Development of a streamlined single-injection workflow for middle-down analysis of protein therapeutics using electron activated dissociation (EAD)

Haichuan Liu, Elliott Jones and Zoe Zhang
SCIEX, USA

ABSTRACT

This poster 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 post-translational modifications (PTMs), such as glycosylation and oxidation. Biologics Explorer software provides powerful tools that enable fast and detailed comparisons of middle-down results to increase confidence in sequence confirmation and PTM localization.

INTRODUCTION

Sequence confirmation and PTM analysis are essential for the comprehensive characterization of therapeutics to ensure drug safety and efficacy. Middle-down mass spectrometry (MS) combines the advantages of bottom-up and top-down approaches and offers high sequence coverages of protein therapeutics following simple sample preparation.[1-3] Traditionally, a middle-down workflow requires extensive method development and often involves multiple fragmentation techniques and/or injections to obtain high sequence coverage.[1-3] This challenge can be addressed using a single-injection, EAD-based middle-down workflow.[4-6] This streamlined workflow provided consistently high sequence coverage of NISTmAb subunits, enabling sequence and PTM confirmations.

MATERIALS AND METHODS

Sample:
The 10-25 µg/µL stock solutions of mAbs, including NISTmAb, adalimumab, bevacizumab, cetuximab and trastuzumab, were diluted and incubated with the IdeS protease (Promega) at 37°C for 2 hours. The mixture was then denatured using guanidine hydrochloride and reduced at 60°C for 30 minutes using dithiothreitol. The final solution contained 0.2-0.5 µg/µL of mAb 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 in digested samples stored in the autosampler for an extended period of time.

HPLC:
The peptides were chromatographically separated with the LC gradients described previously.[4-6] The separation was achieved using a Waters ACQUITY UPLC BEH C4 column (2.1 × 50 mm, 1.7 µm, 300 Å) at a flow rate of 0.3 mL/min. The column was kept at 60°C in the column oven of an ExionLC AD system from SCIEX.

MS/MS:
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 data were analyzed using a new middle-down workflow template in the Biologics Explorer software.[4-6]

ZenoTOF 7600 system

Biologics Explorer software

RESULTS

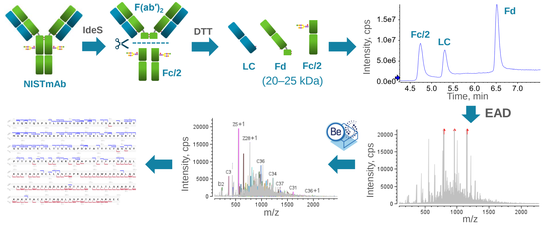

Figure 1. Overview of the EAD-based middle-down workflow. This powerful workflow provides high-quality EAD data of 3 mAb subunits (LC, Fd and Fc/2) in a single injection following simple sample preparation and rapid chromatographic separation. EAD data are automatically analyzed and annotated using a streamlined, optimized workflow template offered by Biologics Explorer software.

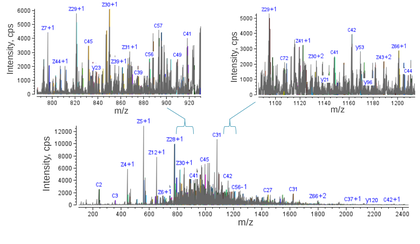

Figure 2. A representative EAD spectrum of the cetuximab LC subunit. EAD using the Zeno trap provided excellent fragmentation of the cetuximab LC subunit and permitted the detection of low-abundant fragments. These factors led to the generation of an information-rich spectrum that was used to achieve high sequence coverage.

Figure 3. The EAD-based middle-down workflow consistently provides high sequence coverages (>70%) of NISTmAb subunits between 6 consecutive injections. The percentage sequence coverages shown on top of the bar chart are average values from 6 injections. A single injection with EAD was sufficient to provide high sequence coverage of mAb subunits for confident sequence confirmation and PTM analysis.

