Collision Energy	*10*

Data Processing

Data were processed using SCIEX OS software 1.4.0.18067 and BioToolKit software 1.0. Reconstruction parameters are shown in Table 3.

Table 3. Reconstruction Parameters for Intact Analysis

Parameter	Setting
Start Mass	*Fc 25000 Da* *LC 22600 Da* *Fd 25200 Da*
Stop Mass	*Fc 26000 Da* *LC 23600 Da* *Fd 26200 Da*
Step Mass	*0.05 Da*
Input Spectrum Isotope Resolution	*30000*

Results and Discussion

Stress studies are commonly executed during the discovery and development of biotherapeutics. These studies ensure that the active pharmaceutical ingredient (API) is stable within the expected stress conditions that may be encountered during manufacture, formulation to drug product, and during shipping and storage.

In some cases, sample volume may be high which can challenge approaches which require extended sample preparation.In addition; sample preparation itself may introduce post-translational modifications if not well controlled. To address this many labs use subunit based studies which provides greater localization of modifications compared to intact analysis while offering more streamlined sample preparation than peptide-based approaches. In particular, the use of Immunoglobulin degrading enzyme of Streptococcus (IdeS) and its variants have emerged for detailed study of mAb subunits.

In this study NISTmAb was subjected to oxidative stress over a time course and aliquots were taken of the sample and quenched with methionine and digested with IdeS. Shown in Figure 2 are overlaid total ion chromatograms (TIC) for each timepoint. As shown digestion and separation of the samples was reproducible.

For each peak in the separation an average spectrum was created as shown in Figure 3. As expected each spectrum demonstrates a typical charge state profile. Raw spectra were reconstructed to the zero-charge mass. The spectra for each peak are shown in Figure 4.

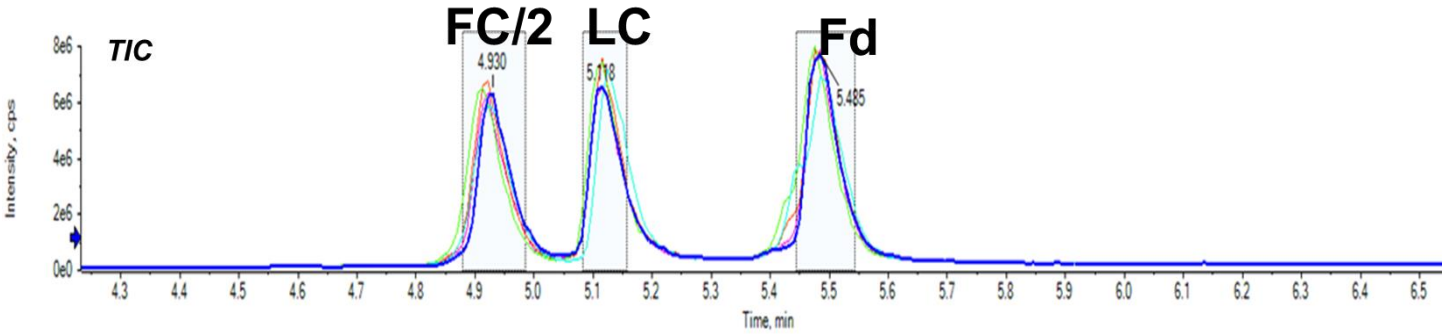

Figure 2. Overlay of total ion chromatograms (TIC) from each timepoint in oxidation stress study.

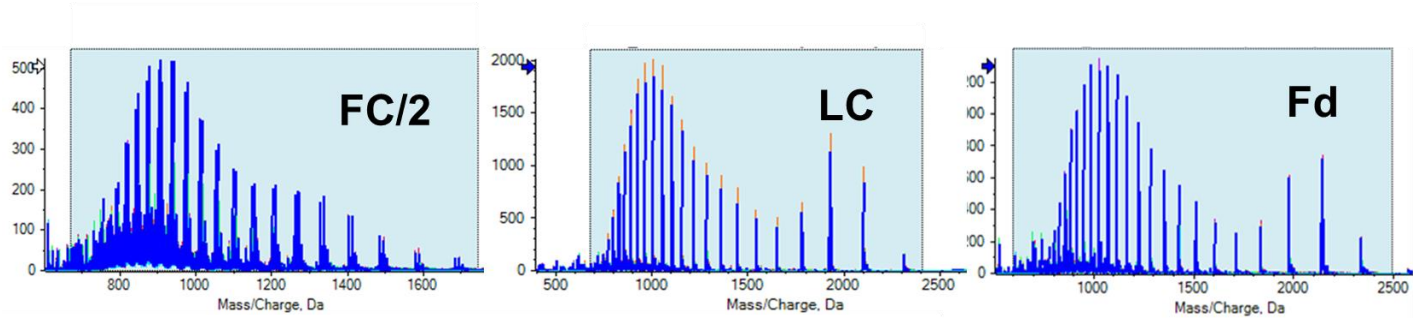

Figure 3. Average mass spectra for each IdeS subunit.

