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Obtaining high sequence coverage and confident post-translational modification (PTM) analysis of biotherapeutics using an electron activated dissociation (EAD)-based middle-down workflow

Featuring the EAD-based middle-down workflow using the ZenoTOF 7600 system and Biologics Explorer software from SCIEX

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This technical note highlights the power of a single-injection, EAD-based middle-down workflow to achieve consistently high sequence coverages (70%-85%) between injections or across different monoclonal antibodies (mAbs). This streamlined workflow leverages information-rich EAD fragmentation with automated data analysis using Biologics Explorer software to offer confident sequence confirmation and accurate localization of PTMs, such as glycosylation and oxidation.

Sequence confirmation and PTM analysis are essential for the comprehensive characterization of therapeutics to ensure drug safety and efficacy.[1] Middle-down mass spectrometry (MS) combines the advantages of bottom-up and top-down approaches and offers high sequence coverages of protein therapeutics following a simple sample preparation.[2-4] Traditionally, a middle-down workflow requires extensive method development and often involves multiple fragmentation techniques and/or injections to obtain high sequence coverage. This challenge can be addressed using a single-injection, EAD-based middle-down workflow.[5] This streamlined workflow provided consistently high sequence coverage of NISTmAb subunits, enabling sequence and PTM confirmations.[5]

In this technical note, the EAD-based middle-down workflow (Figure 1) was used to characterize multiple available biotherapeutics. This powerful workflow achieved high sequence coverages (70%-85%) and enabled the localization of important PTMs, including glycosylation and oxidation.

Key features of the EAD-based middle-down workflow

- **Single-injection platform method:** The EAD-based middle-down workflow provides high sequence coverages in a single injection with 1 fragmentation technique
- **Reproducible high sequence coverage:** Sequence coverages ranging from 70% to 85% are obtained between runs for the subunits of various biotherapeutics
- **Localization of PTMs:** High sequence coverage and preservation of labile modifications by EAD allows for comprehensive PTM analysis
- **Streamlined:** The workflow consists of simple sample preparation, efficient data acquisition and automatic data analysis, with limited method development required

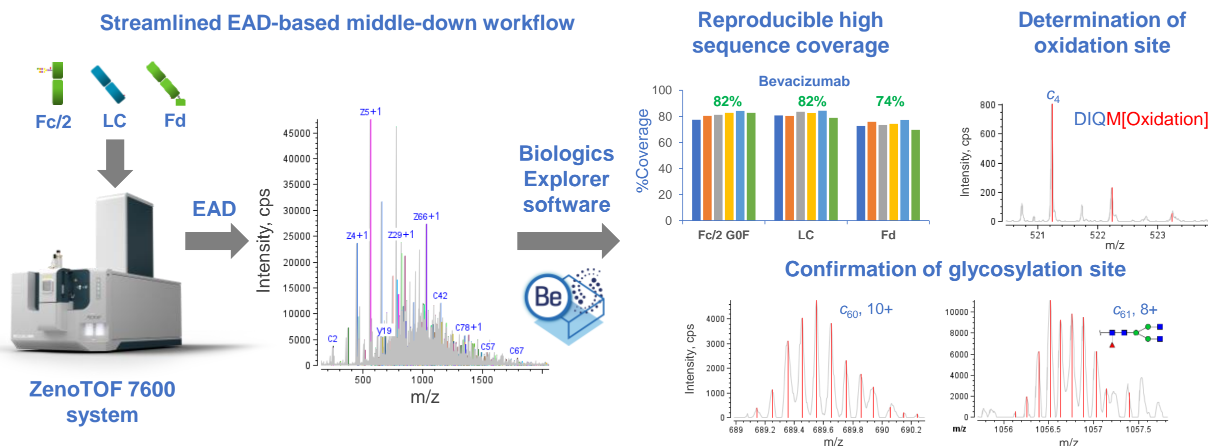

Figure 1. A streamlined, EAD-based middle-down workflow for confident sequence confirmation and PTM analysis. The EAD-based middle-down workflow combines excellent EAD fragmentation of mAb subunits (Fc/2, LC and Fd) provided by the ZenoTOF 7600 system with automated data analysis using Biologics Explorer software. This streamlined workflow provides consistent high sequence coverages between injections or across different protein therapeutics, enabling confirmation and localization of PTMs, such as oxidation and glycosylation.

