# High-confidence disulfide bond mapping 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 power of a single-injection, EAD-based middle-down workflow for rapid disulfide bond mapping of biotherapeutics with high confidence and high fidelity. The mapping of disulfide bonds on the subunit level minimizes the chance of disulfide scrambling, reduces ambiguities or false positives in determining the disulfide linkages and significantly simplifies data interpretation. EAD led to a characteristic fragmentation pattern of disulfide-linked subunits to rapidly confirm or elucidate the expected or unexpected disulfide linkages with high confidence.

Disulfide bonds play a key role in determining the folding, stability and function of biotherapeutics, such as monoclonal antibodies (mAbs).[1] Incorrect formation of disulfide bridges can significantly reduce the efficacy of a biotherapeutic product.[1] Therefore, confirming the disulfide linkages in biotherapeutics is critical to ensure product quality, safety and efficacy. Disulfide bond mapping is traditionally performed using a peptide mapping approach under non-reduced conditions. However, this strategy might lead to ambiguities or false positives in identification due to disulfide scrambling and/or poor MS/MS spectra of low-abundant disulfide-linked peptides. By comparison, the EAD-based middle-down workflow[2-5] provides a clear and direct mapping of intra-chain disulfide linkages on the subunit level (Figure 1) without the challenges mentioned above.

## Key features of the EAD-based middle-down workflow for disulfide bond mapping

- **High confidence and high fidelity:** EAD leads to a distinctive fragmentation pattern of disulfide-linked subunits for disulfide bond mapping with minimal false positives
- **Minimal disulfide scrambling:** Disulfide bond mapping on the subunit level reduces disulfide scrambling and eliminates the need to identify low-abundant disulfide-linked peptides, significantly increasing confidence in mapping results
- **Single-injection method:** The workflow provides high-quality middle-down data in a single injection
- **Streamlined and easy to implement:** The workflow requires minimal method optimization and is streamlined from data acquisition to results review
- **Powerful software tools:** Biologics Explorer software provides optimized workflow templates for improved user experience with data analysis and powerful visualization tools for results review and comparison

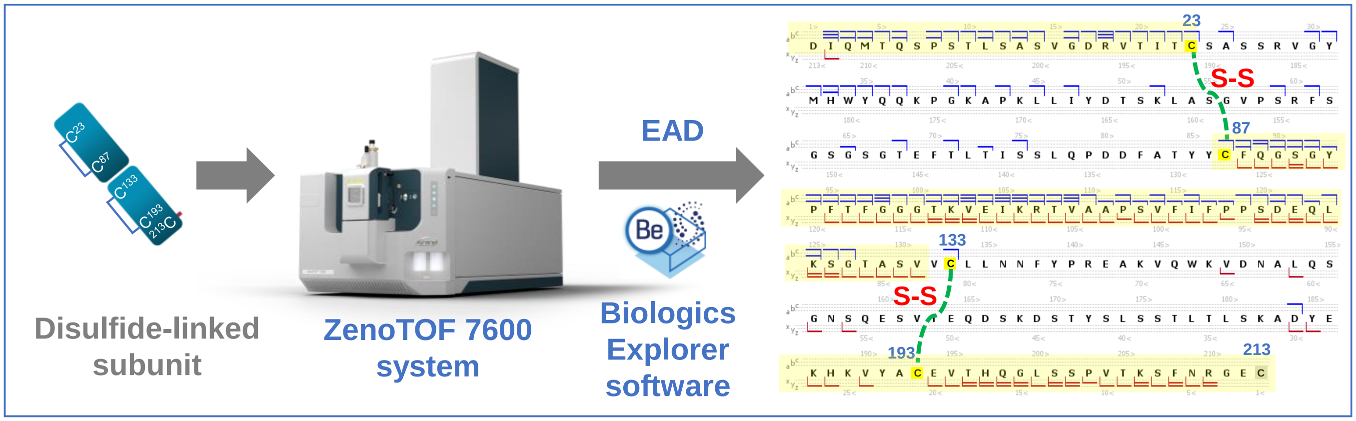


**Figure 1. A single-injection, EAD-based middle-down workflow for high-confident and high-fidelity disulfide bond mapping of biotherapeutics.** The EAD-based middle-down workflow combines excellent EAD fragmentation provided by the ZenoTOF 7600 system with automated data analysis using Biologics Explorer software for confident sequence confirmation, PTM analysis and disulfide bond mapping. EAD of disulfide-linked subunits leads primarily to bond cleavages from outside the disulfide bond-forming regions (highlighted in the right panel). This characteristic fragmentation pattern enables rapid confirmation of known disulfide linkages and also facilitates the elucidation of mispaired disulfide bridges with minimal ambiguities.

