Achieving ultrahigh sequence coverage and 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 an innovative EAD-based middle-down workflow for achieving ultrahigh sequence coverages (85%-93%) of antibody subunits. Additionally, this workflow provides valuable insights into the intra-chain disulfide linkages. The combination of complementary EAD results from the fully reduced and disulfide-linked subunits led to ultrahigh sequence coverage of antibody subunits in 2 injections in addition to high-confidence disulfide bond mapping.

Middle-down mass spectrometry (MS) is emerging as a promising approach for biotherapeutic characterization.[1-5] Middle-down MS provides much higher sequence coverage of biotherapeutics than top-down MS. Compared to bottom-up MS, middle-down MS benefits from simpler sample preparation, a lower degree of artificial modification, easier data interpretation, fewer false positive identifications and higher throughput. One of the limitations of traditional middle-down workflows is the lack of fragmentation in the middle of a subunit sequence. This limitation can be overcome by applying EAD to disulfide-linked subunits.[5]

The state-of-the-art, EAD-based middle-down workflow combines accurate mass measurement and information-rich EAD fragmentation with automated data analysis for rapid sequence confirmation, accurate PTM localization and high-confidence disulfide bond mapping.[1-5] In this technical note, these powerful capabilities of EAD were leveraged to provide a nearly complete sequence coverage of antibody subunits and high-confidence disulfide bond mapping (Figure 1).

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

- **Ultrahigh sequence coverage:** The combined EAD results for the fully reduced and disulfide-linked subunits provide 85%-93% sequence coverage of antibody subunits
- **High-confidence disulfide bond mapping:** EAD leads to a distinctive fragmentation pattern of disulfide-linked subunits for rapid disulfide bond mapping with minimal false positives
- **Accurate localization of PTMs:** Labile PTMs are preserved in the EAD fragments for their accurate localization
- **Streamlined and easy to implement:** The workflow requires minimal method optimization and is streamlined from data acquisition to results review

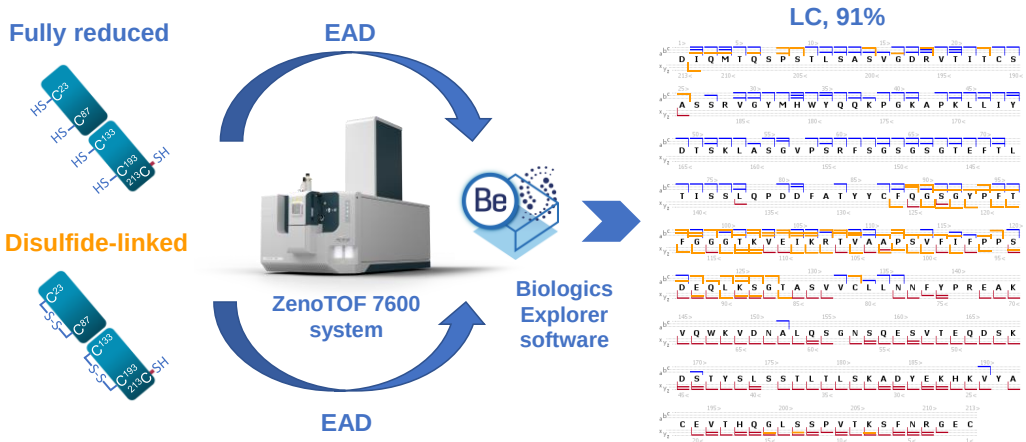

Figure 1. EAD-based middle-down analysis of the fully reduced and disulfide-linked antibody subunits led to a nearly complete sequence coverage (85%-93%) of antibody subunits in 2 injections. EAD provided high sequence coverage (70%-85%) of the fully reduced mAb subunits in a single injection.[1-4] The characteristic fragmentation of the disulfide-linked subunits by EAD led to the detection of unique fragments from the middle of the sequence between 2 central Cys residues, as highlighted by the orange lines in the coverage map.[5] The addition of these fragments offered a significant increase in sequence coverage and provided high-confidence mapping of the intra-chain disulfide bonds.