Methods

Sample preparation: The 10-25 µg/µL stock solutions of mAbs, including NISTmAb, adalimumab, bevacizumab, cetuximab and trastuzumab, were diluted in water to concentrations ranging from 0.5 to 1 µg/µL. The IdeS protease (Promega) with a concentration of 50 units/µL was added to the diluted solutions and the mixture was incubated at 37°C for 2 hours. After IdeS treatment, a solution of 7.6M guanidine hydrochloride (HCl) and 50mM Tris-HCl (pH=7.4) was added, followed by reduction using dithiothreitol. The mixture was incubated at 60°C for 30 minutes. The reaction was terminated by adding 10% formic acid (FA). The final solution contained 0.2-0.5 µg/µL of the Fc/2, LC and Fd subunits. 2-10 µL aliquots of the final solutions (1-2 µg of each subunit) were injected for LC-MS analysis. Oxidation of mAbs occurred during the storage of the digested samples in the autosampler for an extended period of time.

Chromatography: The IdeS subunits of mAbs were separated using an ACQUITY UPLC Protein BEH C4 column (2.1 × 50 mm, 1.7 µm, 300 Å, Waters). The subunits of NISTmAb, adalimumab and trastuzumab were separated using the Gradient 1 parameters shown in Table 1, whereas the cetuximab subunits were separated using the Gradient 2 parameters. Bevacizumab subunits were separated using a modified version of the Gradient 1 parameters (not shown), in which %B was 25% at 2 min and 35% at 9 min. A flow rate of 0.3 mL/min was used for all LC runs. The column was kept at 60°C in the column oven of an ExionLC system (SCIEX). Mobile phase A was 0.1% FA in water and mobile phase B was 0.1% FA in acetonitrile.

Table 1. LC gradient for peptide separation.

Gradient 1			Gradient 2		
Time (min)	A (%)	B (%)	Time (min)	A (%)	B (%)
Initial	80	20	Initial	75	25
2	80	20	2	75	25
9	55	45	14	65	35
10	10	90	15	10	90
12	10	90	17	10	90
12.5	80	20	17.5	75	25
15	80	20	20	75	25

Mass spectrometry: MRM^{HR} EAD experiments were performed in SCIEX OS software using the ZenoTOF 7600 system. Two or 3 charge states were targeted per subunit for EAD fragmentation. The key TOF MS and MRM^{HR} settings used are listed in Tables 2 and 3, respectively.

Data processing: MRM^{HR} data were analyzed using a new top-down workflow template in the Biologics Explorer software, as previously described.[5]

Table 2. TOF MS parameters.

Parameter	Value
Spray voltage	*5500 V*
TOF start mass	*500 m/z*
TOF stop mass	*3000 m/z*
Accumulation time	*0.2 s*
Source temperature	*400°C*
Declustering potential	*80 V*
Collision energy	*10 V*
Time bins to sum	*8*

Table 3. MRM^{HR} parameters using EAD.

Parameter	EAD
Start mass	*100 m/z*
Stop mass	*3000 m/z*
Q1 resolution	*Low*
Zeno trap	*ON*
Zeno threshold	*100,000 cps*
Accumulation time	*0.1 s*
Declustering potential	*80 V*
CE	*12 V*
Time bins to sum	*8*
Electron beam current	*5000 nA*
Electron KE	*1 eV*
ETC	*100%*
Reaction time	*5 ms*
EAD RF	*150 Da*

EAD-based middle-down workflow

The middle-down MS approach combines the advantages of the bottom-up and top-down methods by providing high sequence coverage of mAb subunits with minimal interference from sample preparation-related modification artifacts. Traditionally, the success of middle-down MS relied on extensive method optimization and/or multiple fragmentation techniques,[2-4] limiting its implementation for routine analysis. A single-injection, EAD-based middle-down workflow was developed to achieve consistently high sequence coverage of NISTmAb with minimal effort needed for method development and optimization. As a result, this workflow can be quickly adopted for sequence confirmation and PTM analysis by users with varying levels of LC-MS experience.

