Confident sequence analysis of a trispecific antibody 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 comprehensive sequence analysis of a complex 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 and accurate localization of glycosylation.

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 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 technical note, the EAD-based middle-down workflow (Figure 1) 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.

Key features of the EAD-based middle-down workflow for antibody characterization

- **Single-injection platform method:** The EAD-based middle-down workflow provides high sequence coverage in a single injection with 1 fragmentation technique
- **Confident sequence confirmation:** Zeno EAD leads to fragment-rich spectra of tsAb subunits for confident sequence confirmation and determination of enzymatic cleavage sites
- **Accurate localization of PTMs:** High sequence coverage and preservation of labile modifications by EAD allows for comprehensive PTM analysis
- **Powerful software tools:** Biologics Explorer software provides powerful tools for data visualization, results review and comparison
- **Streamlined:** The workflow consists of simple sample preparation, efficient data acquisition and automatic data analysis, with limited method development required and is easy to adopt by various levels of LC-MS users

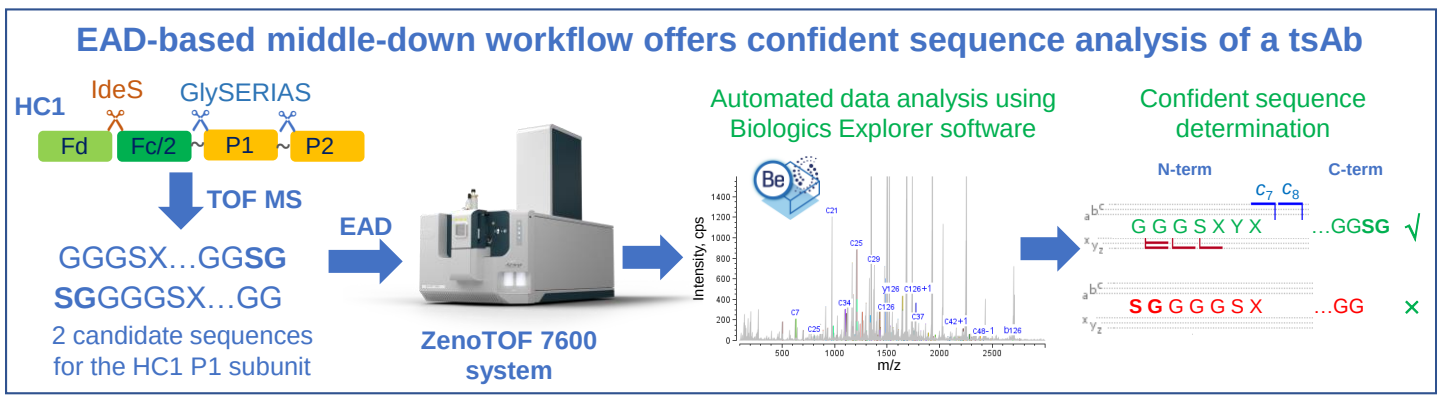

Figure 1. A streamlined, EAD-based middle-down workflow for comprehensive sequence analysis of a tsAb treated with GlySERIAS and IdeS. The EAD-based middle-down workflow combines excellent EAD fragmentation provided by the ZenoTOF 7600 system with automated data analysis using Biologics Explorer software. This streamlined workflow provides high sequence coverage to allow confident sequence confirmation of tsAb subunits, accurate determination of enzymatic cleavage sites on the linkers and precise PTM localization. The highlighted example demonstrates the power of the EAD-based middle-down workflow for confident determination of the sequence of the HC1 P1 subunit.

Methods

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). The gradient used for separation is shown in Table 1. 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.

Time (min)	A (%)	B (%)
Initial	*80*	*20*
2	*80*	*20*
14	*60*	*40*
15	*10*	*90*
17	*10*	*90*
17.5	*80*	*20*
20	*80*	*20*

Mass spectrometry: MRM^{HR} 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. 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 middle-down workflow template in Biologics Explorer software, as previously described.[2-4]

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 or 0.2 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 or 10 ms*
EAD RF	*150 Da*

Intact mass measurement of tsAb subunits

The tsAb used in this work contains 1 light chain (LC) and 2 heavy chains (HC1 and HC2). The composition of these chains is illustrated in Figure 2. Two HCs contain (GGGGS)n linkers that can be cleaved by the GlySERIAS protease. In addition, 2 HCs can be digested by the IdeS protease at the hinge region to produce the respective Fd and Fc/2 subunits. For HC2, the glycine-rich linker is located within the Fd subunit. The cleavage of this linker may lead to the formation of HC2 Fd1 and HC2 Fd2 subunits.

