A single-injection workflow for enhanced peptide mapping using collision-induced dissociation (CID) and electron-activated dissociation (EAD)

Featuring the single-injection CID/EAD data-dependent acquisition (DDA) workflow using the ZenoTOF 7600 system and Biologics Explorer software from SCIEX

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This technical note describes a single-injection CID/EAD DDA workflow for a comprehensive characterization of protein therapeutics with high sensitivity. This workflow combines the advantages of CID and EAD in a single acquisition method with minimal compromise. Compared to the existing CID-based high-resolution mass spectrometry (HRMS) platform methods, there is minimal effect on acquisition rate and sensitivity. The CID/EAD DDA workflow provides high sequence coverage of biotherapeutics (>96%). The addition of EAD fragmentation provides a significant enhancement for the confident identification of short and long peptides, clear differentiation of amino acid isomers and accurate localization of labile post-translational modifications (PTMs), such as N- and O-linked glycosylation.

Peptide mapping is widely used for sequence confirmation and PTM identification for antibody-based therapeutics.[1] Peptide mapping is typically performed with collision-based MS/MS fragmentation methods, such as CID. While CID offers high sensitivity and efficient fragmentation of common peptides, it has limitations in fragmenting long peptides, localizing labile PTMs and differentiating amino acid isomers. By comparison, EAD offers excellent fragmentation of long peptides, accurate localization of labile PTMs and confident isomer differentiation.[2-5]

In this work, a single-injection CID/EAD DDA workflow (Figure 1) was developed to provide enhanced peptide mapping of biotherapeutics.

Key features of the single-injection CID/EAD DDA workflow for peptide mapping

- **Single-injection platform method:** The CID/EAD DDA workflow provides high sequence coverage of protein therapeutics in a single injection
- **Easy to implement:** The workflow involves an easy addition of powerful EAD capabilities while maintaining the advantages of the traditional CID technique
- **Confident identification of a wide range of peptides:** CID and EAD provide excellent fragmentation and confident identification of short and long peptides, respectively
- **Differentiation of amino acid isomers:** EAD generates diagnostic fragments for clear isomer differentiation
- **Accurate localization of PTMs:** EAD preserves labile PTMs, providing site-specific information about these modifications
- **Powerful software tools:** Biologics Explorer software offers optimized workflow templates and powerful visualization tools for analyzing CID and EAD DDA data in the same file

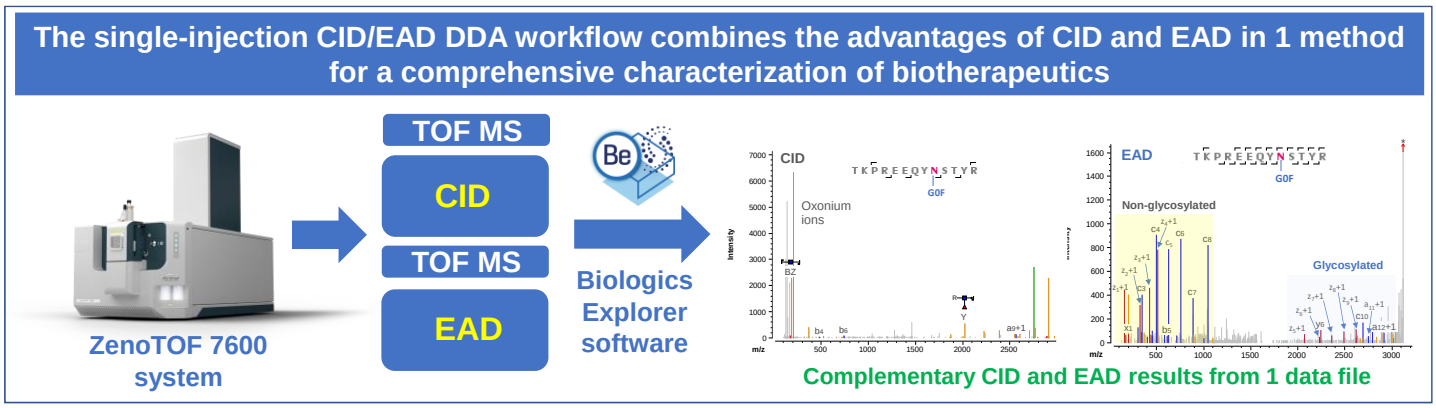

Figure 1. The single-injection CID/EAD DDA workflow combines the advantages of complementary CID and EAD techniques in 1 DDA method. This powerful workflow leverages the fast scanning rate provided by the ZenoTOF 7600 system and streamlined data analysis using Biologics Explorer software. The workflow offers high sequence coverage, confident identification of short and long peptides, clear differentiation of amino acid isomers and accurate localization of labile PTMs while requiring minimal adjustment to the existing CID platform method.