The EAD-based middle-down workflow leverages reproducible and information-rich fragmentation by EAD and automated data analysis by Biologics Explorer software. Figure 2 shows a snapshot of Biologics Explorer software that displays multiple tabs in 1 window, facilitating data inspection and results review. The software provides an easy-to-use pre-built workflow template that is specifically designed for biotherapeutic sequence confirmation and PTM analysis using the middle-down approach. This workflow template includes user instructions and optimized parameters to process middle-down EAD data (Figure 2A).The coverage map (Figure 2B) offers a quick assessment of the sequence coverage, whereas the annotated mass spectrum (Figure 2C) and cluster table (Figure 2D) allow users to perform manual inspection and verification of fragment assignments.

Reproducible middle-down characterization of protein therapeutics

It was demonstrated that the EAD-based middle-down workflow provided reproducible high sequence coverages (70%-80%) of Fc/2, LC and Fd subunits of NISTmAb, enabling confirmation of sequences and the site of N-linked glycosylation on the Fc/2 subunit.[5] In this work, the EAD-based middle-down workflow was extended to characterize multiple protein therapeutics, including adalimumab, bevacizumab, cetuximab and trastuzumab, using the same EAD parameters. Similar to the NISTmAb results,[5] consistently high sequence coverages (70%-85%) were obtained for these biotherapeutics, demonstrating the reproducibility and broad applicability of the EAD-based middle-down workflow for biotherapeutic characterization.

The high sequence coverages of the EAD-based middle-down workflow can be attributed to the information-rich spectra generated by EAD. A representative EAD spectrum of the cetuximab LC subunit with fragment annotation is shown in Figure 3. EAD produced many sequence fragments across the full mass range (Figure 3), leading to high sequence coverage (84%) of the cetuximab LC subunit (Figure 4). High sequence coverages (79% and 73%) were also achieved for cetuximab

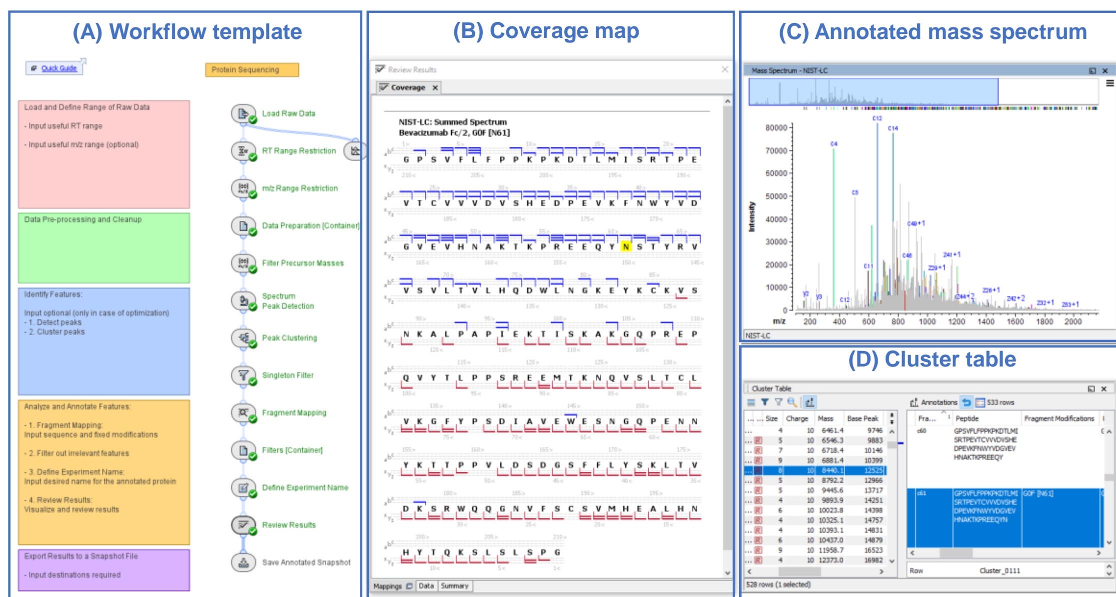

Figure 2. Snapshot of Biologics Explorer software for automated data analysis and results review. Biologics Explorer software provides a pre-built middle-down workflow template with optimized parameters for data processing (A). The easy access to different functions of the software, such as (B) coverage map, (C) annotated mass spectrum and (D) cluster table, in 1 window improves user experience for data processing and results review.