## Methods

***Sample preparation:*** An aliquot of 10 µg/µL of NISTmAb was diluted in water to a concentration of 1 µg/µL. Then, 50 units/µL of FabRICATOR (IdeS) from Genovis and 50mM dithiothreitol (DTT) were added. The NISTmAb mixture was incubated at 37°C for 15 minutes. For trispecific antibody (tsAb), the sample was first treated with GlySERIAS (Genovis) for 2 days at 37°C, then incubated with 20mM DTT for 5 minutes at 37°C. The reduced tsAb subunits were alkylated for 30 minutes at room temperature using 40mM iodoacetamide. Finally, 5 µL of the NISTmAb or tsAb solution (~5 µg) was injected for LC-MS analysis.

***Chromatography:*** The subunits of NISTmAb and tsAb were separated using an ACQUITY UPLC Protein BEH C4 column (2.1 mm × 50 mm, 1.7 µm, 300 Å, Waters). The LC gradient used for the subunit 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% formic acid in water and mobile phase B was 0.1% formic acid 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 charge state (15+ or 17+) per subunit was targeted 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.

**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* | *0.1 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 or 5000 m/z* |
| *Q1 resolution* | *Low* |
| *Zeno trap* | *ON* |
| *Zeno threshold* | *100,000 cps* |
| *Accumulation time* | *0.1 or 0.2 s* |
| *Declustering potential* | *80 V* |
| *Collision energy* | *12 V* |
| *Time bins to sum* | *8* |
| *Electron beam current* | *5000 nA* |
| *Electron KE* | *1 eV* |
| *ETC* | *100%* |
| *Reaction time* | *10 ms* |
| *EAD RF* | *150 Da* |

## Partial reduction of NISTmAb and tsAb

The single-injection, EAD-based middle-down workflow provides consistently high sequence coverages (70%-85%) and accurate localization of PTMs for the fully reduced mAb subunits.[2-5] This powerful workflow was leveraged here to gain insights into the intra-chain disulfide linkages in NISTmAb and a tsAb. To generate disulfide-linked subunits, NISTmAb and tsAb samples were treated with IdeS and GlySERIAS proteases, respectively, then reduced with DTT for a short period of time (5-15 minutes). Under these conditions, the inter-chain disulfide bonds were reduced while the intra-chain linkages remained intact.

For NISTmAb, IdeS treatment and partial reduction led to the formation of LC, Fd and Fc/2 subunits containing 2 intra-chain disulfide bonds (Figure 2A). The LC and Fd subunits also contained 1 and 3 free Cys residues, respectively, from the reduction of inter-chain disulfide bonds. The 3 disulfide-linked subunits were chromatographically separated in an elution order similar to their fully reduced counterparts.[2,3] Accurate mass measurement of these subunits using Biologics Explorer software confirmed the presence of 2 disulfide bonds (-4 Da) in each subunit (Figure 2C). Similarly, intact mass analysis of the partially reduced tsAb sample revealed the presence of 2 disulfide bonds and 1 carbamidomethyl group on the LC subunit (data not shown). To determine how disulfide bonds are connected in these subunits, a representative charge state (15+ or 17+) of each subunit was targeted for EAD fragmentation, as will be described below.

## EAD-based middle-down analysis of disulfide-linked subunits

Figure 3 shows an annotated EAD spectrum of the LC subunit containing 2 intra-chain disulfide bonds. The spectrum is displayed in 3 m/z ranges for a better view of rich fragments across different mass ranges. It was described previously that EAD of the fully reduced LC subunit led to a relatively even distribution of *c*/*z* fragments from the first or second half of the sequence.[2-5] By comparison, the fragments detected in EAD of the disulfide-linked LC subunit were predominantly from the regions not restricted by the disulfide bridges (Figure 3).