Methods

Sample preparation: NISTmAb (RM8671, NIST) and etanercept were denatured by guanidine hydrochloride, reduced with dithiothreitol and alkylated using iodoacetamide. The sample solution was buffer-exchanged into 50mM Tris-HCl (pH = 7.4) using Bio-Spin columns (Bio-Rad), followed by enzymatic digestion at 37°C for 2-4 h using trypsin/LysC (Promega). The etanercept digest was further incubated with SialEXO (Genovis) at 37°C for 4 h to remove sialic acids. A total of 5–10 µL of the final solution (~5–10 µg) was injected for LC-MS analysis.

Chromatography: The peptides were separated with an LC gradient, displayed in Table 1, using a Waters ACQUITY CSH C18 column (2.1 × 150 mm, 1.7 µm, 130 Å). A flow rate of 0.25 mL/min was used for the separation. The column was kept at 60°C in the column oven of an ExionLC AD system from SCIEX. The mobile phases A and B consisted of 0.1% formic acid (FA) in water and 0.1% FA in acetonitrile, respectively.

Table 1. LC gradient for peptide separation.

Time (min)	A (%)	B (%)
Initial	*98*	*2*
2	*98*	*2*
62	*65*	*35*
65	*50*	*50*
67	*10*	*90*
70	*90*	*10*
71	*98*	*2*
75	*2*	*98*

Mass spectrometry: DDA experiments were performed in SCIEX OS software using the ZenoTOF 7600 system. The CID/EAD DDA method was created by adding an EAD DDA event to the existing CID platform method. The key TOF MS and DDA settings are listed in Tables 2 and 3, respectively.

Data processing: CID/EAD DDA data were analyzed using an optimized peptide mapping workflow template in Biologics Explorer software. The fragment ions considered for peptide mapping included the primary fragments from the peptide backbone cleavage, such as *a*/*b*/*c* and *x*/*y*/*z*, and diagnostic fragments for isomer differentiation, such as *c*+57/*z*-57 ions for the differentiation of aspartic acid (Asp) vs. isoaspartic acid (isoAsp) and *w* ions for the differentiation of leucine (Leu) vs. isoleucine (Ile). While N-linked glycosylation was considered for NISTmAb, both N- and O-linked glycosylations were searched when analyzing etanercept DDA data.

Table 2. TOF MS parameters.

Parameter	Value
Spray voltage	*5500 V*
TOF start mass	*400 m/z*
TOF stop mass	*1800 m/z*
Accumulation time	*0.08 s*
Source temperature	*400°C*
Declustering potential	*80 V*
Collision energy	*10 V*
Time bins to sum	*8*

Table 3. DDA parameters using CID or EAD.

Parameter	CID	EAD
Start mass	*100 m/z*	*100 m/z*
Stop mass	*2000 m/z*	*3000 m/z*
Q1 resolution	*Unit*	*Unit*
Zeno trap	*ON*	*ON*
Zeno threshold	*100,000 cps*	*100,000 cps*
Maximum candidate ions	*8*	*6*
Charge state	*1-5*	*2-10*
Accumulation time	*0.05 s*	*0.08 s*
Declustering potential	*80 V*	*80 V*
CE	*Dynamic*	*10 V*
Time bins to sum	*8*	*8*
Electron beam current	*N/A*	*5500 nA*
Electron KE	*N/A*	*7 eV*
ETC	*N/A*	*Dynamic*
Reaction time	*N/A*	*20 ms*
EAD RF	*N/A*	*120 Da*

High sequence coverage obtained using the CID/EAD DDA workflow

The success of the single-injection CID/EAD DDA workflow was built upon the fast scanning rate of the ZenoTOF 7600 system and the enhanced MS/MS sensitivity offered by the Zeno trap, which increases the detection of MS/MS fragments by 5- to 10-fold.[6] Traditional electron-based MS/MS (ExD) approaches suffer from low sensitivity and long reaction times. As a result, these ExD approaches are not suitable for routine peptide mapping as a platform method. By comparison, Zeno EAD benefits from high sensitivity, short reaction times (10-20 ms) and fast scanning rates (up to 20 Hz in DDA mode).[2-6] These advantages allowed the implementation of EAD DDA as a platform method for peptide mapping[2] or a powerful addition to the existing CID platform method to achieve a complete characterization of biotherapeutics. The CID/EAD DDA method combines the strengths of CID in sensitivity and fragmentation of singly charged peptides with the unique capabilities of EAD for fragmentation of long peptides, isomer differentiation and PTM analysis.

