Comparative 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 comparative analysis of biotherapeutics utilizing a streamlined, single-injection EAD-based middle-down workflow to accelerate decision making in biopharmaceutical development. Biologics Explorer software provides powerful tools that enable fast and detailed comparisons of middle-down results to increase confidence in sequence confirmation and localization of post-translational modifications (PTMs).

Middle-down mass spectrometry (MS) combines the advantages of bottom-up and top-down MS approaches and offers high sequence coverages of monoclonal antibody (mAb) subunits following simple sample preparation.[1-3] Traditionally, a middle-down workflow requires time-consuming 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.[4,5] This streamlined workflow provided consistently high sequence coverage of mAb subunits, enabling confident sequence and PTM confirmations.[5]

In this technical note, comparative analyses of the middle-down results of mAb subunits were performed using Biologics Explorer software (Figure 1). These analyses highlight the advantages of tools offered by Biologics Explorer software for sequence confirmation and PTM localization. The potential application of comparative middle-down analysis for the characterization of biosimilars or sequence variants will be discussed.

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 a single 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
- **Comparative data analysis**: Biologics Explorer software provides tools to rapidly compare middle-down results across most stages of protein therapeutic development or stress studies

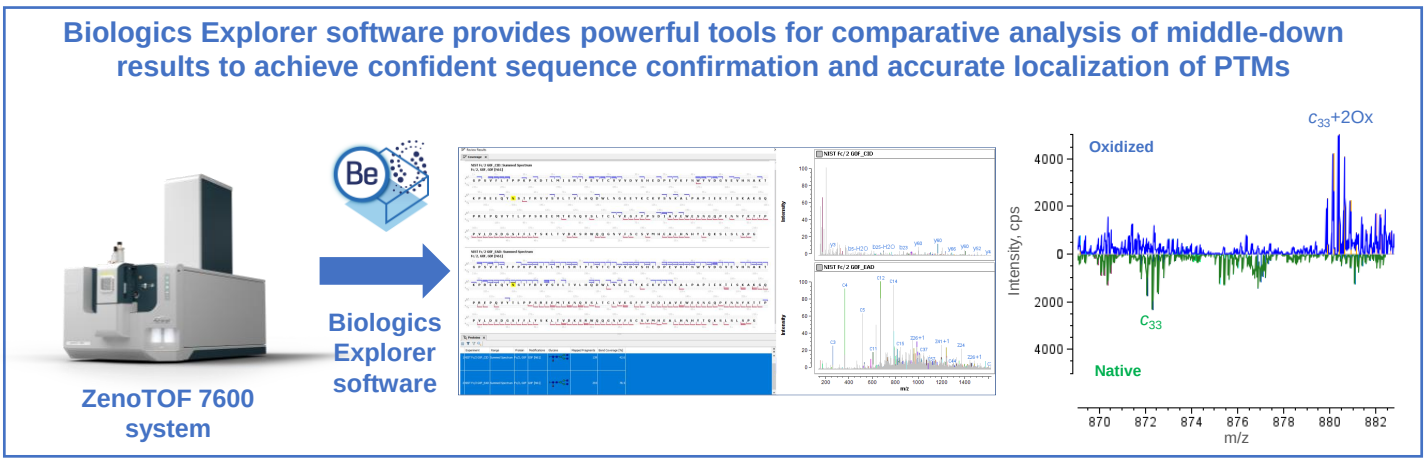

Figure 1. Comparative analysis of middle-down data using Biologics Explorer software enables in-depth sequence and PTM characterizations of biotherapeutics. Biologics Explorer software provides powerful tools for comparative analysis of middle-down results from the ZenoTOF 7600 system, leading to confident sequence confirmation and accurate localization of PTMs.