Methods

Sample preparation: The details of sample preparation for the fully reduced and disulfide-linked subunits of NISTmAb, bevacizumab, trastuzumab and a trispecific antibody (tsAb) were described in previous technical notes.[1-5] Briefly, to prepare the fully reduced subunits, the antibody samples were incubated with FabRICATOR (IdeS) from Genovis at 37°C for 2 hours, then were denatured using guanidine hydrochloride (GuHCl) and reduced at 60°C for 30 minutes using dithiothreitol (DTT). The disulfide-linked subunits were prepared by incubating the IdeS-treated antibodies with DTT at 37°C for 15 minutes in the absence of GuHCl. Finally, 1-5 µL of the final solution (1-2 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 × 50 mm, 1.7 µm, 300 Å, Waters). The LC gradients used for the subunit separation are 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 subunit separation.

Time (min)	A (%)	B (%)
Initial	*80*	*20*
2	*80*	*20*
14	*65 or 60*	*35 or 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. 1-3 charge states were targeted for EAD fragmentation of the fully reduced and disulfide-linked subunits. 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	Value
Start mass	*100 m/z*
Stop mass	*3000-5000 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*
CE	*12 V*
Time bins to sum	*8*
Electron beam current	*5000 nA*
Electron KE	*1 eV*
ETC	*100%*
Reaction time	*5 or 10 ms*
EAD RF	*150 Da*

EAD of the fully reduced and disulfide-linked antibody subunits

The EAD-based middle-down workflow provides consistently high sequence coverages (70%-85%) and accurate localization of PTMs for the fully reduced antibody subunits.[1-4] For the disulfide-linked subunits, EAD leads to a characteristic fragmentation pattern in which the bond cleavages occur primarily outside the disulfide-forming regions, enabling rapid disulfide bond mapping of biotherapeutics on the subunit level with high confidence and high fidelity.[5] The combination of complementary EAD results of the fully reduced and disulfide-linked subunits offers an innovative strategy to achieve a nearly complete characterization of biotherapeutics with minimal modification artifacts and false positives.

Figure 2 compares the EAD MS/MS spectra of the fully reduced and disulfide-linked LC subunit of bevacizumab. EAD led to excellent fragmentation and information-rich spectra in both cases. However, a distinctive difference was observed between EAD fragmentation of the fully reduced and disulfide-linked subunits. EAD of the fully reduced LC subunit resulted in an

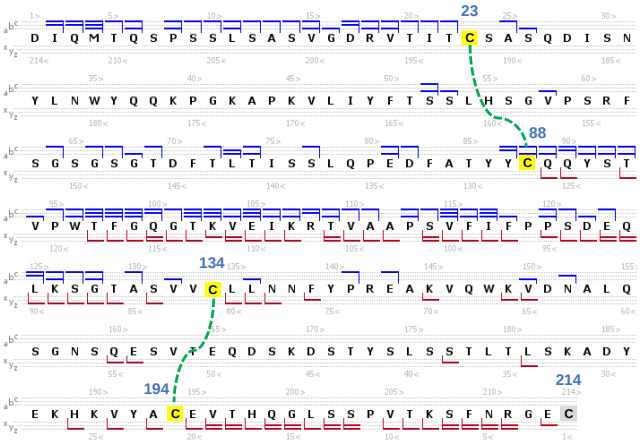

Figure 3. Sequence coverage map of the disulfide-linked LC subunit from bevacizumab. EAD led to the formation of fragments predominantly from outside the 2 disulfide-forming regions between Cys[23]-Cys[88] and Cys[134]-Cys[194], confirming the expected intra-chain disulfide linkages in this subunit. The C-terminal Cys[214] residue was present in its reduced form.

