An enhanced single-injection middle-down workflow to achieve high sequence coverage and disulfide mapping of antibody subunits

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This technical note highlights an electron activated dissociation (EAD)-based middle-down workflow with controlled sample preparation to achieve high sequence coverage (88%) of the light chain (LC) and confident mapping of intra-chain disulfide linkages in a single injection.

Middle-down mass spectrometry (MS) combines the benefits of bottom-up and top-down MS.[1-8] It provides high sequence coverage of biotherapeutics with simple sample preparation, a low degree of artificial modification, no disulfide bond scrambling and easy data interpretation. Traditional middle-down MS workflows often involve multiple fragmentation techniques and/or injections with extensive method optimization to achieve high sequence coverage.[7,8] One of the challenges with middle-down MS is the lack of fragmentation in the middle of a subunit sequence. This limitation can be overcome by applying EAD to disulfide-linked subunits.[5,6] The application of EAD leads to complementary fragmentation patterns of the fully reduced and disulfide-linked subunits.[5,6] The combined EAD results of the fully reduced and disulfide-linked subunits provided high sequence coverage (85-93%) of mAb subunits in 2 injections.[6]

In this work, an EAD-based middle-down workflow with controlled sample preparation is presented to extend the capability of this powerful workflow for biotherapeutic characterization. The controlled denaturation and reduction of NISTmAb leads to the co-presence of the fully reduced and disulfide-linked LC subunits in the same sample. Targeted EAD fragmentation of these subunits provides high sequence coverage and high-confidence disulfide bond mapping from a single injection (Figure 1).

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

- **High sequence coverage in a single injection:** 88% sequence coverage of the LC subunit in a single injection
- **High-confidence disulfide bond mapping:** EAD results in a distinctive fragmentation pattern of disulfide-linked subunits for rapid intra-chain disulfide bond mapping with minimal false positives
- **Accurate localization of labile PTMs:** Labile PTMs are preserved in the EAD fragments
- **Streamlined and easy to implement:** The workflow requires minimal method optimization and is streamlined from data acquisition to results review

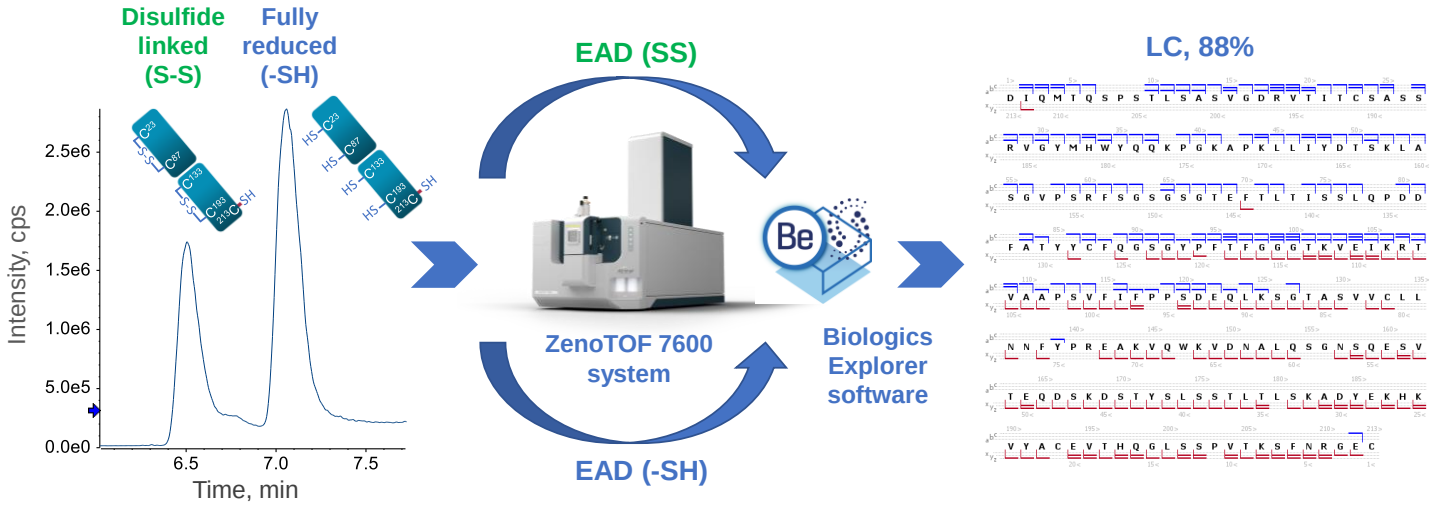

Figure 1. An EAD-based middle-down workflow with controlled sample preparation was developed to achieve high sequence coverage (88%) of antibody subunits in a single injection. EAD leads to complementary fragmentation patterns of the fully reduced (-SH) and disulfide-linked (SS) subunits of biotherapeutics.[1-6] In this work, the fully reduced and disulfide-linked LC subunits were produced in the same sample by fine-tuning the sample preparation condition. EAD fragmentation of these 2 LC subunits provided high sequence coverage (88%) and rapid confirmation of intra-chain disulfide linkages in a single injection.