Combining complementary CID and EAD techniques in the same DDA method led to consistently high sequence coverage. Figure 2 shows that the CID/EAD DDA method provided a nearly complete sequence coverage (>96%) of NISTmAb light chain (LC) and heavy chain (HC) from 1 trypsin/LysC digest in a single injection. This result is comparable to or slightly better than that obtained using CID or EAD DDA alone (data not shown).

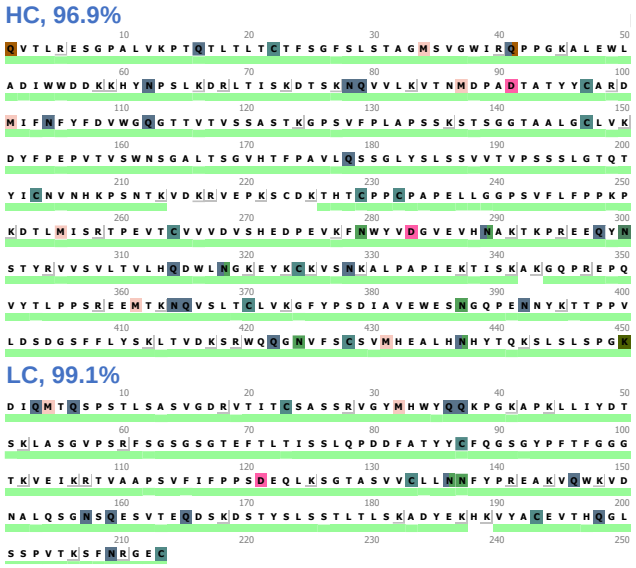

Figure 2. The single-injection CID/EAD DDA workflow offers high sequence coverage (>96%) of NISTmAb LC and HC from 1 trypsin/LysC digest. The CID/EAD workflow provided comparable or slightly better sequence coverages of 2 NISTmAb chains than the CID or EAD method alone.

Confident identification of short and long peptides

Depending on the frequency of the Arg and Lys residues in the sequence, trypsin digestion of protein therapeutics might lead to short peptides containing ≤5 amino acid residues or long peptides larger than >5 kDa. CID and EAD have different strengths in the fragmentation of short vs. long peptides. While CID offers excellent fragmentation of short peptides in low charge states (1+ or 2+), EAD is superior for the characterization of multiply charged long peptides (≥3+). The CID/EAD DDA