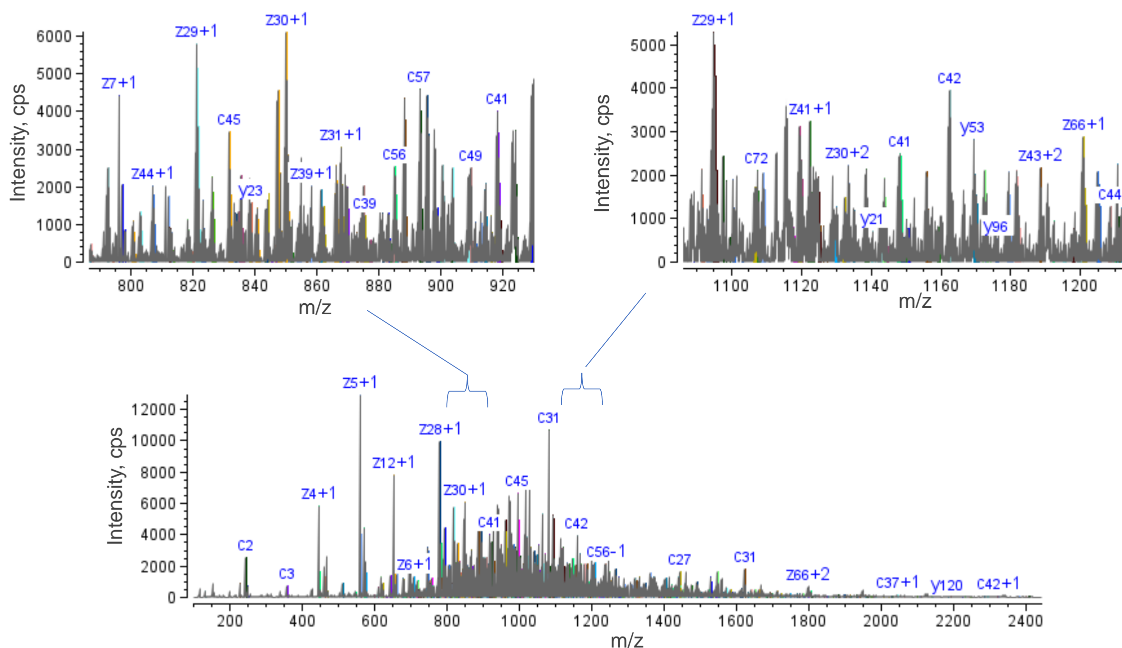

Figure 3. A representative EAD spectrum of cetuximab LC subunit. EAD using the Zeno trap provided excellent fragmentation of the cetuximab LC subunit and permitted the detection of low-abundant fragments. These factors led to the generation of an information-rich spectrum that was used to achieve high sequence coverage. The zoomed-in spectra show the EAD fragments at m/z values ranging from ~790-930 Da and ~1090-1210 Da.