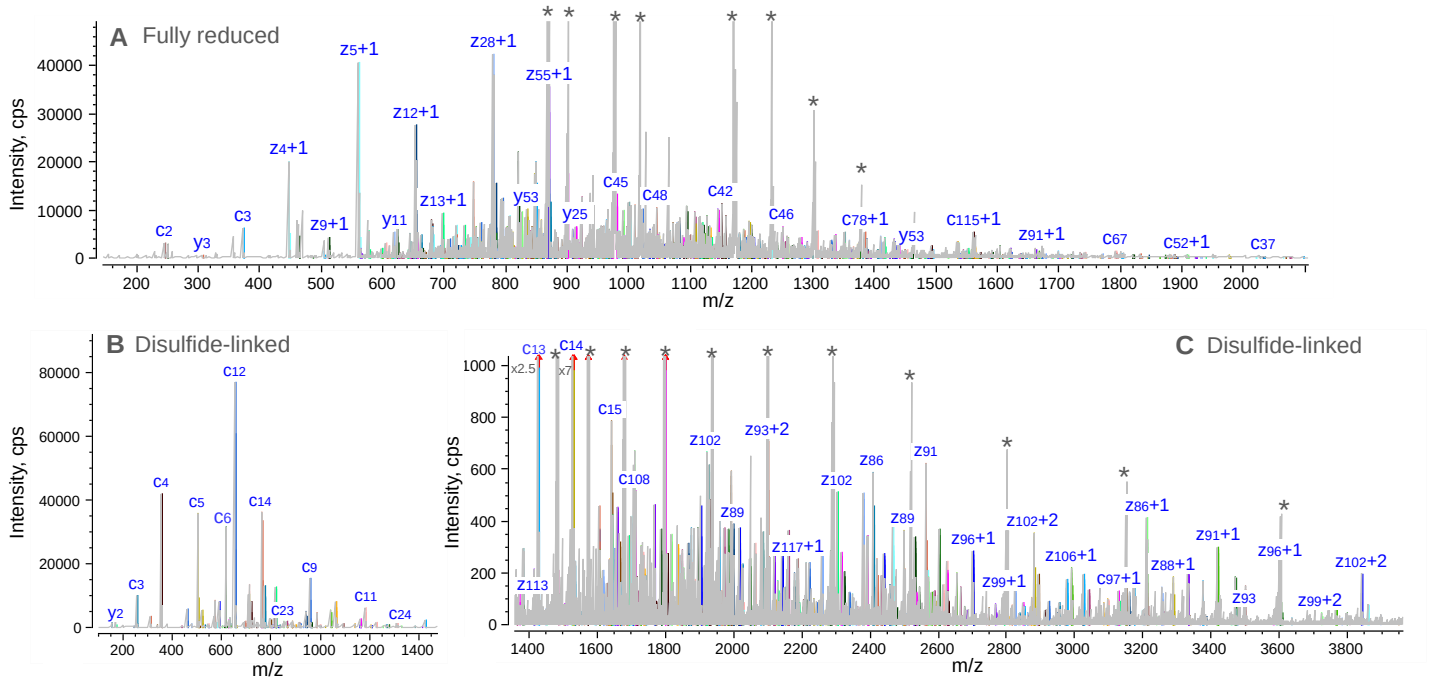

Figure 2. Annotated EAD MS/MS spectra of the fully reduced and disulfide-linked LC subunit of bevacizumab from Biologics Explorer software. The EAD spectrum of the fully reduced LC subunit is displayed in the full m/z range of ~150-2,100 (A), whereas the EAD spectrum of the disulfide-linked subunit is shown in 2 different m/z ranges of ~150-1,400 (B) and ~1,400-3,850 (C) for a better spectral view. EAD provided excellent fragmentation and information-rich MS/MS spectra for the fully reduced and disulfide-linked LC subunits. Complementary results were obtained from the 2 experiments. EAD of the fully reduced subunit led to extensive fragmentation of the N- and C-terminal regions, with limited cleavages in the middle of the sequence. By comparison, EAD fragmentation of the disulfide-linked subunit occurred primarily outside the disulfide-forming regions, including the central region between the Cys[88] and Cys[134] residues in the LC subunit of bevacizumab, leading to the detection of rich large *c/z* fragments across the high m/z range (C). Not all fragments are displayed for spectral clarity. The peaks labeled with asterisks are the precursors or charge-reduced species.