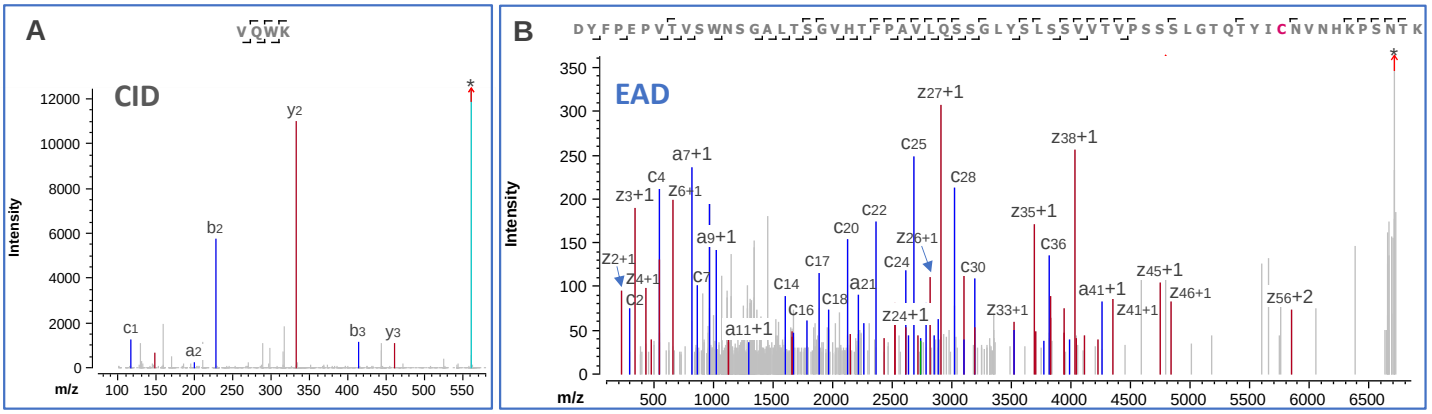

Figure 3. Confident identification of short peptides using CID and long peptides using EAD. The CID/EAD DDA workflow allowed excellent fragmentation and confident identification of a singly charged peptide VQWK from NISTmAb LC by CID (A) and a multiply charged long peptide (~6.7 kDa) from NISTmAb HC by EAD (B). Not all fragments in the spectra are labeled for spectral clarity. All the MS/MS spectra in this technical note are the deisotoped spectra taken directly from Biologics Explorer software. The peaks labeled with * are the deisotoped precursors.

workflow provides the combined advantages of these 2 techniques to cover a full spectrum of peptides with various sizes or charge states.

Figure 3 shows the CID spectrum of a singly charged peptide VQWK and the EAD spectrum of a long tryptic peptide (~6.7 kDa). Excellent fragmentation of these 2 peptides using CID or EAD led to their confident identification in 1 experiment. The ability of the CID/EAD DDA method to confidently identify short and long peptides enables consistently high sequence coverage of protein therapeutics from single enzymatic digestion.

Differentiation of amino acid isomers

It is challenging to differentiate amino acid isomers, such as Leu vs. Ile or Asp vs. isoAsp, using CID MS/MS. By comparison, EAD produces diagnostic fragments to enable a clear differentiation between these amino acid isomers.[2,4] Therefore, including EAD in the CID/EAD DDA method significantly enriches the information for peptide mapping to achieve a complete characterization of biotherapeutics in a single injection.

Figure 4 shows the zoomed-in views of signature fragments for the confirmation of Leu and Ile residues in the peptide ALPALIEK from the NISTmAb HC. The detection of z_7+1-43 and z_3+1-29 fragments confirmed the presence of 2 amino acid residues in their respective positions in this peptide.

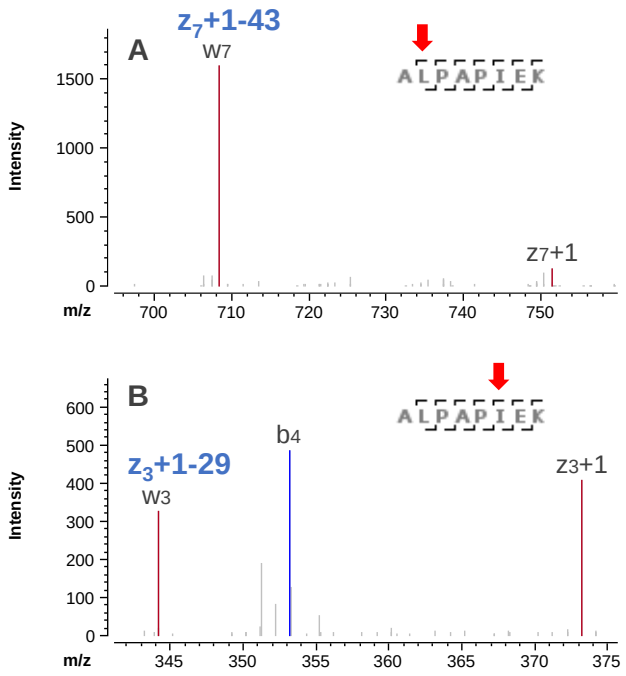

Figure 4. Confirmation of Leu vs. Ile residues using diagnostic EAD fragments. The Leu[2] and Ile[6] residues in the peptide ALPAPIEK can be confirmed based on the detection of the signature w_7 or z_7+1-43 ion for Leu (A) and the w_3 or z_3+1-29 ion for Ile (B), respectively, in the EAD DDA data.