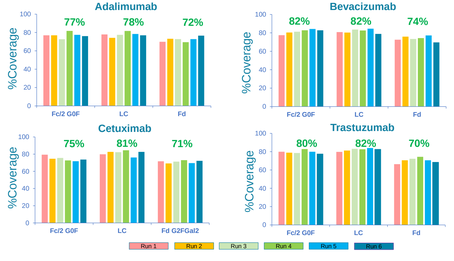

Figure 4. Percent sequence coverages of the Fc/2, LC and Fd subunits of multiple mAbs from 6 replicate injections. The EAD-based middle-down workflow provided consistently high sequence coverages (70%-85%) between runs or across different mAbs, including adalimumab, bevacizumab, cetuximab and trastuzumab. The percent sequence coverages shown above the bar charts are averaged across 6 replicate injections.

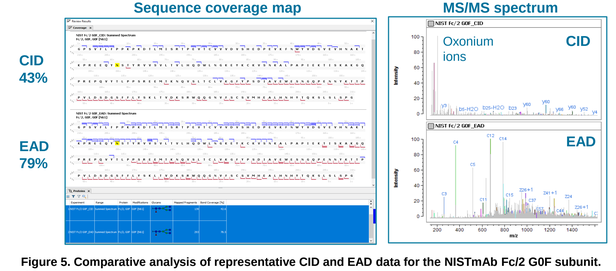

Figure 5. Comparative analysis of representative CID and EAD data for the NISTmAb Fc/2 G0F subunit. Biologics Explorer software provides powerful tools for comparative analysis of middle-down results to facilitate biotherapeutic development.

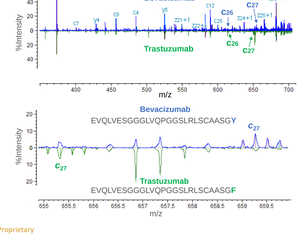

Figure 6. Comparative analysis using the mirror plot facilitated sequence differentiation and confirmation. The mirror plot can be leveraged to compare middle-down results of mAbs with high sequence similarities, such as biosimilars and sequence variants. Bevacizumab and trastuzumab Fd subunits have 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 c26 at the same m/z (A). The comparison of the m/z of the c27 fragment (B) indicated that the 2 subunits had different amino acid residues in position 27 (Tyr for bevacizumab and Phe for trastuzumab).

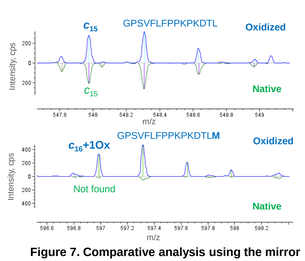

Figure 7. Comparative analysis using the mirror plot facilitated confident localization of an oxidation site in the NISTmAb Fc/2 subunit.

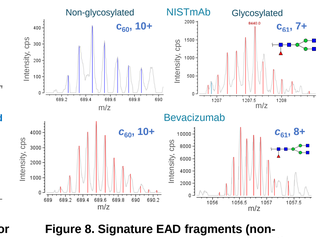

Figure 8. Signature EAD fragments (non-glycosylated c_{60} and glycosylated c_{61}) of the Fc/2 G0F subunit confirmed the site of N-linked glycosylation.

CONCLUSIONS

- A streamlined, single-injection EAD-based middle-down workflow enables confident sequence confirmation, accurate PTM localization and powerful comparative analysis of biotherapeutics.
- High sequence coverages ranging from 70% to 85% were obtained for mAb subunits consistently between injections or across biotherapeutics.
- Biologics Explorer software offers an easy-to-use middle-down workflow and provides automated data analysis with high accuracy and efficient results review and comparison for improved user experience.
- The workflow requires minimal method development and can be rapidly adopted by users with various levels of LC-MS experience, ideal for biopharma scientists.
- The EAD-based middle-down workflow might benefit biosimilar, stability and sequence variant analyses.

REFERENCES

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