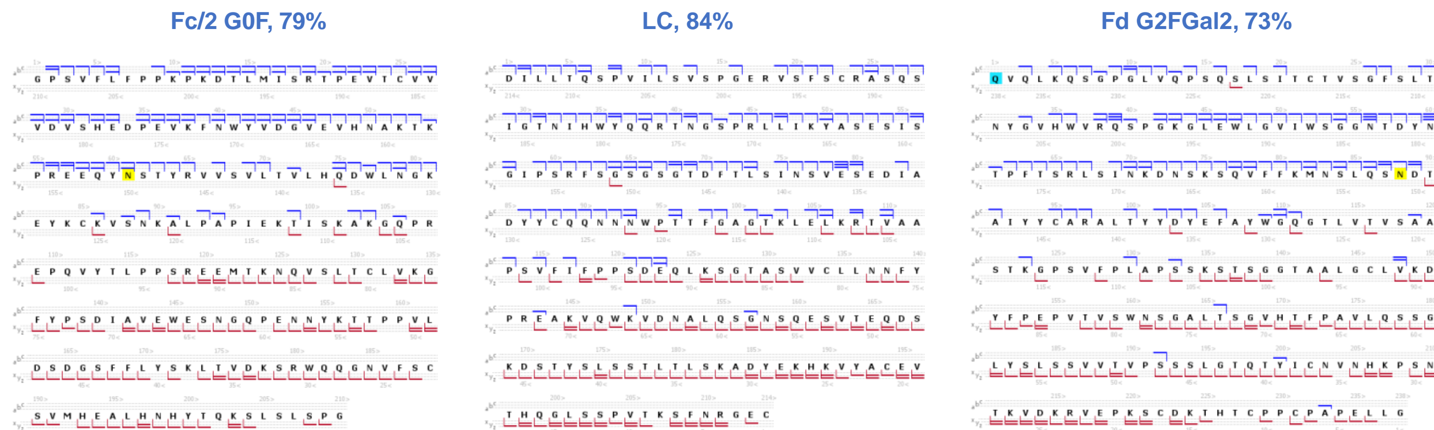

Figure 4. Percent sequence coverages of cetuximab Fc/2, LC and Fd subunits from a single injection. The high-quality data generated by the EAD-based middle-down workflow led to high sequence coverages (70%-85%) of cetuximab Fc/2, LC and Fd subunits in a single injection. The G0F and G2FGal2 glycoforms of Fc/2 and Fd, respectively, were targeted for EAD fragmentation. The blue and red lines indicate the backbone cleavages that correspond to *a*/*b*/*c* and *x*/*y*/*z* fragments, respectively. The Asn residues carrying glycosylation were highlighted with a yellow background.

Fc/2 and Fd subunits containing glycans G0F and G2FGal2, respectively (Figure 4).

The EAD-based middle-down workflow provided reproducible high sequence coverages between runs or across different protein therapeutics. Figure 5 shows the high sequence coverages (70-85%) of Fc/2, LC and Fd subunits that were obtained consistently between 6 runs or across 4 different mAbs.

This result demonstrates that the EAD-based middle-down workflow can be employed as a single-injection method for confident biotherapeutic characterization.

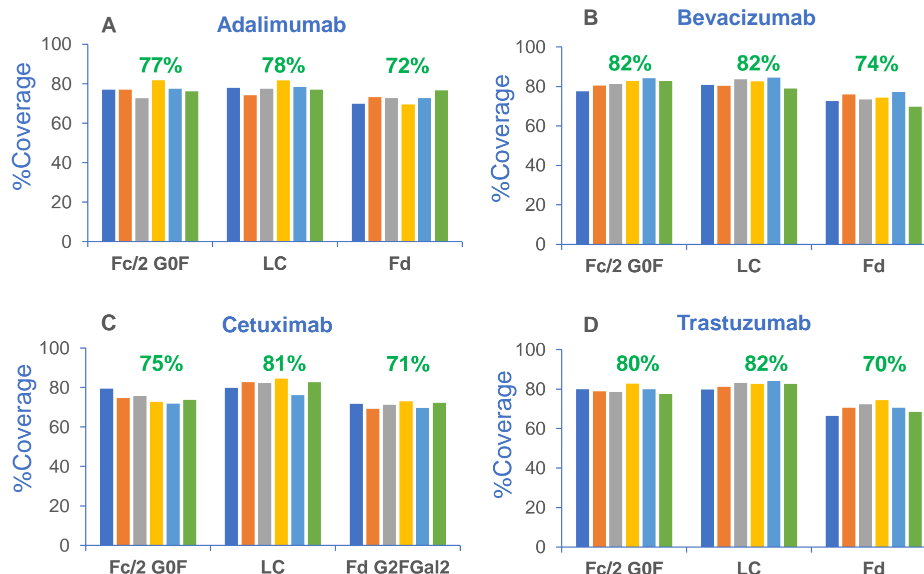

Figure 5. Percent sequence coverages of the Fc/2, LC and Fd subunits of multiple mAbs from 6 replicate injections. The EAD-based middle-down workflow provided consistently high sequence coverages (70%-85%) between runs or across different mAbs, including adalimumab (A), bevacizumab (B), cetuximab (C) and trastuzumab (D). The percent sequence coverages above the bar charts are averaged values measured across 6 replicate injections.