To generate 1 sample containing all subunits for middle-down analysis using EAD, the tsAb was incubated with GlySERIAS and IdeS proteases simultaneously, followed by the reduction of the disulfide bonds using DTT. This enzymatic treatment led to the formation of tsAb subunits with sizes ranging from 10 kDa to 30 kDa (Figure 2). Most of these subunits were baseline separated chromatographically using the short gradient shown in Table 1.

An intact mass measurement workflow using Biologics Explorer software was employed to identify major subunits detected in the chromatogram (Figure 3A). Figures 3B and 3C show an example of using an ion map for data visualization and performing protein deconvolution to obtain the accurate mass of the HC1 Fd subunit. The sequence of this subunit was further confirmed by middle-down analysis, as will be described in the next section. The combination of accurate mass measurement and middle-down analysis led to confident assignments of major tsAb subunits produced from GlySERIAS and IdeS treatment.

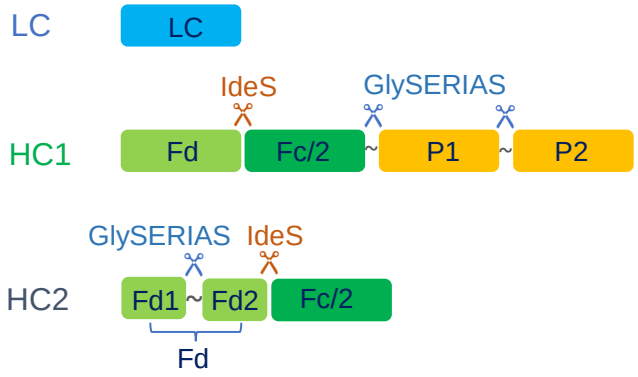

Figure 2. Major tsAb subunits from GlySERIAS and IdeS treatments followed by DTT reduction. The IdeS reaction produced LC and Fc/2 and Fd from 2 HC subunits (HC1 and HC2). The GlySERIAS treatment produced P1 and P2 subunits from HC1 and further cleaved HC2 Fd to Fd1 and Fd2. The "✂" symbols indicate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

Sequence confirmation of tsAb subunits in a single injection using the middle-down workflow

The single-injection, EAD-based middle-down workflow leverages reproducible and information-rich fragmentation by EAD and automated data analysis by Biologics Explorer software. It provides high sequence coverage for confident sequence confirmation and accurate localization of PTMs.[2-4] This powerful workflow was used in this work to confirm the sequences of tsAb subunits with or without glycosylation.

Figure 4 shows the EAD spectrum and sequence coverage map of the HC1 P2 subunit (13.5 kDa), which was produced from the cleavage of a glycine-rich linker on HC1 (Figure 2). EAD resulted in extensive fragmentation of this subunit, leading to high sequence coverage (80%) and confident confirmation of the sequence.

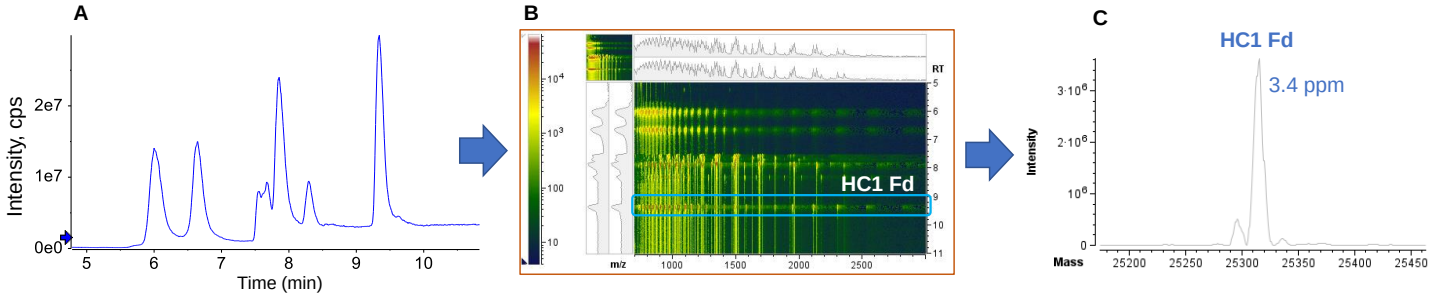

Figure 3. Intact mass measurement of major tsAb subunits from GlySERIAS and IdeS treatment. The incubation of the tsAb with GlySERIAS and IdeS proteases followed by DTT reduction produced multiple subunits, most of which were chromatographically separated using the gradient shown in Table 1 (A). Biologics 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.