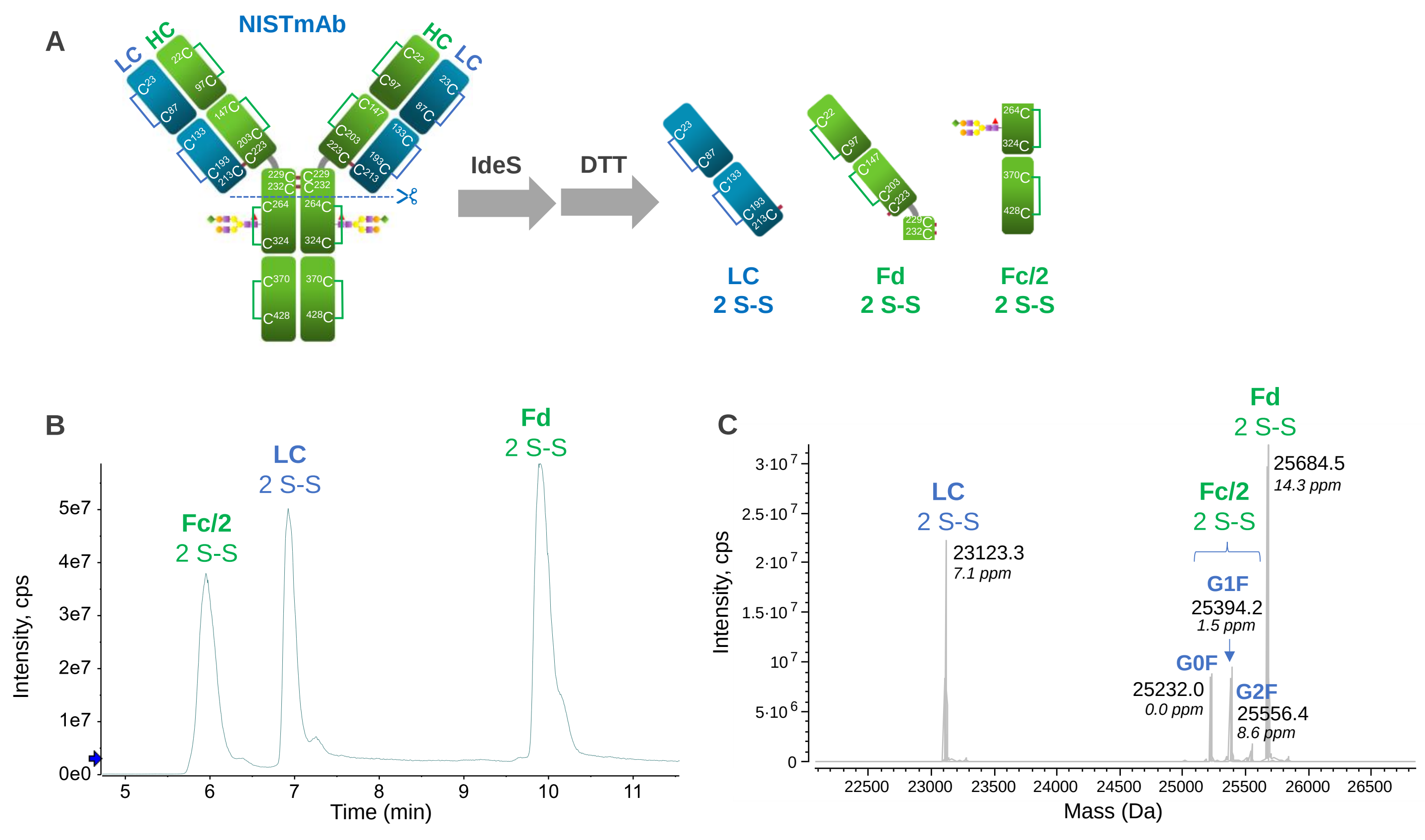


**Figure 2. Intact mass measurement of the partially reduced NISTmAb subunits.** IdeS treatment of NISTmAb followed by partial DTT reduction led to the formation of LC, Fd and Fc/2 subunits containing 2 intra-chain disulfide bonds (A). Three disulfide-linked subunits were baseline separated chromatographically using the LC gradient shown in Table 1 (B). Deconvolution of the intact TOF MS data using Biologics Explorer software confirmed the presence of 2 disulfide bonds in all 3 subunits (C). The symbol "✂" indicates the cleavage site of IdeS in the hinge region.