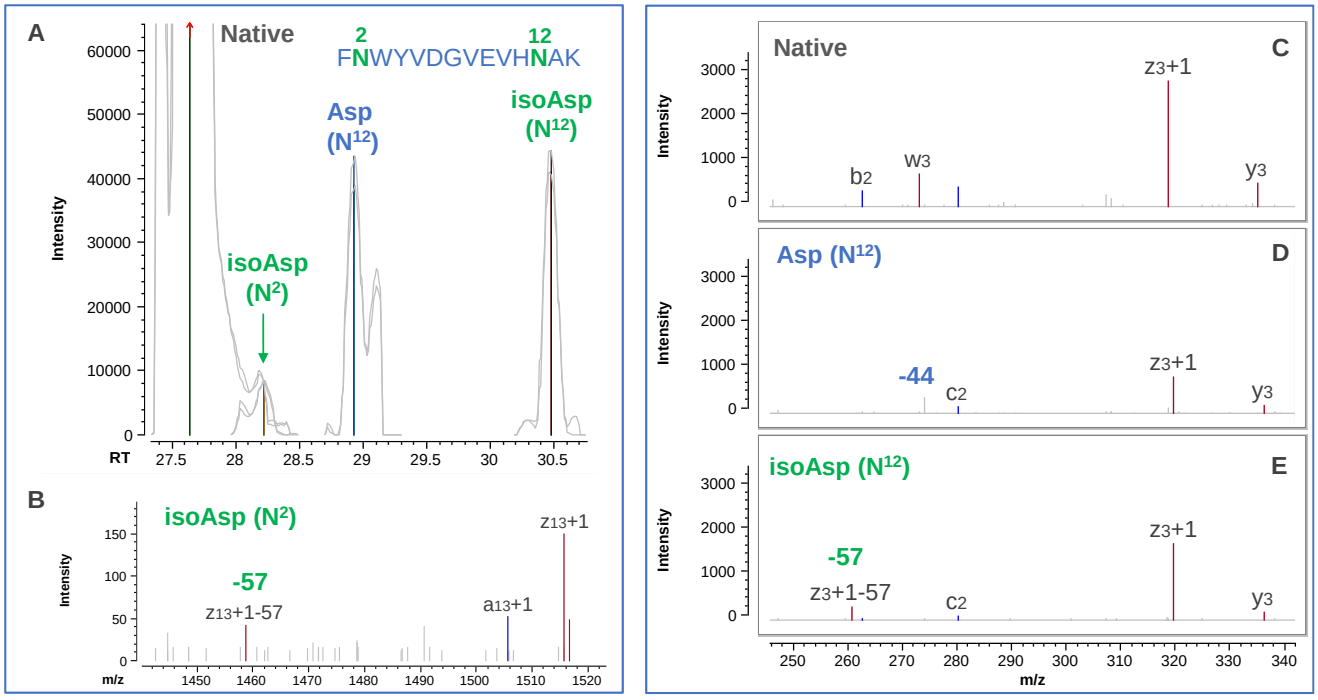

Figure 5. Identification and differentiation of Asp vs. isoAsp isomers using EAD. Three deamidation isomers were identified in the extracted ion chromatogram (XIC) of the peptide FNWYVDGYEVHNAK (A). EAD led to the differentiation of these 3 isomers based on the detection of *z*-57 or *z*-44 fragments (B-E). The detection of a z_{13}+1-57 ion (B) confirmed the isoAsp isomer from N^2 deamidation (B), while the detection of a z_3+1-44 or z_3+1-57 fragment enabled the differentiation of Asp vs. isoAsp isomers from N^{12} deamidation (D and E).

Figure 5 shows an example of using EAD to identify and differentiate 3 deamidation isomers of the peptide FNWYVDGYEVHNAK from the NISTmAb HC. The 3 isomers identified in the XIC (Figure 5A) were produced from the deamidations of the Asn^2 (N^2) or Asn^{12} (N^{12}) residue in this peptide. The detection of a z_{13}+1-57 fragment in EAD of the N^2-deamidated species led to the confident assignment of this species as the isoAsp isomer (Figure 5B). Similarly, the N^{12}-deamidated species eluting at ~30 min was an isoAsp isomer based on the detection of a z_3+1-57 ion (Figure 5E). The absence of this diagnostic fragment and the detection of a z_3+1-44 ion for the N^{12}-deamidated species eluting at ~29 min confirms that this species was an Asp isomer (Figure 5D).

Comprehensive characterization of glycosylation

The CID/EAD DDA workflow combines the complementary capabilities of CID and EAD for the comprehensive characterization of glycopeptides. While CID preferentially cleaves the glycan to produce diagnostic oxonium ions, EAD preserves this labile modification for its confident identification and accurate localization.

Figure 6 shows the CID and EAD spectra of an N-linked glycopeptide TKPREEQYNSTYR carrying a G0F moiety. The detection of abundant oxonium ions, such as m/z 204 Da, in the CID spectrum (Figure 6A) confirms the presence of glycosylation in this peptide. However, CID led to insufficient sequence coverage and a lack of information about the site of glycosylation. By comparison, EAD produced a nearly complete series of sequence fragments with or without glycosylation, enabling the accurate localization of the G0F glycan.