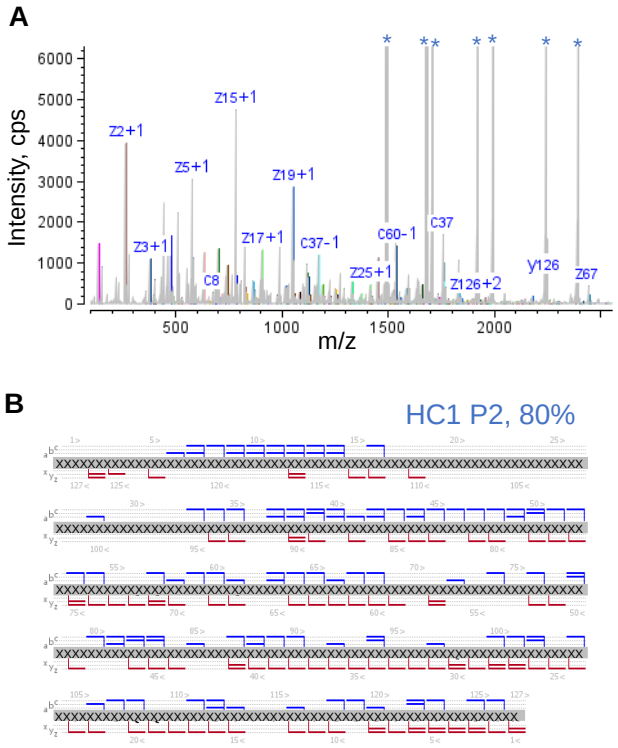

Figure 4. EAD spectrum and sequence coverage of the HC1 P2 subunit (13.5 kDa). Fragment-rich EAD spectrum (A) of the HC1 P2 subunit led to high sequence coverage (80%, B) for confident sequence confirmation of this subunit. The peaks labeled with "*" panel A are the remaining precursors or charged reduced species.

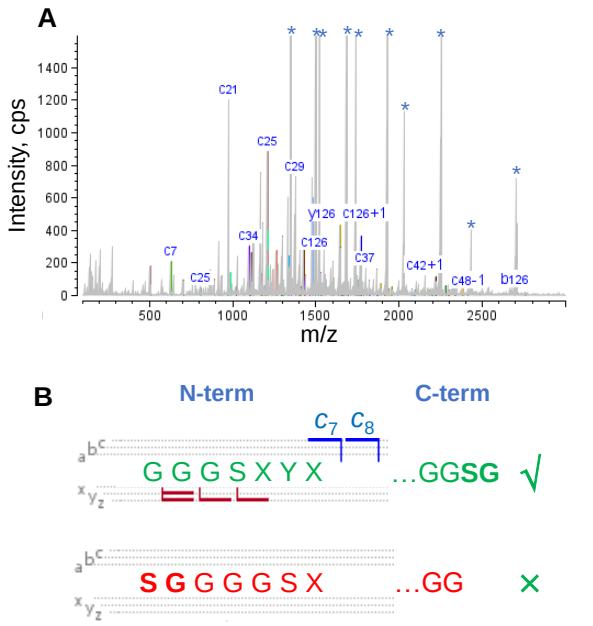

Figure 5. Sequence confirmation for the HC1 P1 subunit (13.5 kDa). Intact mass measurement revealed 2 possible sequences with identical masses for the HC1 P1 subunit. The detection of *c*-series ions, such as c_7 and c_8, in the high-quality EAD spectrum (A) confirmed the N-terminal sequence of the HC1 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.

The presence of 2 (GGGGS)n linkers on both ends of the HC1 P1 subunit (Figure 2) can result in 2 different sequences with identical masses (13.5 kDa) due to cleavage by GlySERIAS at different residues on the linkers. While 1 candidate sequence contains "GGGSX" and "GGSG" at the N- and C-terminus, respectively, another sequence can occur in which the SG moiety instead appears at the N-terminus (Figure 5). The 2 candidate sequences cannot be differentiated using TOF MS alone. The EAD-based middle-down workflow provided a fragment-rich MS/MS spectrum for confident determination of the sequence (Figure 5). Specifically, the detection of *c*-series ions, such as c_7 and c_8 (Figure 5B), showed that the HC1 P1 subunit carried an N-terminal sequence of "GGGSX" instead of "SGGGGSX". This sequence determination also allowed for accurate determination of the GlySERIAS cleavage sites on HC1.

Figure 6 shows EAD spectra and sequence coverage maps of HC1 Fd and HC2 Fc/2 subunits (25.3 kDa) from IdeS cleavages of HC1 and HC2, respectively. Excellent EAD fragmentation of the 2 subunits led to >70% sequence coverages, similar to the data obtained for various mAbs in previous studies.[2-4] This result demonstrates the power of the EAD-based middle-down workflow for achieving high sequence coverage consistently across different mAbs. The high sequence coverage of the HC2 Fc/2 subunit also enabled the accurate localization of the G0F glycan on the Asn[61] residue (Figure 6D).