Methods

Sample preparation: The 10-25 µg/µL stock solutions of mAbs, including NISTmAb, bevacizumab 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. Finally, 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 and trastuzumab were separated using the Gradient 1 parameters shown in Table 1, whereas the bevacizumab subunits were separated using the Gradient 2 parameters. 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*	*80*	*25*
2	*80*	*20*	*2*	*80*	*25*
9	*55*	*45*	*9*	*55*	*35*
10	*10*	*90*	*10*	*10*	*90*
12	*10*	*90*	*12*	*10*	*90*
12.5	*80*	*20*	*12.5*	*80*	*25*
15	*80*	*20*	*15*	*80*	*25*

Mass spectrometry: MRM^{HR} 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} EAD settings used are listed in Tables 2 and 3, respectively. CID data for the NISTmAb Fc/2 G0F subunit were acquired using collision energies of 25-30 eV, 33-37 eV and 45-50 eV for charge states 29+, 24+ and 20+, respectively.

Data processing: MRM^{HR} data were analyzed using a new middle-down workflow template in the Biologics Explorer software, as previously described.[4,5]

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*

Comparative analysis of middle-down results using Biologics Explorer software

The EAD-based middle-down workflow leverages reproducible and information-rich fragmentation by EAD and automated data analysis by Biologics Explorer software.[4,5] This powerful workflow requires minimal effort in method development because of the reproducibility of EAD fragmentation and the capability of Biologics Explorer software for rapid comparative analysis. The Biologics Explorer software offers easy-to-use middle-down templates optimized for therapeutic characterization from data processing to results review and comparison. Figure 2 shows the streamlined process of using the snapshot review template for the comparative analysis of 2 EAD "snapshots" (results files). All results files saved from the middle-down workflow template are simultaneously loaded into the review template (Figures 2A and 2B). The results can be compared in detail using the sequence coverage map, summary table and/or combined MS/MS spectrum. Furthermore, MS/MS spectra can be compared in an overlaid or stacked view or using a mirror plot for confident sequence confirmation or PTM localization.

Figure 3 shows the middle-down results of the NISTmAb Fc/2 G0F subunit obtained using CID and EAD fragmentation approaches. It is evident from the sequence coverage maps and summary table (Figure 3A) that EAD provided more extensive fragmentation and higher sequence coverage than CID. Detailed comparison of 2 MS/MS spectra (Figure 3B) showed that EAD led to an information-rich spectrum with fragments detected across the full mass range. In contrast, the CID spectrum was dominated by the fragments generated from preferential cleavages, such as the oxonium ions from the fragmentation of the glycan moiety (for example, m/z 204) and the fragments associated with the cleavage of the N-terminus of proline residues (for example, y_{60}). These results highlight the advantage of EAD over CID for middle-down analysis and the benefit of Biologics Explorer software for rapid comparative analysis.

Comparative analysis for confident sequence confirmation and PTM localization

The mirror plot provided by Biologics Explorer software enables an in-depth comparison of 2 middle-down results files in an intuitive manner. This functionality can be leveraged to compare the results of native and forced degradation samples to localize PTMs or to compare 2 highly similar sequences, such as biosimilars or sequence variants, for sequence confirmation or differentiation. Figure 4 shows an example of using the mirror plot to localize an oxidation site in the oxidized NISTmAb Fc/2 subunit. The detection of a non-oxidized c_{15} fragment in the middle-down results of the native and oxidized Fc/2 subunit indicated the absence of oxidation for the first 15 amino acid residues (Figure 4A). A c_{16} ion containing 1 oxidation was detected for the oxidized Fc/2 subunit. However, the oxidized c_{16} ion was absent in the EAD spectrum of the native species (Figure 4B), indicating that Met[16] was oxidized in the oxidized Fc/2 subunit.

The mirror plot can also be employed to compare the middle-down results of mAb subunits with highly similar sequences, such as biosimilars and sequence variants. The middle-down results from bevacizumab and trastuzumab Fd subunits with highly similar sequences were compared in Figure 5 using the mirror plot. The comparative analysis confirmed that the first 26 amino acid residues are shared between the 2 Fd subunits (Figure 5A). The amino acid residue in position 27 differs between the 2 subunits, with Tyr present in bevacizumab and Phe present in trastuzumab (Figure 5B) based on different m/z values measured for the c_{27} fragments. These results