Methods

Samples preparation: An aliquot of 10 µg/µL NISTmAb was mixed with guanidine hydrochloride (Gnd) and dithiothreitol (DTT) at a final concentration of 2M and 50mM, respectively. The mixture was incubated at 45°C for 30 minutes to generate LC and HC subunits. 4 µL of the final solution (~4 µg) was injected for LC-MS analysis immediately following the completion of the incubation.

Chromatography: The subunits of NISTmAb 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 AD 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	*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 (SCIEX). 2 charge states of the fully reduced (24+ and 19+) and disulfide-linked (18+ and 15+) LC subunits were targeted for EAD fragmentation in this work. The key TOF MS and MRMHR settings used are listed in Table 2 and 3, respectively.

Data processing: MRM^{HR} data were analyzed using a middle-down workflow template in Biologics Explorer software. EAD spectra of the disulfide-linked LC subunit were processed separately for disulfide bond mapping, or jointly with those of the fully reduced counterpart in the same data file to obtain high sequence coverage.

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	*4000 m/z*
Q1 resolution	*Low*
Zeno trap	*ON*
Zeno threshold	*100,000 cps*
Accumulation time	*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-based middle-down analysis of the fully reduced and disulfide-linked subunits

It was demonstrated previously that the EAD-based middle-down workflow is a highly effective approach for obtaining high sequence coverage and confident disulfide-bond mapping of antibody subunits (Figure 2).[1-6] EAD leads to complementary fragmentation patterns from the fully reduced and disulfide-linked subunits.[5,6] EAD fragmentation of the fully reduced subunits occurs preferentially in the regions near the 2 termini (Figure 2A).[1-4] By comparison, EAD of the disulfide-linked subunits results in a characteristic fragmentation pattern, where the middle of the sequence outside the disulfide-forming areas is extensively cleaved (Figure 2B).[5,6] The distinctive fragmentation pattern of the disulfide-linked subunits provides additional sequence coverage and confident mapping of intra-chain disulfide linkages without false positives.[5,6] By combining EAD results of the fully reduced and disulfide-linked subunits high sequence coverages (85-93%) of antibody subunits can be obtained in 2 injections.[6]

To further streamline the EAD-based workflow, the condition of sample preparation was fine-tuned to generate the fully reduced and disulfide-linked LC subunits in the same sample. EAD fragmentation of these 2 forms of the LC subunit provided high sequence coverage (88%) and rapid disulfide mapping in a single injection (Figure 2C).

Co-formation of the fully reduced and disulfide-linked subunits

Fine-tuning the denaturation and reduction conditions produced the fully reduced and disulfide-linked LC subunits of NISTmAb in the same sample. The concentration of Gnd, the temperature and duration of incubation were the key factors in determining the ratio of the fully reduced *vs.* disulfide-linked subunits. In this study, a final concentration of 2M Gnd and an incubation temperature and time of 45°C and 30 minutes, respectively, led to the co-formation of the fully reduced and disulfide-linked LC subunits of NISTmAb (Figure 3). These 2 forms of the LC subunit can be

