Top/middle-down protein sequencing: A novel automated data processing tool for the top-down/middle-down analysis of biological therapeutics

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ABSTRACT

This poster describes a powerful, streamlined middle-down protein sequencing workflow in Biologics Explorer software to process the MS/MS data of monoclonal antibody (mAb) IdeS subunits acquired in the electron activated dissociation (EAD) mode. The middle-down data processing workflow, *Top_Middle-Down_ProteinSequencing workflow,* offers optimized parameters as the default settings. With powerful visualization tools, the workflow offers an accelerated path for biopharmaceutical laboratories to achieve an evidence-based decision-making approach to mAb characterization.

INTRODUCTION

Middle-down mass spectrometry (MS) offers several advantages over conventional bottom-up and top-down MS of biological therapeutics. Bottom-up MS provides complete sequence coverage of protein therapeutics. However, this technique involves multiple steps of sample preparation, in which modification artifacts are often introduced.[1] In contrast, top-down MS requires minimal sample preparation but typically results in low sequence coverage of fully intact mAbs.[1] Middle-down MS combines the advantages of these two approaches, offering high sequence coverage of subunits following simple sample preparation.[1-3] To date, commercial software designed for biopharmaceutical middle-down data analysis has been lacking in the market. This challenge is addressed by a new streamlined workflow optimized specifically for middle-down data, incorporated as an automated, user-friendly and robust data processing tool in Biologics Explorer software.

MATERIALS AND METHODS

Sample: 50 units of IdeS protease (Promega) was added to 50 µg NISTmAb solution (1 µg/µL) and the mixture was incubated at 37˚ C for 2 hours. After IdeS treatment, NISTmAb was denatured using 7.2M guanidine-hydrochloride in 50mM Tris-HCl solution (pH=7.4), followed by reduction using dithiothreitol. The mixture was incubated at 60˚ C for 30 minutes. The reaction was terminated by adding 0.1% formic acid (FA).

HPLC: The separation was accomplished on a Shimadzu LC system using an Agilent Poroshell 300SB-C8, Micro Bore 1.0 x 75 mm, 5 µm column kept at 80˚ C. Mobile phase A was 0.1% FA in water and mobile phase B was 0.1% FA in acetonitrile. A gradient system was used at a flow rate of 300 µL/min, in which mobile phase B increased from 10% to 90% over 9 minutes. The injection volume was set to 10 µL, which corresponds to approximately 1 µg of Fc/2, LC and Fd' subunits on column.

MS/MS: MRM[HR] EAD experiments were performed in SCIEX OS software using the ZenoTOF 7600 system. Three charge states per subunit were selected for EAD fragmentation in the MRM[HR] experiments (LC: 25+, 22+, 20+, Fc/2: 30+, 28+, 25+, Fd': 28+, 25+, 22+). Raw data (Wiff2) was processed with Biologics Explorer software using the *Top_Middle-Down_ProteinSequencing* workflow.

Figure 1. A streamlined workflow for middle-down analysis.

RESULTS

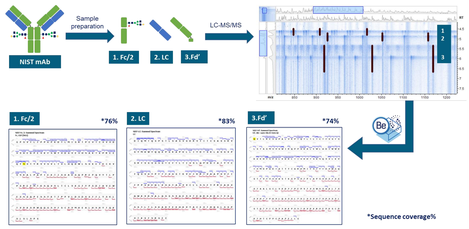

Figure 2. Overview of the middle-down workflow. With minimal user intervention, consistently high sequence coverage was observed for all NISTmAb subunits using the pre-optimized default settings.

Middle-down MS produces complex MS/MS spectra, in which isotope clusters overlap with each other. Therefore, correct identification relies not only on high mass accuracy, but also on recognition of correct isotope patterns.

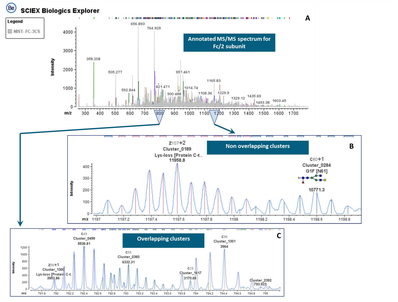

Figure 3. Representative EAD spectrum of NISTmAb Fc/2 G1F subunit. Figure 2B illustrates a confident identification of c_{80} fragment (z=9, 6.3 ppm), a signature fragment confirming the site of G1F N-linked glycosylation, and z_{107} fragment (z=10, -1.3 ppm), a fragment confirming C-terminus lysine loss on the NISTmAb Fc/2 subunit. Figure 2B demonstrates a more complex case in which multiple isotopic clusters of EAD-type fragments are overlapping. All clusters were correctly annotated with a mass accuracy of less than 10 ppm.