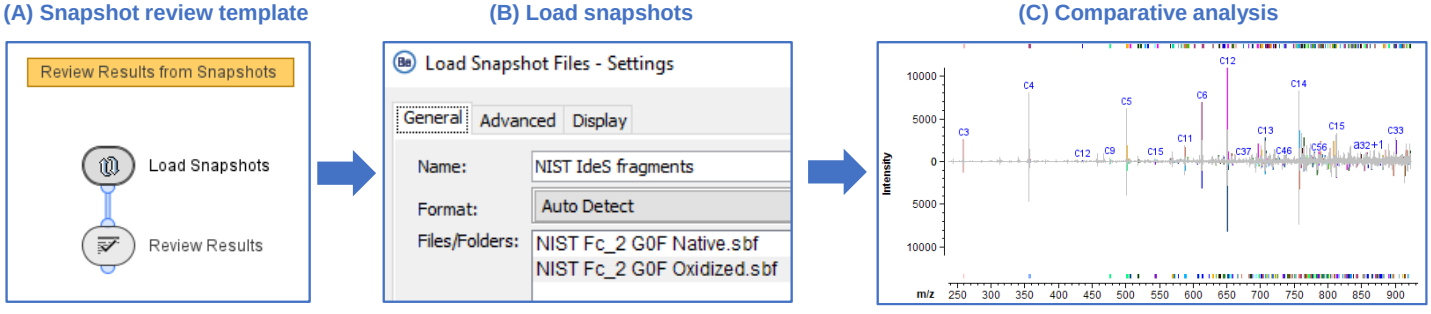

Figure 2. Biologics Explorer software provides tools for comparative analysis of middle-down results. The snapshot review template (A) in Biologics Explorer software allows the selection of multiple "snapshots" (results files, B) for comparative analysis of the sequence coverage maps, percentage of bond coverages and combined MS/MS spectra (C). This strategy can be applied to rapidly compare the results obtained between different samples (for example, native and oxidized) or using different methods (for example, CID and EAD).