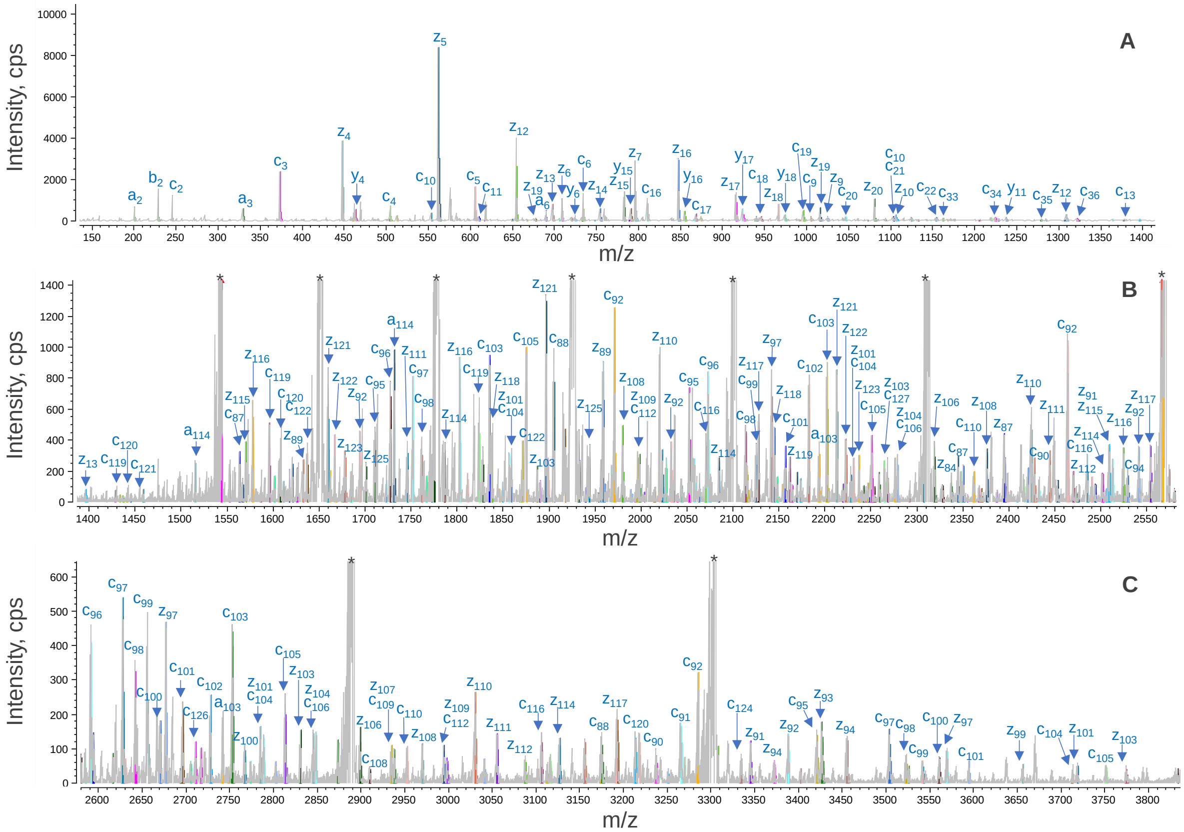


**Figure 3. Annotated EAD spectrum of a disulfide-linked LC subunit from NISTmAb.** The spectrum is displayed in 3 m/z ranges, including ~150-1,400 (A), ~1,400-2,600 (B) and ~2,600-3,800 (C), for a better view of rich fragments across different mass ranges. EAD led to extensive fragmentation of the LC sequence beyond the disulfide-forming regions. The peaks at m/z<1,500 correspond mainly to the fragmentation occurring near the N- and C-termini (A), while most of the fragments above m/z=1500 (B and C) were produced from the sequence region between Cys$^{87}$ and Cys$^{133}$ residues (Figure 2A). In this technical note, the number of extra hydrogen atoms, if any, in the fragments was not specified for spectral clarity. For example, all the "$z$+1" fragments were labeled as "$z$". The peaks labeled with * are the precursor or charged reduced species. Not all fragments were labeled in the spectra.