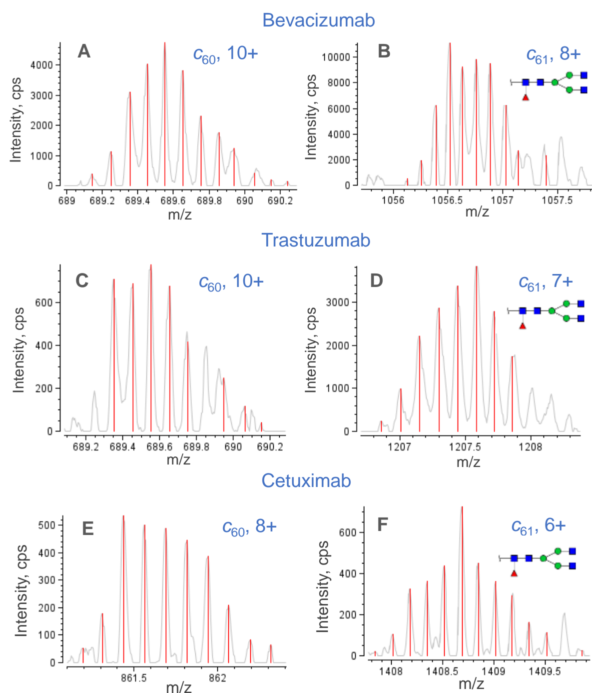

Figure 6. Signature EAD fragments of the Fc/2 G0F subunit confirmed the site of N-linked glycosylation. The detection of non-glycosylated c_{60} and N-glycosylated c_{61} fragments confirmed Asn^{61} glycosylation in bevacizumab (A and B), trastuzumab (C and D) and cetuximab (E and F).

EAD-based middle-down workflow for comprehensive PTM analysis

PTMs are important for the function of protein therapeutics and are often considered critical quality attributes of protein therapeutics.[1] PTMs occurring in unexpected locations or at undesirable levels might have a significant impact on the safety and efficacy of therapeutics. Although the bottom-up MS approach offers a complete characterization of PTMs, modification artifacts might be introduced during complicated sample preparations. Top-down MS requires little or no sample preparation, however, its low sequence coverage is often insufficient to analyze modifications that are not near the termini. Middle-down MS allows superior PTM analysis compared to top-down MS while reducing the artificial modifications from sample preparation compared to bottom-up MS.

The EAD-based middle-down workflow was used to confirm the location of N-linked glycosylation in the Fc/2 subunits of all mAbs and the Fd subunit of cetuximab. Figure 6 shows the signature fragments (c_{60} and c_{61}) generated for the Fc/2 subunits of bevacizumab, trastuzumab and cetuximab for the localization of G0F. The detection of non-glycosylated c_{60} and glycosylated c_{61} ions confirmed the glycosylation of Asn^{61} (Figure 6). The detailed