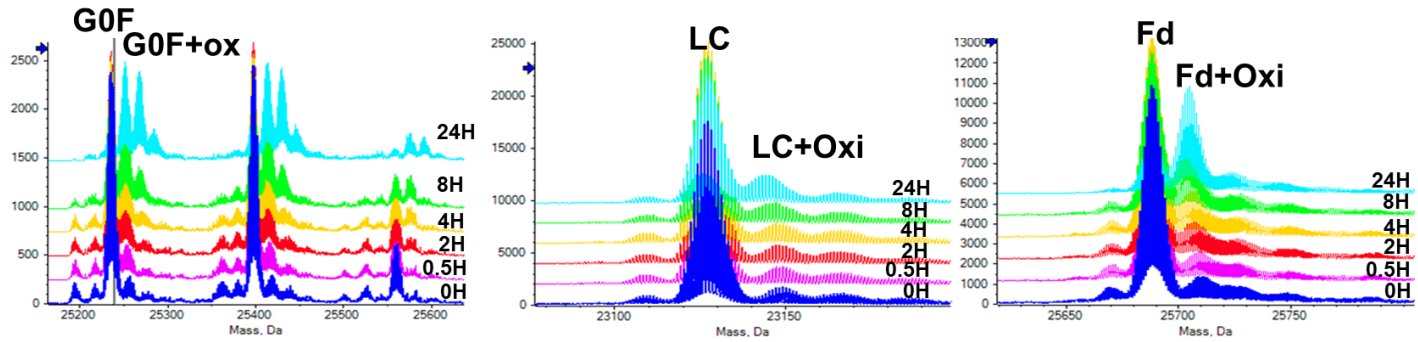

Figure 4. Reconstructed spectra for each protein subunit showing isotopic resolution for each component.

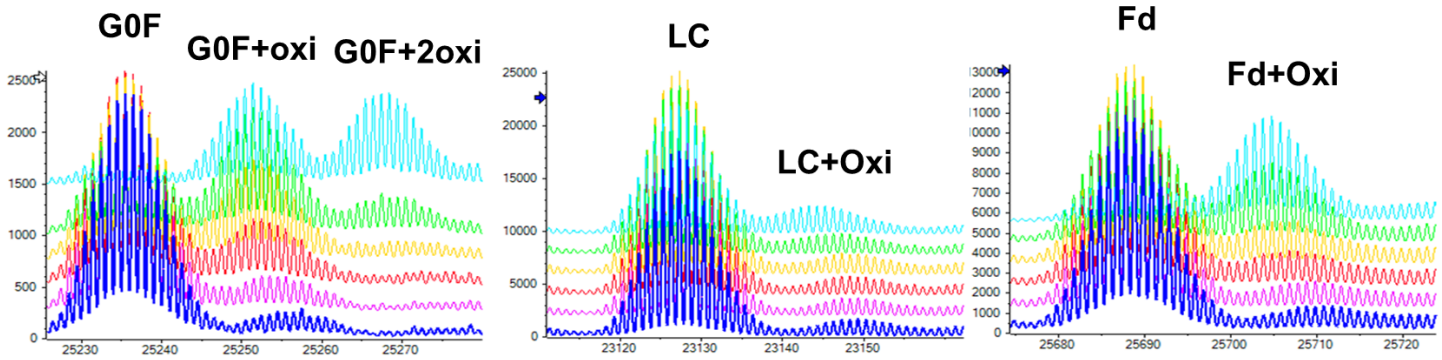

Figure 5. Zoomed reconstructed spectra for each component from IdeS digest.

As shown, oxidation increases for each component over the course of oxidative stress. The data indicate that the heavy chain is more susceptible that the light chain to oxidative stress. In particular, the Fc/2 portion of the protein is susceptible to multiple oxidations as evidenced by mass shifts of +16 and +32 Da for each glycoform compared to the unstressed sample.

To focus on the level of oxidation and resolution of the data zoomed spectra are shown in Figure 5. As shown, each component and its oxidized form is clearly isotopically resolved. In each series, there is a clear increase in oxidized species. At early time points, there is evidence for a sodium adduct. It is unlikely that sodium adduct levels decrease and rather the oxidized species overlaps with the sodium adduct.

Conclusions

- A time-course oxidative stress study was performed to monitor the oxidation levels on NISTmAb
- Isotopically resolved IdeS reduced subunit analysis on the X500B QTOF system provides accurate assessment of post-translational modification with high mass accuracy
- The assay is highly reproducible at both the chromatographic and mass spectral level enabling accurate tracking of oxidized species

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