Figure 7 highlights the power of EAD for the characterization of 2 O-linked glycopeptides in etanercept.[3] EAD provided extensive fragmentation of these 2 glycopeptides while preserving the labile O-glycan in the fragments, enabling confident peptide identification and glycosylation site determination. Specifically, the O-glycan site in the peptide THTCPPCPAPELLGGPSVFLFPPKPK was pinpointed to the Thr^3 residue, which is 1 of 3 potential O-glycosylation sites in this peptide, based on the detection of non-glycosylated c_2 and glycosylated c_3 fragments (Figure 7A). Similarly, the 2 O-glycans in the peptide SMAPGAVHLPQPVSTR were confidently localized to the Ser and Thr residues near the C-terminus based on the glycosylation status of z_1-z_3 fragments (Figure 7B).

In summary, the CID/EAD DDA workflow provides the combined benefits of 2 complementary MS/MS techniques, as summarized in Table 4, to achieve a complete characterization of protein therapeutics. The workflow offers high sequence coverage, confident identification of short and long peptides, clear differentiation of amino acid isomers and accurate localization of

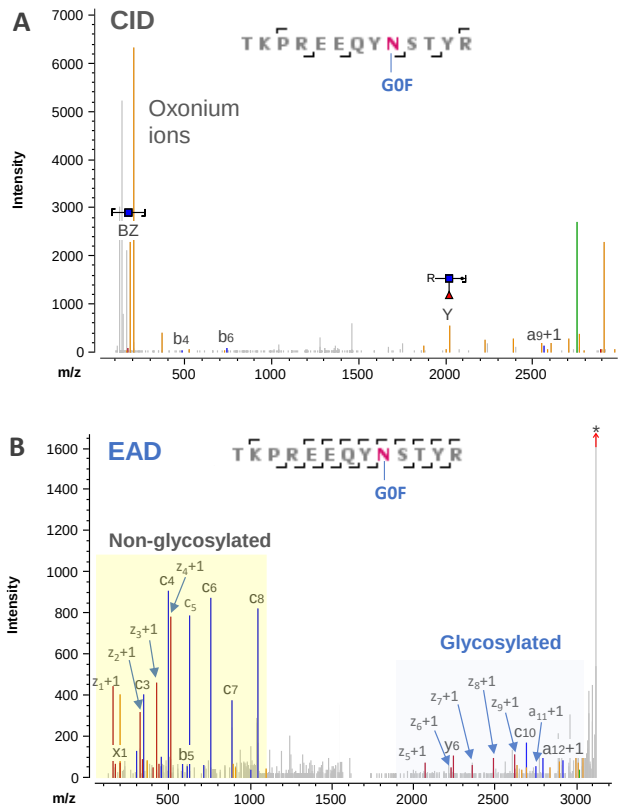

Figure 6. Characterization of N-linked glycosylation in NISTmAb using CID and EAD. The CID/EAD workflow provided complementary DDA data for confident identification and localization of N-linked glycosylation. While CID generated abundant oxonium ions to confirm the presence of glycosylation in the peptide TKPREEQYNSTYR (A). EAD provided a nearly complete series of *c*/*z* ions with (in blue background) or without (in yellow background) glycosylation (B). Specifically, the detection of non-glycosylated z_1-z_4 and glycosylated z_5-z_9 fragments allowed the accurate localization of the G0F glycan to the Asn residue. The peak labeled with * in B is the deisotoped precursor.

Table 4. The CID/EAD workflow provides a comprehensive characterization of protein therapeutics.

Characterizations	
High sequence coverage	√
Singly charged short peptides	√
Long peptides (>5 kDa)	√
Isomer differentiation	√
Localization of labile PTMs	√
Disulfide bond mapping	√