Specifically, a series of large $c/z$ fragments at m/z values >1500 (Figures 3B and 3C) were produced from the backbone cleavages between the Cys$^{87}$ and Cys$^{133}$ residues, as depicted in the sequence coverage map shown in Figure 4. The fragments detected at m/z values <1500 (Figure 3A) are mainly from the N- or C-terminal regions beyond the Cys$^{23}$ and Cys$^{193}$ residues (Figure 4). A similar fragmentation pattern of the disulfide-linked LC subunit was also observed for the Fd and Fc/2 subunits (Figure 5). These results show that disulfide bridges restricted the fragmentation to the regions not 'protected' by the disulfide bridges. The characteristic EAD fragmentation pattern of the disulfide-linked subunits can be leveraged to confirm the expected disulfide linkages or elucidate the mispaired connections.

The powerful capability of the EAD-based middle-down workflow for disulfide bond mapping was employed to characterize a partially reduced tsAb treated with GlySERIAS. Figure 6 shows the EAD spectrum and sequence coverage map of the disulfide-

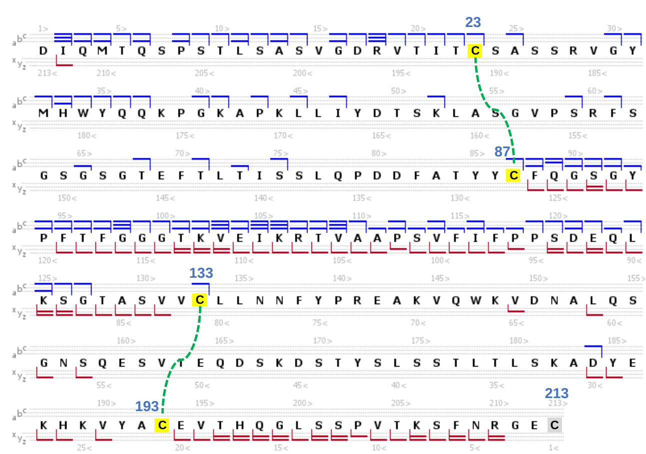


**Figure 4. Sequence coverage map of the disulfide-linked LC subunit from NISTmAb.** EAD led to the formation of fragments predominantly from outside 2 disulfide-forming regions between Cys$^{23}$-Cys$^{87}$ and Cys$^{133}$-Cys$^{193}$. This distinctive fragmentation pattern can facilitate rapid confirmation of intra-chain disulfide linkages in mAb subunits. The C-terminal Cys$^{213}$ residue was present in the reduced form.

linked LC subunit from the tsAb. Similar to the NISTmAb results described above (Figures 3-5), EAD provided excellent fragmentation of the sequence regions that were not covered by the disulfide bridges, allowing for confident confirmation of the disulfide connections in this LC subunit. In another example, the