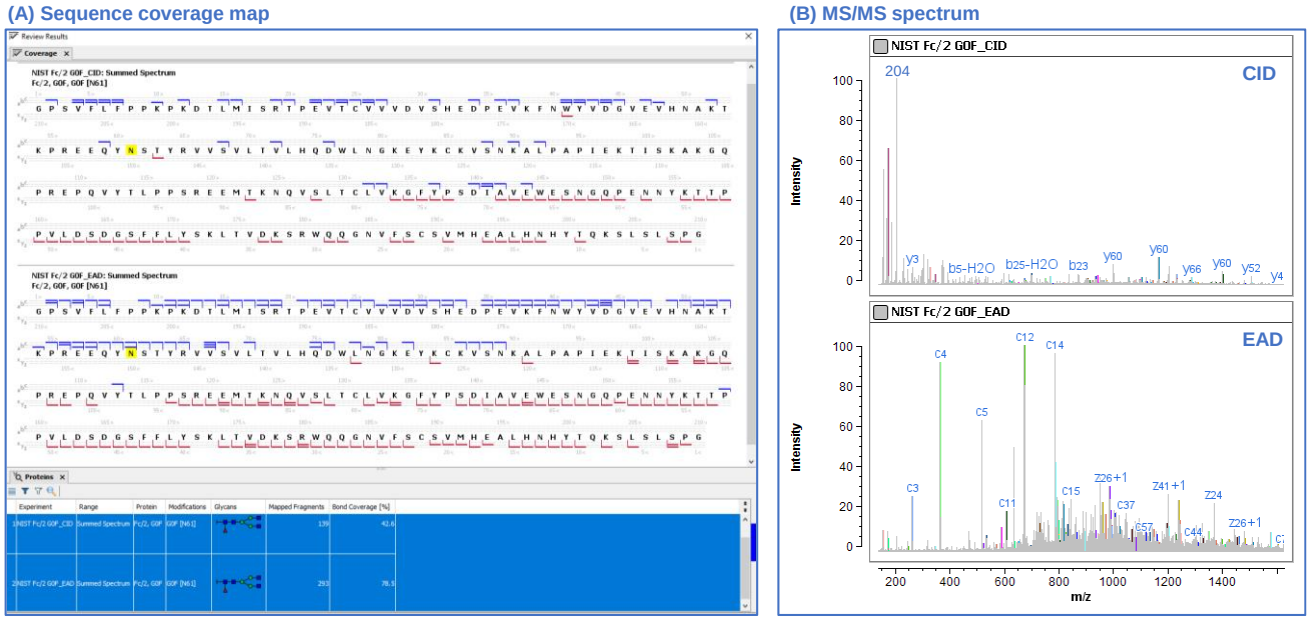

Figure 3. Comparative analysis of representative CID and EAD data for the NISTmAb Fc/2 G0F subunit. The sequence coverage maps (A) and combined MS/MS spectra (B) from CID and EAD data indicate that EAD is better for middle-down analysis of mAb subunits. EAD led to more extensive fragmentation of the subunit backbone to achieve a higher sequence coverage while preserving the labile G0F glycan for its accurate localization. In contrast, CID resulted in preferential cleavage of the glycan moiety and the formation of abundant oxonium ions (for example, m/z 204).

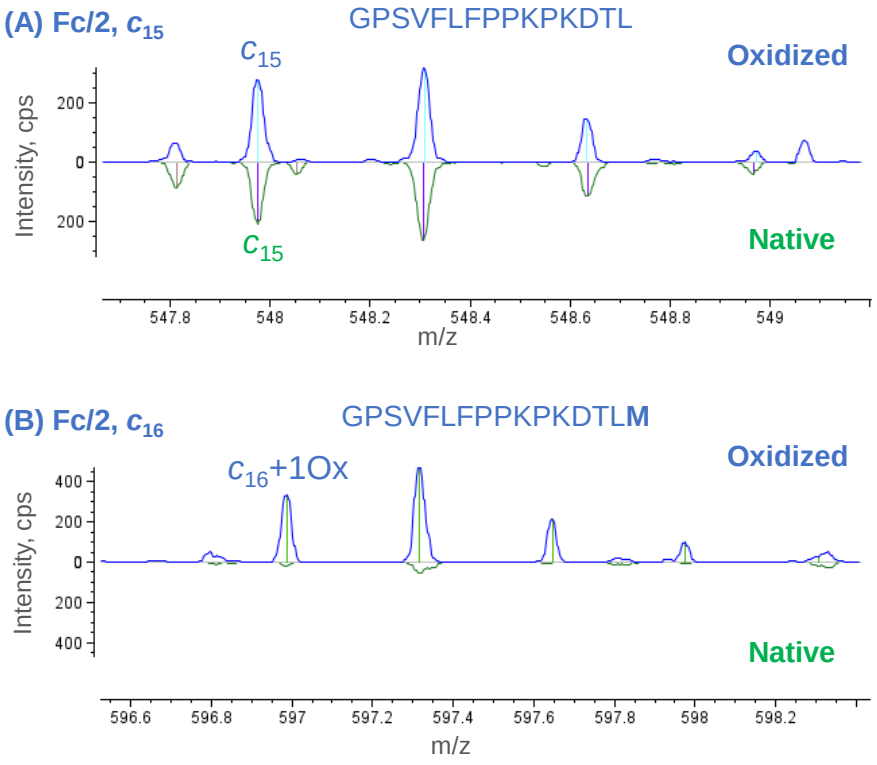

Figure 4. Comparative analysis using the mirror plot facilitated confident localization of an oxidation site in the NISTmAb Fc/2 subunit. The comparison of the EAD-based middle-down results of the native and oxidized NISTmAb Fc/2 subunits using the mirror plot enabled rapid determination of an oxidation site in this subunit. The detection of a non-oxidized c_{15} fragment for both species (A) and a singly oxidized c_{16} ion (c_{16}+1Ox in B) for the oxidized form confirmed the oxidation of Met[16]. The blue and green traces correspond to the EAD spectra of the oxidized and native Fc/2 subunits, respectively.