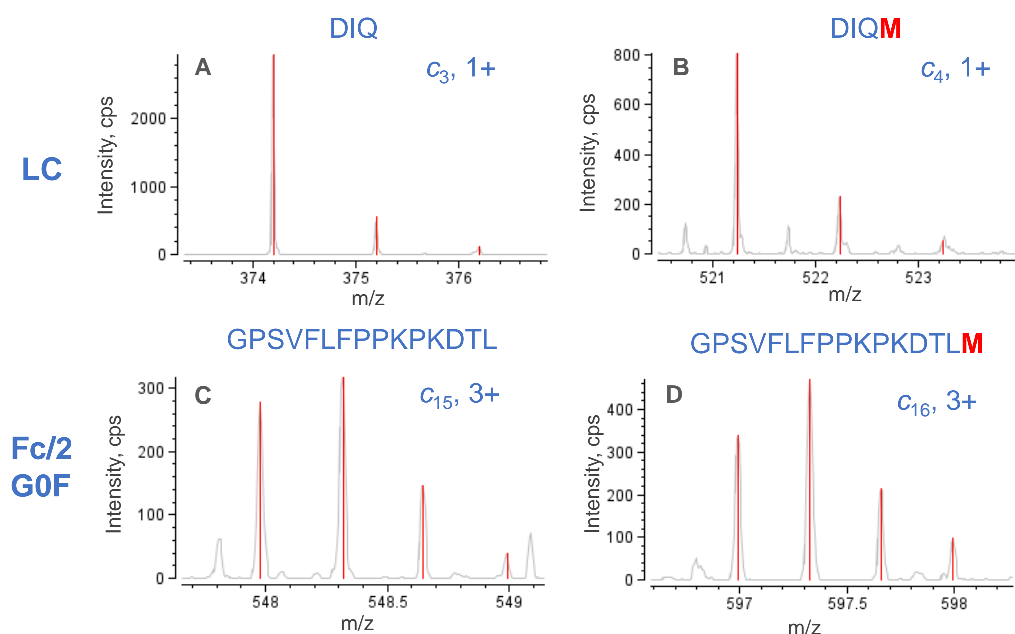

Figure 7. Signature fragments of NISTmAb LC and Fc/2 subunits for the localization of the main oxidation sites. EAD of the oxidized LC (A and B) and Fc/2 subunits (C and D) of NISTmAb led to the formation of signature fragments (c_3/c_4 for LC subunit and c_{15}/c_{16} for Fc/2 subunit) for the localization of 2 main oxidation sites (Met^4 for LC subunit and Met^{16} for Fc/2 subunit).

characterization of N-linked glycosylation in Fc/2 and Fd subunits of cetuximab will be described in a separate technical note.

Oxidation might occur at different stages of biotherapeutic manufacturing, storage or characterization. The localization of oxidation can facilitate understanding the hot spots in a protein therapeutic. Figure 7 shows 2 examples in which the EAD-based middle-down workflow localized 2 main oxidation sites in NISTmAb LC and Fc/2 subunits. The detection of an oxidized c_4 (Figure 7B) confirmed the oxidation of Met^4 in the LC subunit. The detection of non-oxidized c_{15} and oxidized c_{16} fragments (Figures 7C and 7D) indicated that the Met^{16} residue was the main oxidation site in the Fc/2 subunit. In-depth characterization of oxidation in different protein therapeutics by the EAD-based middle-down workflow will be demonstrated in a separate technical note.

In summary, these results demonstrate that the streamlined, EAD-based middle-down workflow can achieve consistent high sequence coverages of mAb subunits and confident PTM analysis in a single injection.

Conclusions

- A streamlined EAD-based middle-down workflow enabled confident sequence confirmation and PTM analysis in a single injection
- High sequence coverages ranging from 70% to 85% were obtained for the Fc/2, LC and Fd subunits consistently between injections or across multiple protein therapeutics
- Middle-down MS allows for more accurate characterization of a protein therapeutic with a lower risk of introducing artificial modifications compared to a complete protein digestion
- The detection of signature fragments enabled the confirmation of N-linked glycosylation on the Fc/2 and Fd subunits
- The EAD-based middle-down workflow confirmed the 2 main oxidation sites in the LC and Fc/2 subunits of NISTmAb
- Biologics Explorer software offers an optimized middle-down workflow and provides automated data analysis with high accuracy and efficient results review for improved user experience

References

1. Anna Robotham and John Kelly. (2020) LC-MS characterization of antibody-based therapeutics: recent highlights and future prospects. *Approaches to the Purification, Analysis and Characterization of Antibody-Based Therapeutics.* Chapter 1: 1-33.
2. Milos Cejkov et al. (2021) Electron transfer dissociation parameter optimization using design of experiments increases sequence coverage of monoclonal. *J. Am. Soc. Mass Spectrom.* 32(3): 762-771.
3. Luca Fornelli *et al.* (2018) Accurate sequence analysis of a monoclonal antibody by top-down and middle-down Orbitrap mass spectrometry applying multiple ion activation techniques. Anal. Chem. 90(14): 8421-8429.
4. Kristina Srzentic *et al.* (2020) Interlaboratory study for characterizing monoclonal antibodies by top-down and middle-down mass spectrometry. *J. Am. Soc. Mass Spectrom.* 31(9): 1783-1802.
5. A streamlined single-injection middle-down workflow using electron activated dissociation (EAD) for biotherapeutics characterization. SCIEX technical note, MKT-26997-A.

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