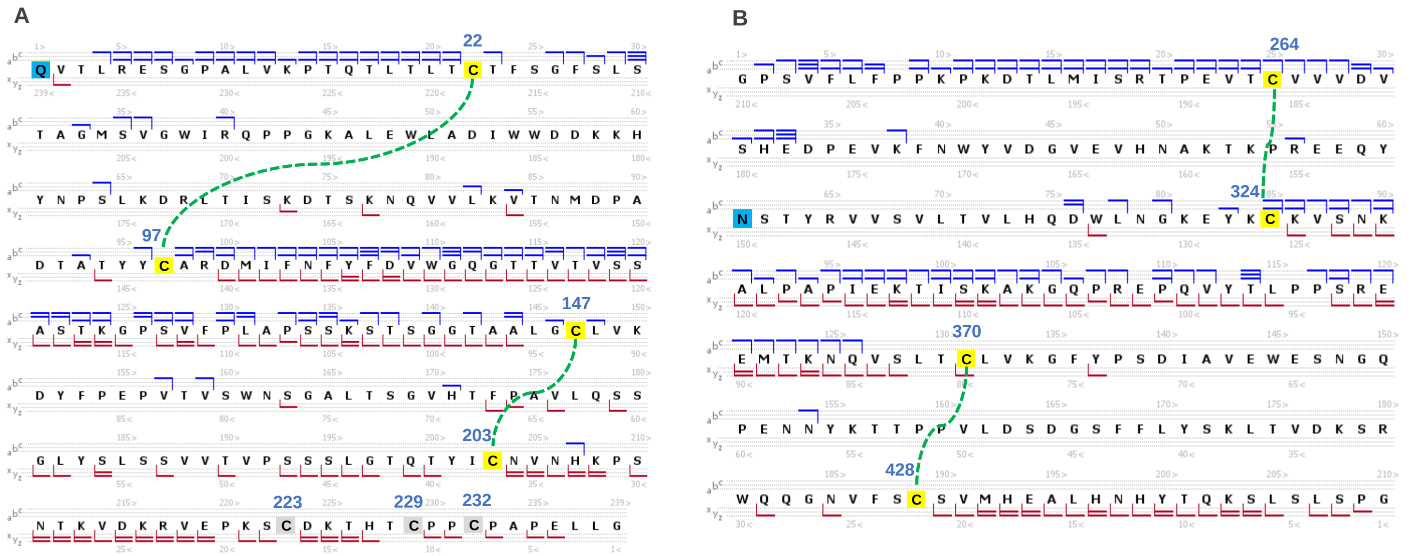


**Figure 5. Sequence coverage maps of disulfide-linked Fd and Fc/2 subunits from NISTmAb.** The distinctive fragmentation patterns of these 2 disulfide-linked subunits enabled a confident confirmation of the expected linkages between Cys[22]-Cys[97] and Cys[147]-Cys[203] in the Fd subunit (A) and Cys[264]-Cys[324] and Cys[370]-Cys[428] in the Fc/2 subunit (B). The N-terminal Gln residue in the Fd subunit (A) is a pyro-glutamic acid, while the Asn residue highlighted for the Fc/2 subunit (B) carried an N-linked G0F glycan. The 3 Cys residues (Cys[223], Cys[229] and Cys[232]) near the C-terminus of the Fd subunit (A) have the free -SH end.