extensive fragmentation across the N- and C-terminal regions of the sequence, producing a relatively even distribution of the fragments across the m/z range of ~400-1600 (Figure 2A). By comparison, EAD fragmentation of the disulfide-linked LC subunit was concentrated on the sequences outside the 2 disulfide-forming regions. This led to the detection of the low m/z fragments from the 2 termini (Figure 2B and Figure 3) and rich high m/z fragments from the central region between the Cys^{88} and Cys^{134} residues (Figure 2C and Figure 3). This characteristic fragmentation pattern of the disulfide-linked subunits allowed confident confirmation of the intra-chain disulfide bonds in the LC subunit of bevacizumab (Figure 3). In this technical note, the complementary EAD results of the fully reduced and disulfide-linked subunits were leveraged to achieve a nearly complete sequence coverage of biotherapeutics in 2 injections.

Achieving ultrahigh sequence coverage of biotherapeutics

As mentioned above, EAD resulted in complementary fragmentation of the fully reduced and disulfide-linked subunits of biotherapeutics. EAD of the disulfide-linked subunits led to extensive fragmentation of the middle of the sequence, a region that is challenging to cleave in the fully reduced subunits. As a result, many unique bond cleavages were detected in EAD of the disulfide-linked subunits. The combination of these cleavages with those detected by EAD of the fully reduced subunits led to an ultrahigh sequence coverage of antibody subunits in 2 injections.

Figure 4 and 5 show the sequence coverage maps of the LC, Fd and Fc/2 subunits from NISTmAb and bevacizumab, respectively, based on the combined EAD results of the fully reduced and disulfide-linked subunits. An ultrahigh sequence coverage of ≥90% was achieved for all subunits except the NISTmAb Fd subunit, for which 85% sequence coverage was obtained. The unique bond cleavages detected by EAD of the disulfide-linked subunits, as highlighted by the orange lines in Figure 4 and 5, contributed to an absolute increase of 10%-16% in sequence coverage. Ultrahigh sequence coverages were also obtained for the LC (92%), Fd (90%) and Fc/2 (88%) subunits of trastuzumab (data not shown).

Figure 6 shows the combined sequence coverage of a tsAb LC subunit from 2 EAD experiments. Similar to the results described above, EAD of the disulfide-linked LC subunit of the tsAb contributed to an absolute increase in sequence coverage by ~10%, leading to an ultrahigh sequence coverage (88%) of the tsAb LC subunit.

