Confident sequence analysis of a trispecific antibody using an electron-activated dissociation (EAD)-based middle-down workflow

Featuring alternative fragmentation and streamlined data analysis software

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ABSTRACT

This poster describes the use of a single-injection, EAD-based middle-down workflow to achieve comprehensive sequence and disulfide-bond analysis of a trispecific antibody (tsAb). This powerful workflow leverages accurate mass measurement, information-rich EAD fragmentation and automated data analysis using Biologics Explorer software to offer confident sequence confirmation, disulfide bond mapping, and accurate localization of glycosylation.

Introduction

Sequence confirmation and post-translational modification (PTM) analysis are important for the comprehensive characterization of antibody-based therapeutics to ensure drug safety and efficacy.[1] Biotherapeutic characterization faces growing challenges due to the increasing complexity and variety of therapeutic modalities, such as fusion proteins and multispecific antibodies. A single-injection, EAD-based middle-down workflow was recently developed to provide consistently high sequence coverage of monoclonal antibody (mAb) subunits across mAbs.[2-4] This powerful, streamlined workflow offered confident sequence confirmation and accurate localization of PTMs, such as glycosylation and oxidation.[2-4] In this work, the EAD-based middle-down workflow was leveraged to confirm the sequences of major subunits of a tsAb treated with GlySERIAS and IdeS proteases, to determine the enzymatic cleavage sites on the linkers and to localize the glycosylation. Additionally, this single injection workflow also provides disulfide linkage confirmation.

MATERIALS AND METHOD

Sample preparation: The tsAb sample was incubated overnight with GlySERIAS (Genovis) and IdeS protease (Promega) at 37°C. The resulting solution was diluted using a mixture of 7.6M guanidine hydrochloride (HCl) and 50mM Tris-HCl (pH=7.4). Then, dithiothreitol (DTT) was added to a final concentration of 50mM to reduce disulfide bonds. The mixture was incubated for 1 hour at 60°C. The reaction was terminated by adding 10% formic acid (FA). The final solution contained ~1 µg/µL of the subunits.

Chromatography: TsAb subunits were separated using an ACQUITY UPLC Protein BEH C4 column (2.1 mm × 50 mm, 1.7 µm, 300 Å, Waters). 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 AD system (SCIEX). Mobile phase A was 0.1% FA in water and mobile phase B was 0.1% FA in acetonitrile.

Mass spectrometry: MRMHR EAD experiments were performed in SCIEX OS software using the ZenoTOF 7600 system. One or 2 charge states were targeted per subunit for EAD fragmentation.

Data processing: MRMHR data were analyzed using a middle-down workflow template in Biologics Explorer software, as described previously.

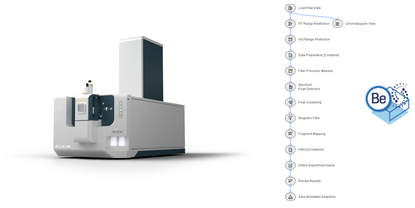

Figure 1. The ZenoTOF 7600 system (SCIEX) and middle-down workflow template provided by Biologics Explorer software (SCIEX).

RESULTS & DISCUSSION

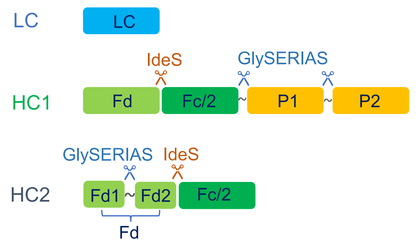

Figure 1. Major tsAb subunits from GlySERIAS and IdeS treatments followed by DTT reduction. The IdeS reaction produced LC and Fc/2 and Fd from 2 HC subunits (HC1 and HC2). The GlySERIAS treatment produced P1 and P2 subunits from HC1 and further cleaved HC2 Fd to Fd1 and Fd2. The "✂" symbols indicate the cleavage sites by GlySERIAS or IdeS. The "~" symbols indicate the $(GGGGS)_n$ linkers where GlySERIAS cleavages occur.