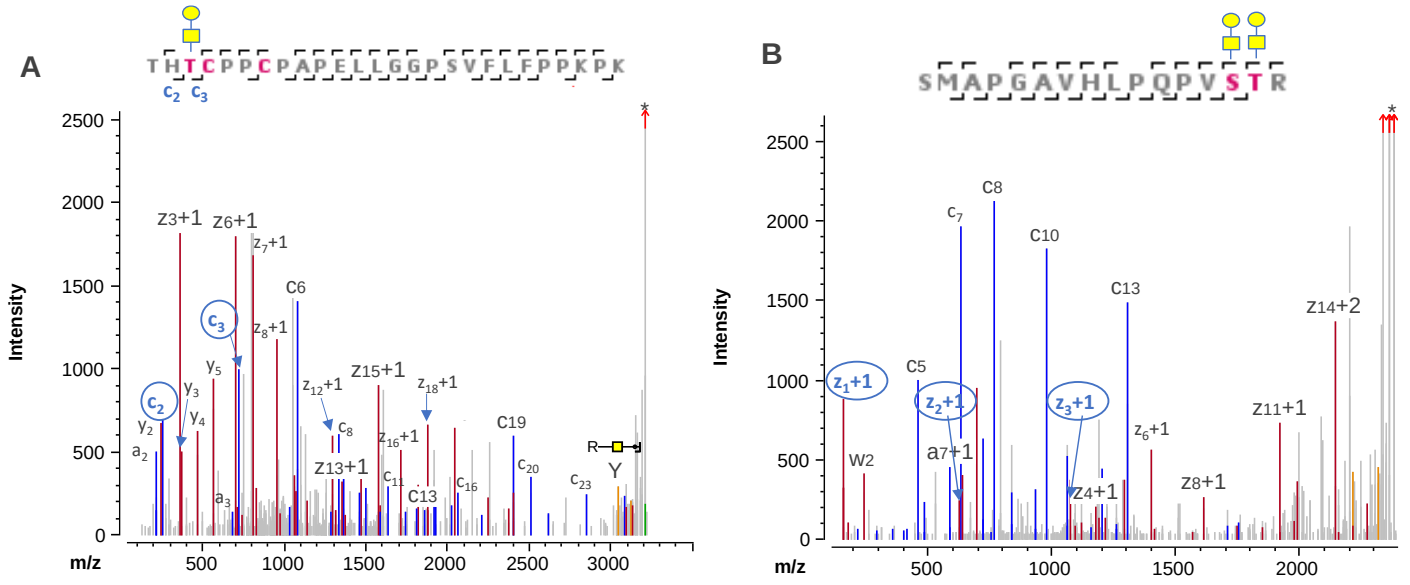

Figure 7. Accurate localization of O-linked glycosylation in etanercept using EAD. The location of 1 HexHexNAc moiety in the glycopeptide THTCPPCPAPELLGGPSVFLFPPKPK was pinpointed to the Thr[3] residue, out of 3 potential O-linked glycosylation sites including 2 Thr and 1 Ser, based on the detection of non-glycosylated c_2 and glycosylated c_3 fragments (circled in A). Similarly, the sites of 2 O-glycans in the glycopeptide SMAPGAVHLPQPVSTR were confidently determined based on the detection of z_1-z_3 (circled in B) with 0, 1 and 2 HexHexNAc moieties, respectively. The peaks labeled with * are the deisotoped precursors.