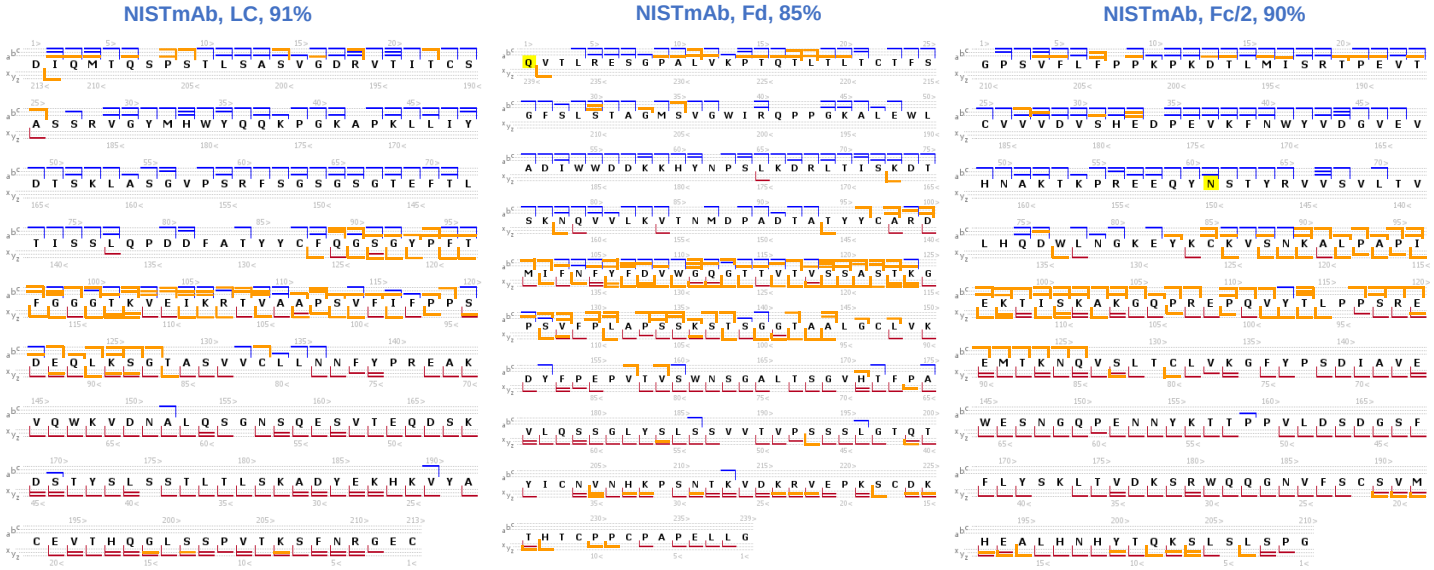

Figure 4. The combined sequence coverage maps of NISTmAb LC, Fd and Fc/2 subunits from 2 EAD injections. EAD of the fully reduced NISTmAb LC, Fd and Fc/2 subunits led to 70%-80% sequence coverage of the 3 subunits in a single injection.[1] The corresponding bond cleavages are indicated by the blue and red lines in the 3 sequence coverage maps. The distinctive EAD fragmentation patterns of the disulfide-linked subunits enabled the detection of many unique fragments from the regions not restricted by the disulfide bonds, including the middle of the sequence. These unique fragments are highlighted by the orange lines in the 3 coverage maps. The combined EAD results from the 2 injections led to 85%-91% sequence coverage of the 3 NISTmAb subunits, with an absolute increase of ~10%-16% contributed by unique fragments produced from EAD of the disulfide-linked subunits. The N-terminal glutamine residue highlighted in the sequence of the Fd subunit (middle panel) was converted into a pyroglutamic acid. The Asn residue highlighted in the sequence of the Fc/2 subunit (right panel) was modified with a G0F glycan.