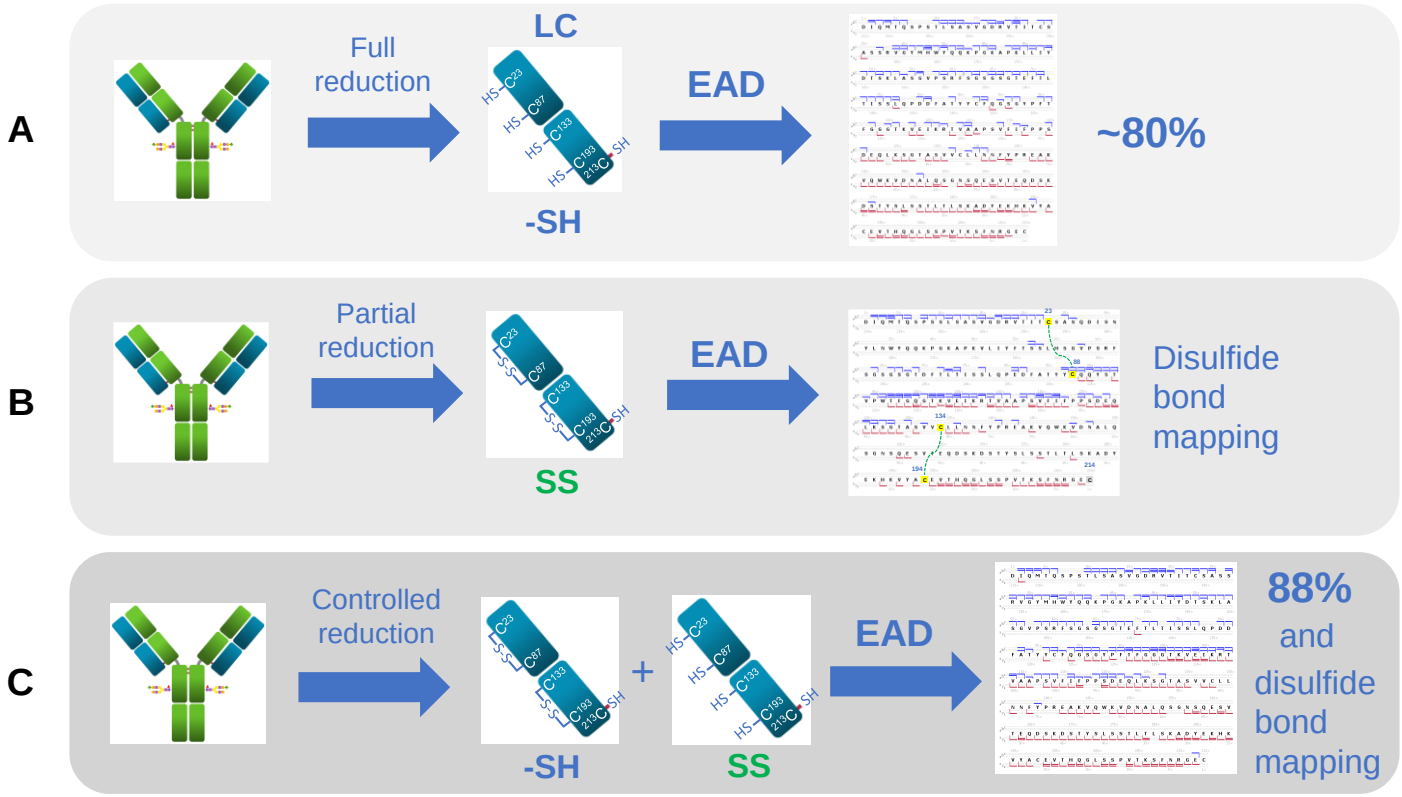

Figure 2. Development of EAD-based middle-down workflows for comprehensive characterization of biotherapeutic subunits. EAD provided a sequence coverage of ~80% for the fully reduced LC subunit in a single injection (A).[1-4] EAD of the LC subunit containing 2 intra-chain disulfide bonds led to a distinct fragmentation pattern where the sequences outside the disulfide-forming regions were preferentially cleaved (B).[5,6] This strategy provided additional coverage for the middle of the sequence that is difficult to fragment in the traditional middle-down workflow (A). In addition, it allows rapid mapping of intra-chain disulfide linkages.[5,6] In this work, the sample preparation condition was fine-tuned to produce a mixture of fully reduced and disulfide-linked LC subunits. EAD fragmentation of these 2 LC forms in the same sample led to a high sequence coverage (88%) of the subunit and rapid mapping of intra-chain disulfide linkages in a single injection. -SH: fully reduced subunit. SS: disulfide-linked subunit.