The major tsAb subunits identified from intact mass measurement and middle-down analysis were annotated in Figure 7. The confident assignment of these species provides several benefits for tsAb characterization, including the sequence confirmation of LC, HC1 and HC2 chains and the characterization of the linkers within HCs. Additionally, the cleavage sites of GlySERIAS and IdeS were determined and accurate localization of PTMs such as glycosylation was acheived.

In summary, the EAD-based middle-down solution provides comprehensive sequence and PTM analyses of the tsAb in a streamlined manner. This powerful workflow can be rapidly adopted to characterize other types of multispecific antibodies.

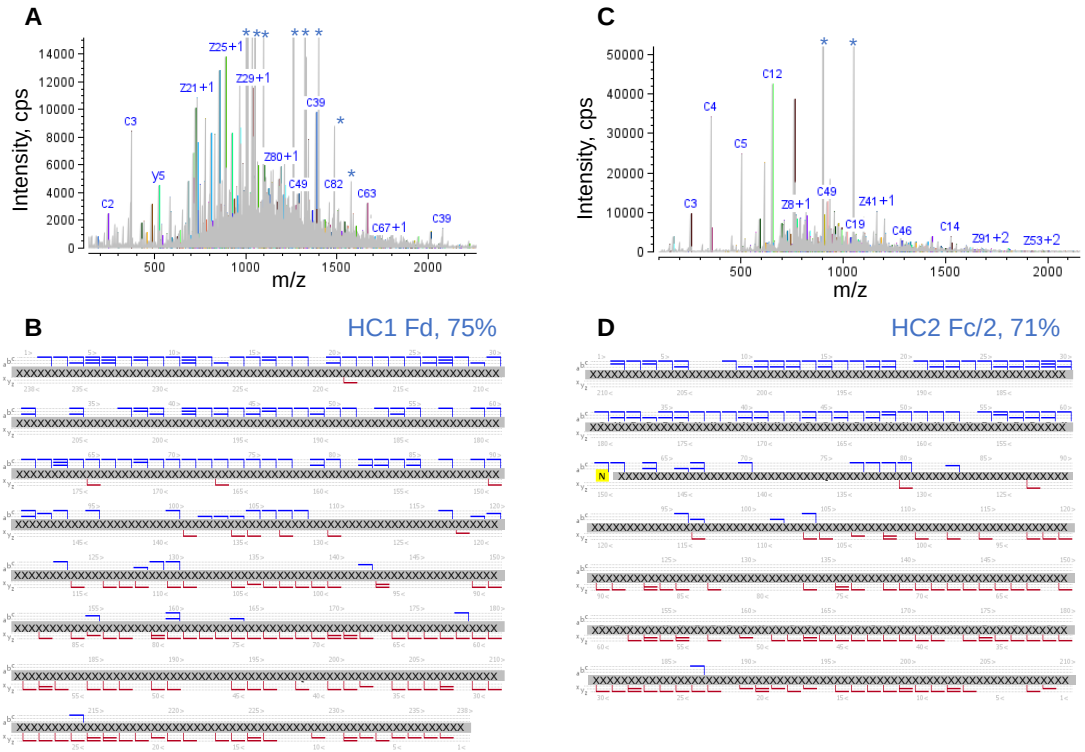

Figure 6. EAD-based middle-down analysis of the HC1 Fd and HC2 Fc/2 G0F subunits (25.3 kDa). The EAD-based middle-down workflow provided fragment-rich spectra and high sequence coverages (70%-75%) to enable confident sequence confirmation of HC1 Fd (A and B) and HC2 Fc/2 (C and D) subunits. The result also allowed for accurate localization of the G0F glycan on Asn[61] (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.

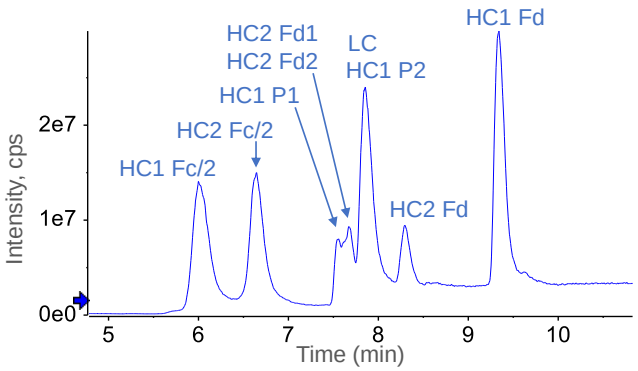

Figure 7. Major subunits identified in the tsAb sample treated with GlySERIAS and IdeS followed by DTT reduction. These tsAb subunits were confidently assigned based on the results of intact mass measurement and EAD-based middle-down analysis.

Conclusions

- 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
- 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
- Excellent EAD data enabled the differentiation of 2 potential sequences with identical masses for the HC1 P1 subunit
- The glycosylation in the Fc/2 subunit was accurately localized based on the high-quality EAD data

References

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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

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