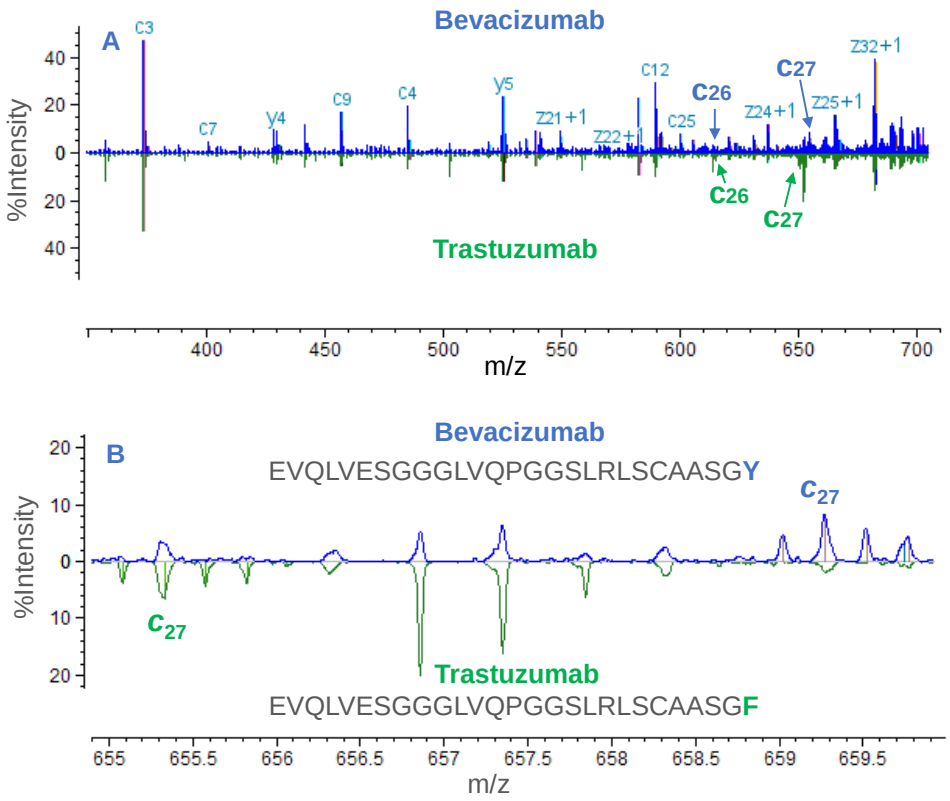

Figure 5. Comparative analysis using the mirror plot facilitated sequence differentiation and confirmation. The mirror plot can be leveraged to compare middle-down results of mAbs with high sequence similarities, such as biosimilars and sequence variants. Bevacizumab and trastuzumab Fd subunits have highly similar sequences and their middle-down results were compared using the mirror plot. This comparison showed that these 2 subunits share the sequence of their first 26 amino acid residues, as confirmed by the detection of c_{26} at the same m/z (A). The comparison of the m/z of the c_{27} fragment (B) indicated that the 2 subunits had different amino acid residues in position 27 (Tyr for bevacizumab and Phe for trastuzumab). The blue and green traces correspond to the EAD spectra of bevacizumab and trastuzumab Fd subunits, respectively.

highlight the power of the single-injection, EAD-based middle-down workflow for confident differentiation between similar sequences and accurate localization of PTMs. Further, these results indicate that this approach could be beneficial for performing comparative analysis to facilitate data analysis during biotherapeutic characterization.

In summary, Biologics Explorer software provides powerful functions for the comparative analysis of middle-down results acquired for different subunits or using different methods. The approach enabled confident sequence confirmation and accurate localization of PTMs. These tools can significantly benefit comparative middle-down analysis of the native and forced degradation samples, biosimilars or sequence variants in high abundance.

Conclusions

- The streamlined, single-injection, EAD-based middle-down workflow can be a valuable addition to the biopharmaceutical toolkit to accelerate biotherapeutic development
- Biologics Explorer software provides powerful tools for comparative analysis of middle-down results to enable confident sequence confirmation and accurate PTM localization
- The comparison between EAD and CID middle-down results demonstrated the advantage of EAD over CID for the fragmentation of mAb subunits
- Comparative analysis of the native and oxidation samples using the mirror plot led to accurate localization of an oxidation site
- The mirror plot facilitated the confirmation and differentiation of 2 mAb subunits with highly similar sequences

References

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