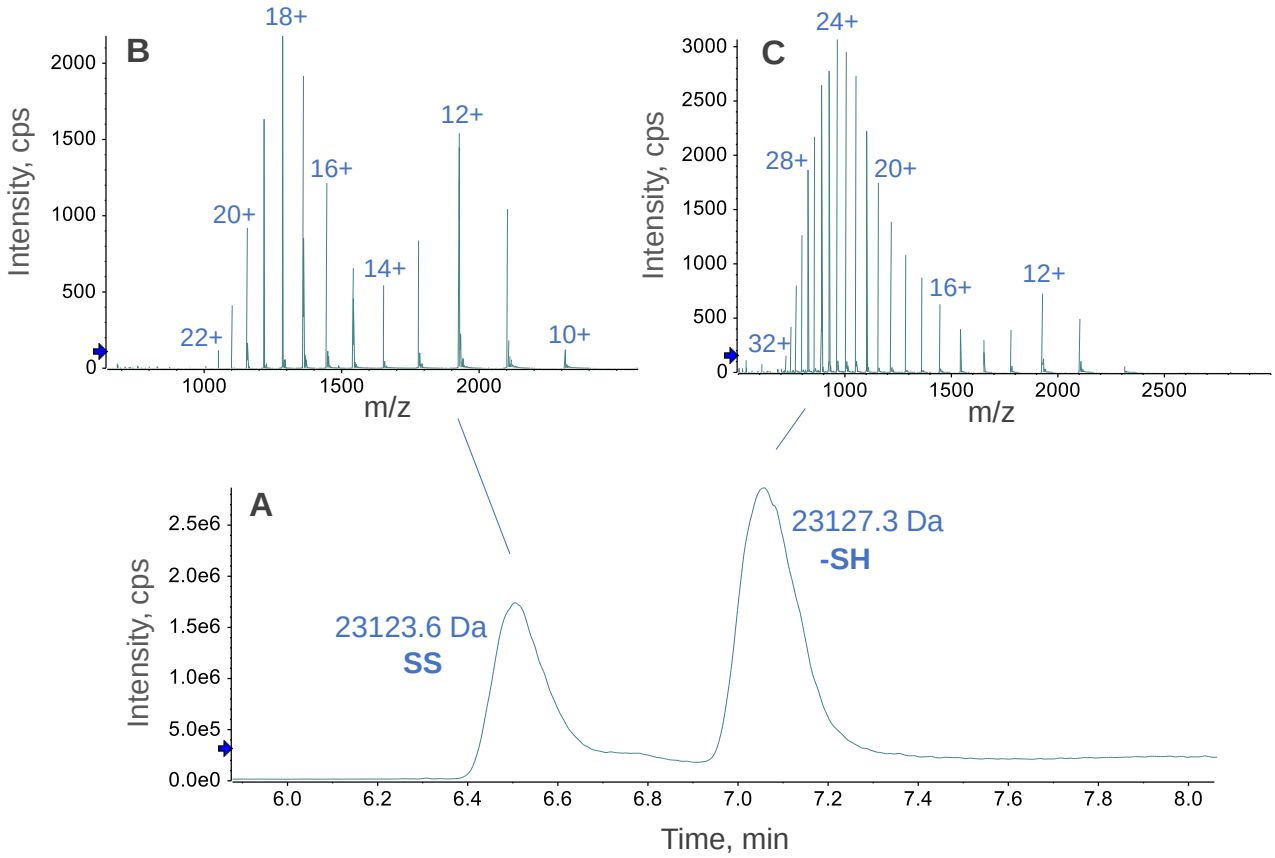

Figure 3. The controlled denaturation and reduction of NISTmAb produced the fully reduced and disulfide-linked LC subunits of NISTmAb in the same sample. The incubation of NISTmAb with 2M Gnd and 50mM DTT at 45°C for 30 minutes led to the co-presence of the fully reduced (-SH) and disulfide-linked (SS) LC subunits that can be chromatographically resolved (A). The accurate mass measurement confirmed that the peak eluting at a retention time (RT) of 7.05 min corresponds to the fully reduced subunit (23,127.3 Da), while the subunit detected at RT = 6.50 min contained 2 intra-chain disulfide bonds (23,123.6 Da). A distinctive difference was observed between the charge state distributions of 2 subunits (B and C). The disulfide-linked LC subunit carried fewer charges than the fully reduced counterpart due to a structural restriction posed by the 2 intra-chain disulfide bonds.

chromatographically resolved (Figure 3A). The mass spectra of the 2 species (Figure 3B and 3C) show a distinct difference in charge state distribution. The disulfide-linked LC subunit carried fewer charges on average compared to the fully reduced counterpart due to a lesser degree of sequence unfolding because of the presence of 2 intra-chain disulfide bonds. Automated protein deconvolution performed using Biologics Explorer software confirmed the masses of the fully reduced (23,127.3 Da) and disulfide-linked (23,123.6 Da) LC subunits. 2 charge states from each form of the LC subunit were targeted for EAD fragmentation in a single MRM^{HR} experiment. The processing of these EAD data separately or jointly led to high sequence coverage and rapid confirmation of intra-chain disulfide linkages for the LC subunit, as described below.