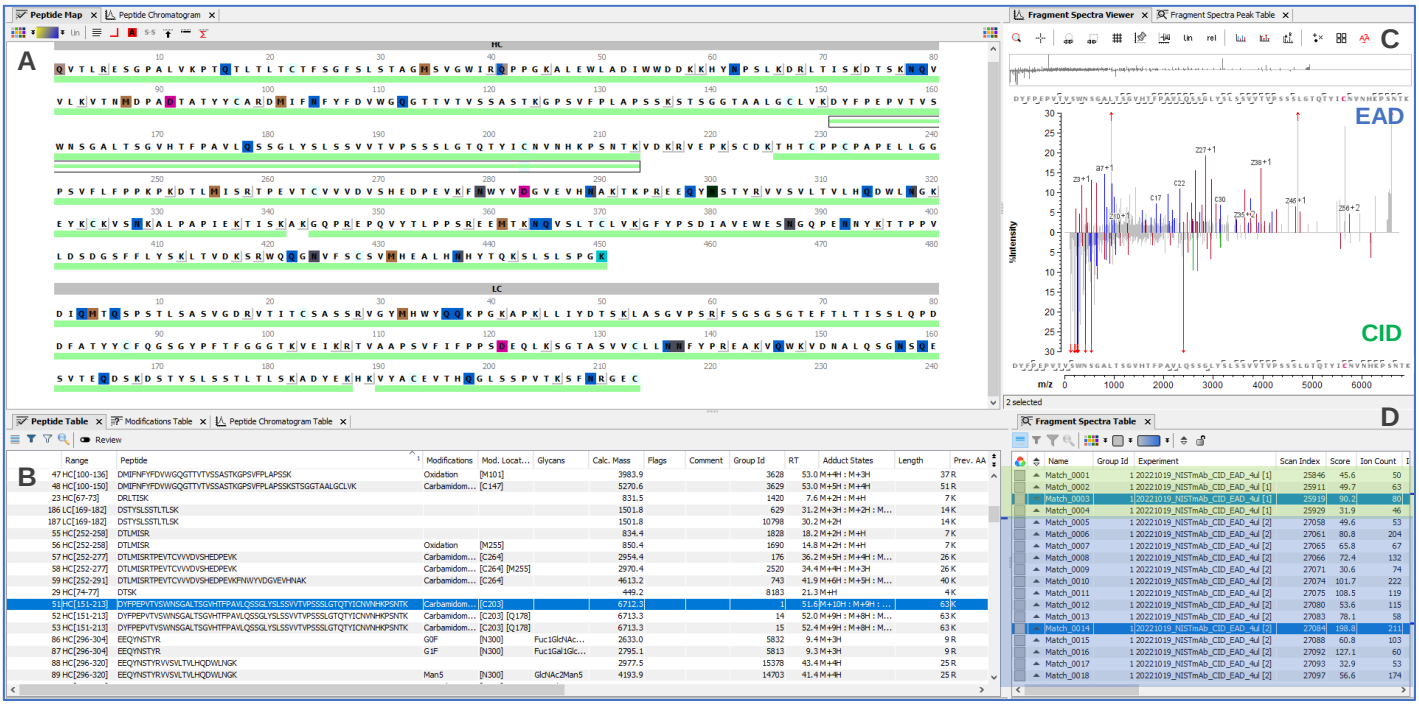

Figure 8. Biologics Explorer software provides user-friendly interfaces for reviewing and comparing the CID/EAD DDA results. Biologics Explorer software offers a streamlined and automatic workflow template for processing the CID and EAD data in the same raw file. The software provides the combined sequence coverage (A) based on the identified peptides, which are listed in the peptide table (B). The fragment spectra viewer (C) displays the individual or combined CID or EAD spectra with annotations, depending on the spectral selection in the fragment spectra table (D). The spectra can be visualized or compared using different tools such as a stacked view or mirror plot (C). The fragment spectra table compiles all the annotated CID and EAD spectra, as highlighted in the green and blue backgrounds, respectively (D). The peptide highlighted in this figure is the long peptide described in the previous section (Figure 3B). It is evident from the annotated spectra (C) that EAD provided more extensive fragmentation and a higher sequence coverage of this long peptide compared to CID.

labile PTMs while requiring minimal adjustment to the existing CID platform method (Table 4). The advantage of the CID/EAD workflow for disulfide bond mapping will be highlighted in a future technical note.

Powerful software tools for data processing and results review

Biologics Explorer software offers streamlined peptide mapping templates optimized for DDA data from the ZenoTOF 7600 system. In addition, the software provides an array of effective tools to facilitate results review and comparison for improved user experience.

Figure 8 shows the default view of the peptide mapping result from the CID/EAD DDA analysis of NISTmAb. The peptide map tab (Figure 8A) provides a quick overview of the sequence coverage of the HC and LC. The peptide table (Figure 8B) lists all peptides identified using CID and/or EAD. The annotated MS/MS spectra of the highlighted peptide in the peptide table can be visualized and compared in the fragment spectra viewer (Figure 8C), depending on the spectral selection in the fragment spectra table (Figure 8D).

Conclusions

- The single-injection CID/EAD DDA workflow combines the advantages of complementary CID and EAD techniques and offers a comprehensive characterization of biotherapeutics
- This powerful workflow maintains the capabilities of the traditional CID DDA platform method while offering additional benefits from EAD in the same data file
- The CID/EAD DDA workflow led to high sequence coverage (>96%) of LC and HC from 1 trypsin digest of NISTmAb in a single injection
- The workflow couples the ultrahigh sensitivity and confident identification of short peptides offered by CID with excellent fragmentation and improved confidence in the characterization of long peptides using EAD
- EAD enabled confident differentiation of amino acid isomers, such as Leu vs. Ile and Asp vs. isoAsp, and accurate localization of N- and O-linked glycosylation
- Biologics Explorer software provides optimized peptide mapping workflow templates and powerful visualization tools for streamlined data processing and improved user experience with results review and comparison

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