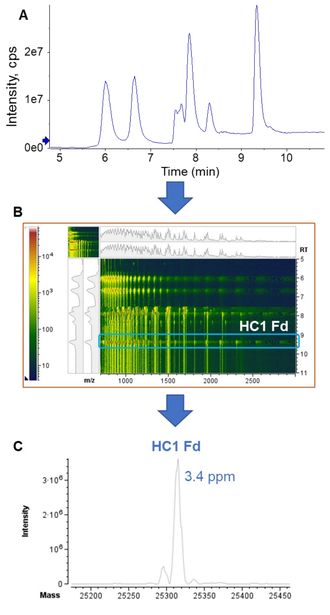

Figure 2. Intact mass measurement of major tsAb subunits from GlySERIAS and IdeS treatment. The incubation of the tsAb with GlySERIAS and IdeS proteases followed by DTT reduction produced multiple subunits, most of which were chromatographically separated. Biologics Explorer software provides powerful tools, such as ion map (B), for data visualization and advanced algorithms for protein deconvolution (C). An example of intact mass measurement for the HC1 Fd subunit is shown in panels B and C.

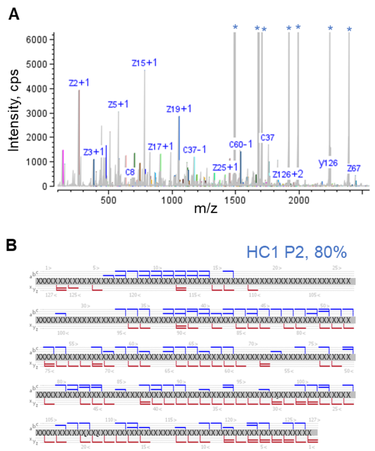

Figure 3. EAD spectrum and sequence coverage of the HC1 P2 subunit (13.5 kDa). Fragment-rich EAD spectrum (A) of the HC1 P2 subunit led to high sequence coverage (80%, B) for confident sequence confirmation of this subunit. The peaks labeled with "*" panel A are the remaining precursors or charged reduced species.

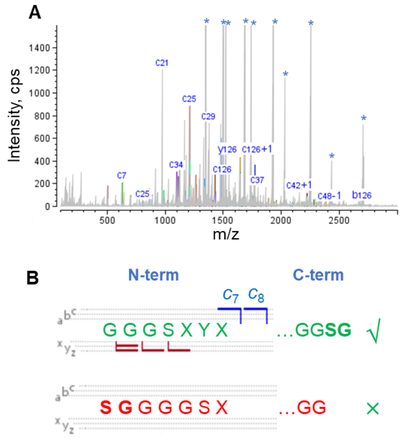

Figure 4. Sequence confirmation for the HC1 P1 subunit (13.5 kDa). Intact mass measurement revealed 2 possible sequences with identical masses for the HC1 P1 subunit. The detection of c-series ions, such as c7 and c8, in the high-quality EAD spectrum (A) confirmed the N-terminal sequence of the HC1 P1 subunit as "GGGSX" instead of "SGGGGSX" (B). The amino acid residues "X" and "Y" are not specified for proprietary reasons. The peaks labeled with "*" in panel A are the remaining precursors or charged reduced species.