Achieving high sequence coverage and confident disulfide bond mapping of the LC subunit from a single injection

The formation of the fully reduced and disulfide-linked LC subunits of NISTmAb in the same sample enabled the EAD fragmentation of these 2 species in a single injection. The combined EAD spectrum of the 2 LC forms is composed of rich fragments across a wide m/z range of 100-4,000 (Figure 4A). Highlighted in Figures 4B and 4C are the zoom-ins of 2 selected spectral regions in the low and high m/z range where a large number of *c*/*z* fragments were detected. An abundant level of *c*/*z* fragments was also present in the rest of the spectral regions (data not shown). EAD spectra of the fully reduced and disulfide-linked LC subunits can be processed separately or jointly—depending on the RT range restriction—using an intuitive middle-down workflow template within Biologics Explorer software. The interpretation of the EAD result of the disulfide-linked LC subunit led to a characteristic sequence coverage that is indicative of 2 intra-chain disulfide

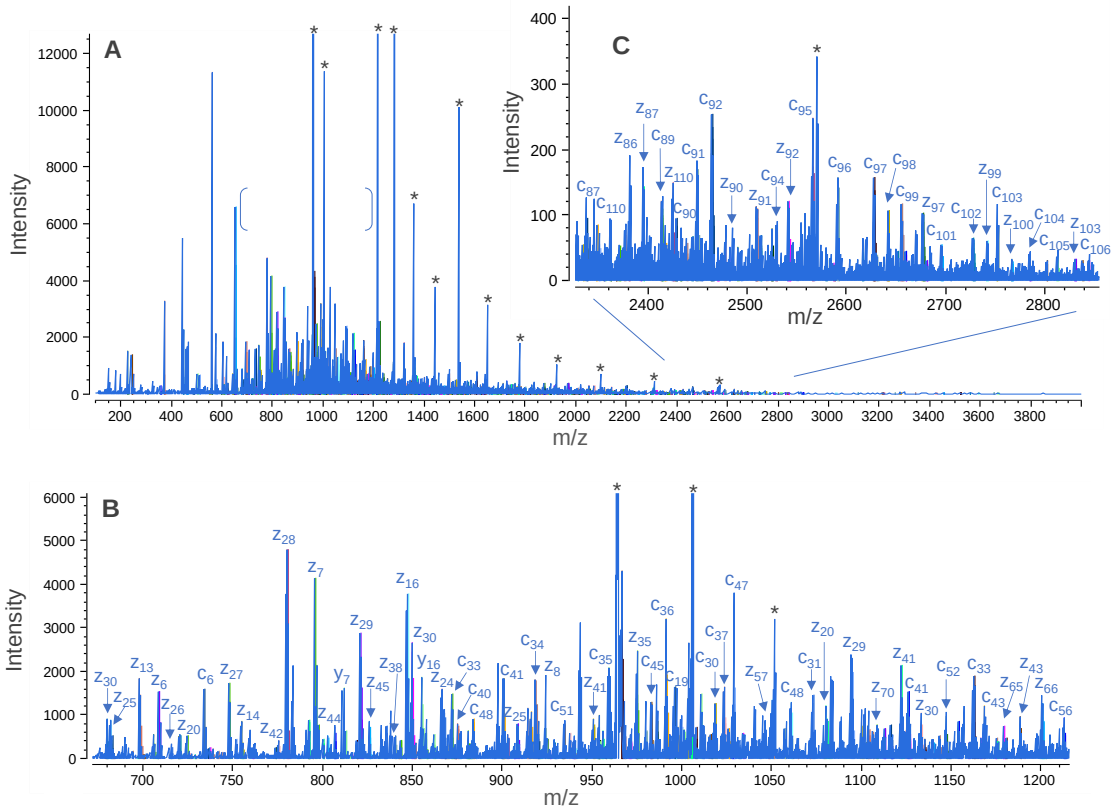

Figure 4. Combined EAD spectrum of the fully reduced and disulfide-linked LC subunits from NISTmAb. The combined EAD spectrum of the fully reduced and disulfide-linked LC subunits consists of rich fragments across the entire m/z range of 100-4,000 (A). The 2 selected spectral regions, including m/z ranges of ~650-1,200 (B) and ~2,350-2,850 (C), were zoomed in for a better view of rich *c*/*z* fragments in these 2 regions. The number of extra hydrogen atoms, if any, in the annotated fragments was not specified for spectral clarity. For example, all the "*z*+1" fragments were labeled as "*z*". The peaks labeled with * are the precursors or charged reduced species. Not all fragments were labeled in the spectra.