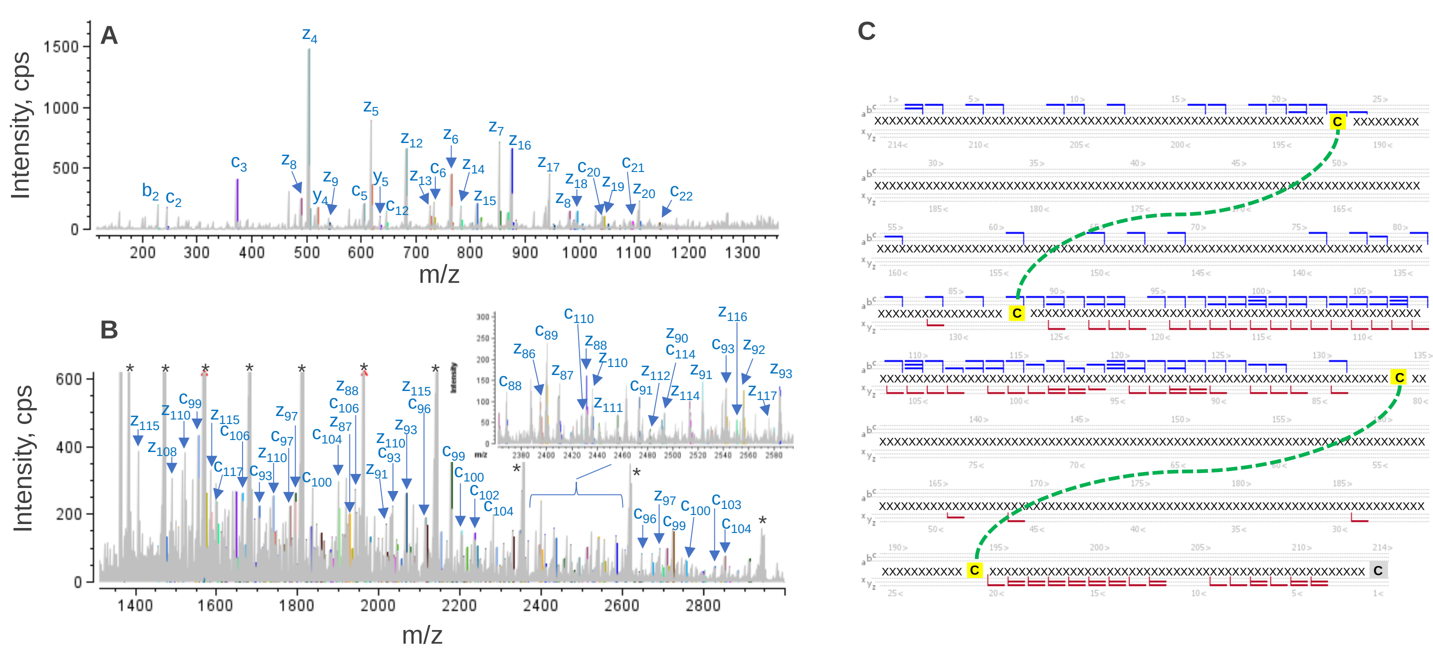


**Figure 6. Disulfide bond mapping of the LC subunit from the tsAb.** EAD produced rich fragments of the disulfide-linked LC subunit from a tsAb across a wide m/z range (A and B). The inset in B highlights the annotated fragments detected in the m/z range of ~2,350-2,600. The peaks labeled with * are the precursor or charged reduced species. Not all fragments were labeled in the spectra. The detection of extensive fragmentation outside the disulfide-forming regions confirmed the expected disulfide linkages between 2 Cys pairs (C). The C-terminal Cys residue was modified with a carbamidomethyl group (B).

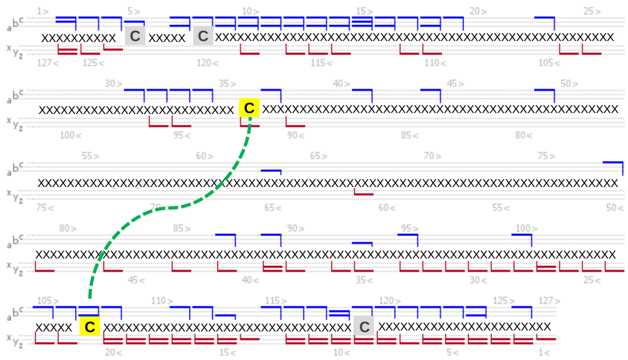

**Figure 7. Sequence coverage map of a disulfide-linked subunit (~13.7 kDa) from GlySERIAS cleavage of the HC1 subunit of the tsAb.** The disulfide linkage in this subunit was established based on the detection of rich fragments from 2 ends of the sequence. The 3 Cys residues in the gray background were modified with a carbamidomethyl group.

disulfide linkage in a tsAb subunit (~13.7 kDa) from GlySERIAS cleavage of the HC1 subunit was confirmed based on the detection of the fragments primarily from the N- and C-terminal ends of the sequence (Figure 7). EAD of the disulfide-linked subunits revealed that the disulfide bond was reduced in some fragments detected in the high m/z range, resulting in a 2 Da increase in their masses. Similar results were obtained for the fragmentation of disulfide-linked peptides.[6,7] The mechanism for the formation of these fragments will be discussed in a future technical note.

In summary, the EAD-based middle-down workflow provides a characteristic fragmentation pattern for rapid disulfide bond mapping of biotherapeutics on the subunit level with high confidence and high fidelity. This workflow involves simple sample preparation that avoids disulfide scrambling and minimizes ambiguities or false positives in the determination of the disulfide bridges. In addition, the complexity of data interpretation is significantly reduced using the EAD-based middle-down workflow. This powerful workflow can be leveraged to confirm expected disulfide linkages or to elucidate the formation of mispaired disulfide bonds with confidence and throughput that are unrivaled by traditional peptide mapping approaches.

## Conclusions

- A streamlined, EAD-based middle-down workflow led to a characteristic fragmentation pattern of disulfide-linked subunits for rapid disulfide bond mapping with high confidence and high fidelity
- This workflow minimizes the chance of disulfide scrambling and reduces the ambiguities or false positives in determining the intra-chain disulfide bridges
- EAD produced rich fragments across the full m/z range from the sequence regions not restricted by the disulfide bridges
- The EAD-based middle-down workflow combines accurate mass measurement, reproducible EAD fragmentation and automated data analysis and provides an in-depth characterization of fully or partially reduced biotherapeutics
- Biologics Explorer software offers easy-to-use middle-down workflow templates and powerful visualization tools for improved user experience with data analysis

## References


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