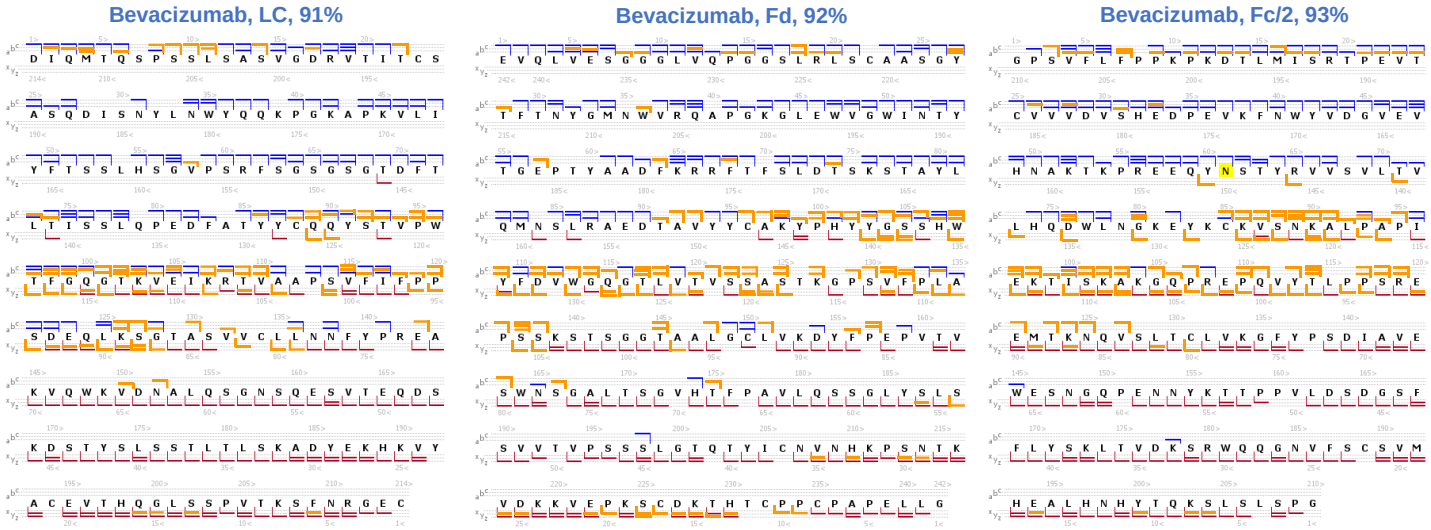

Figure 5. The combined sequence coverage maps of bevacizumab LC, Fd and Fc/2 subunits from 2 EAD injections. The combined EAD results for the fully reduced and disulfide-linked subunits from 2 injections led to >90% sequence coverage of the 3 bevacizumab subunits. The unique fragments produced from EAD of the disulfide-linked subunits, as highlighted by the orange lines in the 3 coverage maps, contributed to a ~10-16% increase in the sequence coverage. The Asn residue highlighted in the sequence of the Fc/2 subunit carried a G0F glycan.

In addition to providing unique bond cleavages, EAD of the disulfide-linked subunits offered rapid disulfide bond mapping and confirmation of the N- and C-terminal cleavages detected by EAD of the fully reduced subunits. These benefits significantly improved confidence in antibody sequence characterization.

In summary, the innovative EAD-based middle-down strategy described in this technical note led to ultrahigh sequence coverage (85%-93%) of biotherapeutics in 2 injections by leveraging the complementary EAD results of the fully reduced and disulfide-linked subunits. This powerful strategy also enabled high-confidence disulfide bond mapping at the subunit level based on the EAD results for the disulfide-linked subunits. This innovative middle-down strategy provides a balanced approach for the comprehensive characterization of biotherapeutics by overcoming the shortcomings of top-down MS in fragmentation efficiency and sequence coverage, and the limitations of bottom-up MS in modification artifacts, data complexity, false positives and throughput. The strategy can greatly improve the efficiency and effectiveness of middle-down MS for the comprehensive characterization of biotherapeutics.

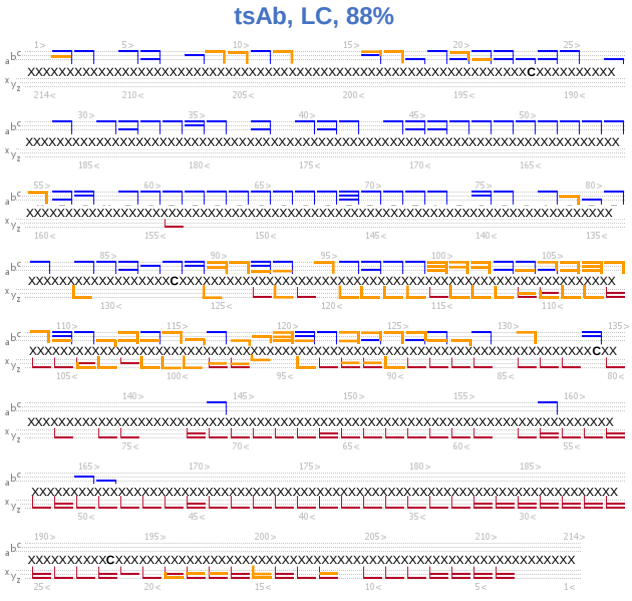

Figure 6. The combined sequence coverage map of the LC subunit from the tsAb. The unique cleavages detected by EAD of the disulfide-linked subunit, as highlighted by the orange lines, contributed to a 10% increase in sequence coverage.

Conclusions

- An innovative EAD-based middle-down MS strategy led to ultrahigh sequence coverages (85%-93%) for antibody subunits in 2 injections
- The characteristic fragmentation pattern offered by EAD for the disulfide-linked subunits enabled rapid disulfide bond mapping with high confidence and high fidelity
- Complementary fragmentation was achieved from EAD of the fully reduced and disulfide-linked subunits
- The powerful strategy 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

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