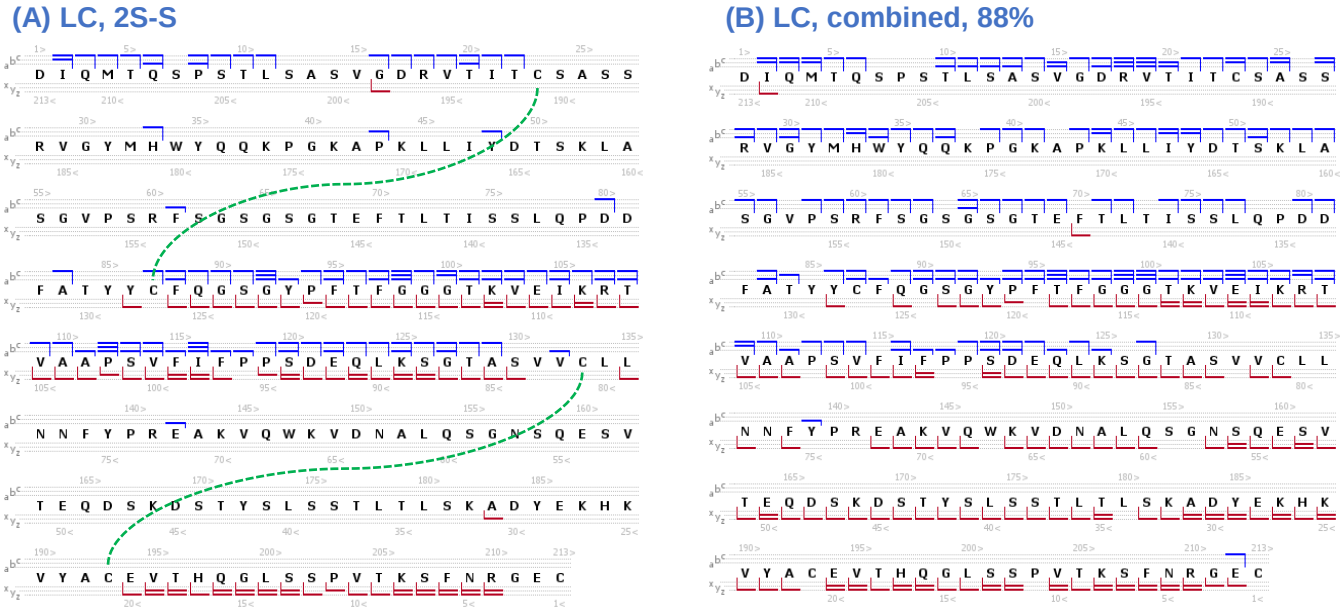

Figure 5. Sequence coverage maps obtained from EAD of the fully reduced and/or disulfide-linked LC subunit of NISTmAb. EAD of the disulfide-linked LC subunit resulted in a characteristic fragmentation pattern that enabled rapid disulfide bond mapping in high confidence and high fidelity (A). The combined EAD result of the fully reduced and disulfide-linked LC subunits co-present in the same sample led to high sequence coverage (88%) of this subunit in a single injection (B).

linkages (Figure 5A), in agreement with the previous results.[5,6] The analysis of the combined EAD data from the 2 species produced high sequence coverage (88%) of the LC subunit in a single injection (Figure 5B).

In summary, an EAD-based middle-down workflow with controlled sample preparation was developed to provide high sequence coverage and disulfide bond mapping of antibody subunits in a single injection. The controlled sample preparation led to the formation of the fully reduced and disulfide-linked LC subunits in the same sample. The complementary EAD fragmentation patterns of these 2 species resulted in high sequence coverage (88%) of the LC subunit. In addition, EAD enabled the confident determination of 2 intra-chain disulfide linkages in the disulfide-linked subunit. The strategy described in this technical note can improve the efficiency and effectiveness of using the middle-down workflow for the comprehensive characterization of biotherapeutics with increasing diversity and complexity.

Conclusions

- An EAD-based middle-down workflow with controlled sample preparation provides high sequence coverage (88%) of the LC subunit of NISTmAb in a single injection
- The characteristic fragmentation pattern offered by EAD for the disulfide-linked subunits enables confident determination of intra-chain disulfide bonds
- 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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