Intermediate results can be viewed and assessed at each step of data processing to offer transparency and traceability during analysis.

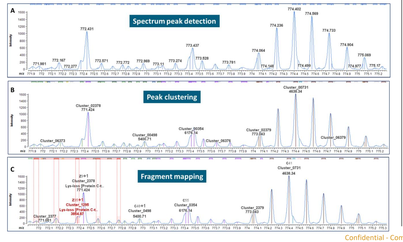

Figure 4. MS/MS synchronized view across different activity nodes. A zoomed view of the MS/MS spectrum of NISTmAb Fc/2 G1F subunit, in which the selection of a specific range can be synchronized across *Spectrum Peak Detection* (A), *Peak Clustering* (B) and *Fragment Mapping* (C) activity nodes.

All charge states of each annotated fragment can be simultaneously reviewed, adding confidence in results and allowing data review to follow an evidence-based decision-making approach.

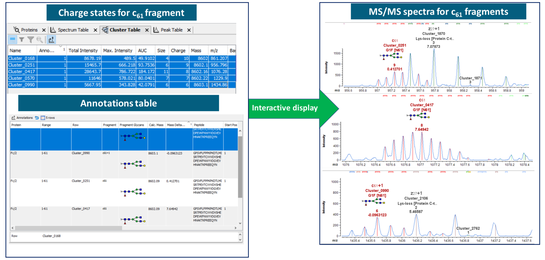

Figure 5. Overview of *Review Results* output. As a representative example, all charge states of c_{61} diagnostic fragment can be viewed simultaneously for easy data review. The annotation table displays details on each charge state, such as size, ppm, mass and range. The tables are interactive, such that the selection of any entry automatically zooms on the selected clusters in the MS/MS spectrum.

A new preconfigured instrument profile is introduced in the Fragment Mapping activity node to annotate different c- and z- ions observed in middle-down analysis using EAD fragmentation.

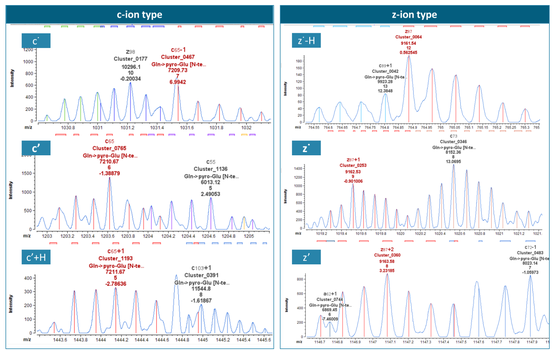

Figure 6. Representative EAD spectra of NISTmAb Fd' subunit showing different c- and z- ion types that can be currently annotated in *Top_Middle-Down_ProteinSequencing* workflow.

Sequence coverage % and number of unique mapped fragments are significantly increased with the annotation of different c- and z- ions observed during EAD fragmentation.

Figure 7. Sequence coverage map of NISTmAb LC subunit using different instrument profiles. c', c'+H, z'-H and z' ions are essential for the annotation of larger peptides observed during middle-down analysis using EAD fragmentation.

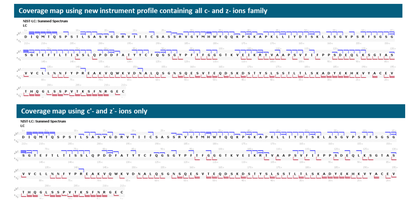

Figure 8. Impact of the new instrument profile containing additional c- and z- ions observed during EAD fragmentation on sequence coverage (right) and number of unique mapped fragments (left) of NISTmAb subunits.

CONCLUSIONS

- A streamlined EAD-based middle-down workflow enables confident sequence confirmation and PTM analysis using the default settings
- The powerful algorithm, diverse visualization tools and high traceability in Biologics Explorer software provide evidence-based review and confident identification of complex middle-down data
- A new preconfigured instrument profile of different ion types observed in EAD fragmentation mode maximizes the sequence coverage during middle-down analysis by correctly annotating all relevant fragment types

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