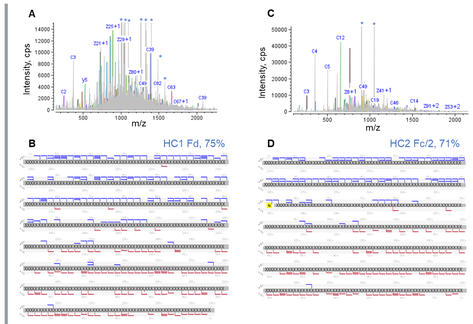

Figure 5. EAD-based middle-down analysis of the HC1 Fd and HC2 Fc/2 G0F subunits (25.3 kDa). The EAD-based middle-down workflow provided fragment-rich spectra and high sequence coverages (70%-75%) to enable confident sequence confirmation of HC1 Fd (A and B) and HC2 Fc/2 (C and D) subunits. The result also allowed for accurate localization of the G0F glycan on Asn61 (highlighted in D) in the HC2 Fc/2 subunit. The peaks labeled with "*" in panels A and C are the remaining precursors or charged reduced species.

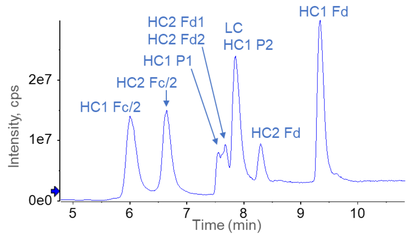

Figure 6. Major subunits identified in the tsAb sample treated with GlySERIAS and IdeS followed by DTT reduction. These tsAb subunits were confidently assigned based on the results of intact mass measurement and EAD-based middle-down analysis.

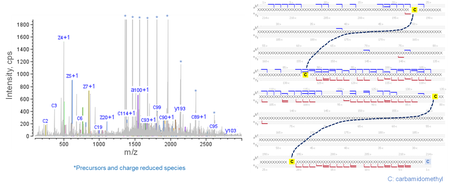

Figure 7. EAD showed a distinct fragmentation pattern for the disulfide-linked LC subunit. The detection of rich fragments that correspond to fragmentation in the middle of the LC sequence facilitated the determination of the 2 disulfide linkages, as illustrated on the sequence coverage map (right panel). The C-terminal Cys residue was carbamidomethylated.

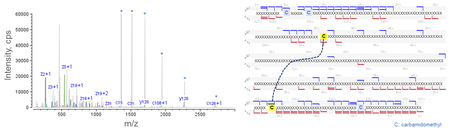

Figure 8. Determination of the disulfide linkage in the partially reduced HC1 P2 subunit. Intact mass measurement showed that this subunit contains 1 disulfide bond and 3 Cys residues carrying the carbamidomethyl modification. Middle-down analysis using EAD confirmed the carbamidomethylation of 3 Cys residues near the C-/N-terminus and a disulfide linkage between other two Cys residues (right panel).

CONCLUSION

- **Singe-injection:** A single-injection, EAD-based middle-down workflow combined accurate mass measurement, information-rich EAD fragmentation and automated data analysis to achieve comprehensive sequence and PTM analyses of a tsAb
- **Higher sequence coverage:** Fragment-rich EAD spectra led to high sequence coverages (>70%) of tsAb subunits from GlySERIAS and IdeS treatment, enabling confident sequence confirmation of the tsAb
- **Confident sequence confirmation:** Excellent EAD data enabled the differentiation of 2 potential sequences with identical masses for the HC1 P1 subunit
- **Localization of PTMs:** The glycosylation in the Fc/2 subunit was accurately localized based on the high-quality EAD data
- **Disulfide bond mapping:** The distinct fragmentation pattern of EAD facilitated the determination of disulfide bond linkages
- **Streamlined and easy-to-use:** Biologics Explorer software provides an optimized middle-down workflow template and powerful tools for data visualization, results review and comparison

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. A streamlined single-injection middle-down workflow using electron activated dissociation (EAD) for biotherapeutics characterization. SCIEX technical note, MKT-26997-A.
3. Obtaining high sequence coverage and confident post-translational modification (PTM) analysis of biotherapeutics using an electron activated dissociation (EAD)-based middle-down workflow. SCIEX technical note, MKT-27223-A.
4. Comparative analysis of biotherapeutics using an electron-activated dissociation (EAD)-based middle-down workflow. SCIEX technical note, MKT-27427-A.

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