Evaluation of electron kinetic energies for advanced characterization of protein therapeutics using electron-activated dissociation (EAD)

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ABSTRACT

A single injection platform method using electron activated dissociation (EAD)[1-6] offers superior capability over traditional fragmentation techniques for detailed biopharmaceutical characterization. In this poster, a comprehensive evaluation of the electron kinetic energy (KE) of EAD was performed to determine the optimal condition for peptide mapping and characterization of labile modifications. Furthermore, the results highlight the ability to adjust electron KE, a key advantage of EAD, to facilitate the fragmentation of a wide range of peptides, including extremely labile sulfated peptides.

INTRODUCTION

Next-gen biologics, such as fusion and multi-specific protein therapeutics, are becoming more complex in their composition and more diverse in their modifications. As a result of this complexity, the comprehensive characterization of biopharmaceutical modalities might not be covered by traditional analytical approaches. The collision-based MS/MS approaches are ineffective with respect to differentiation of amino acid isomers, such as aspartic acid vs. isoaspartic acid or leucine vs. isoleucine and localization of labile post-translational modifications (PTMs), such as, glycosylation and sulfation. Traditional electron-based methods (ExD) suffer from high duty cycles and low sensitivity and are rarely used for routine characterization of biotherapeutics.

The EAD platform method[1-6] addresses these gaps of traditional collision-based and ExD approaches. EAD is not only able to differentiate isomers and localize labile PTMs, but it is also fast and sensitive and can be used as a single injection method for routine and confident peptide mapping as well as enhanced characterization of protein therapeutics with increasing variety and complexity. EAD can be performed in a data-dependent manner at up to 20 Hz to maintain compatibility with analytical flow liquid chromatography. Additionally, the ability to fine-tune electron KE in EAD enables characterization of various peptides, including singly charged and sulfated peptides, that are a challenge for traditional ExD approaches.

MATERIALS AND METHODS

Sample preparation: The leucine (Leu) and isoleucine (Ile) isomers of human leptin (93–105, NVIQISND-X-ENLR, where X = Leu or Ile) were obtained from AnaSpec Inc. Sulfated leucine enkephalin (LES) and cholecystokinin fragment 26–33 (CLKS) were purchased from Sigma Aldrich. These peptides were characterized using different KEs in EAD MRM^{HR} experiments. NISTmAb (reference material #8671, NIST) and etanercept were denatured, reduced and alkylated, followed by tryptic digestion. Tryptic digests of etanercept were further treated with N-glycanase (PNGase F, Agilent Technologies Inc.) for removal of all N-glycans and with SialEXO (Genovis Inc.) to cleave sialic acids. Selected N-linked glycopeptides and singly charged peptides from NISTmAb and O-linked glycopeptides from etanercept were subjected to EAD MRM^{HR} analysis using different KEs.

Chromatography: Peptides were separated using an ACQUITY CSH C18 column (2.1 x 150 mm, 1.7 µm, 130 Å, Waters), which was kept at 60°C in the column oven of an ExionLC system (SCIEX). A 20 min and 60 min gradient with a flow rate of 0.25 mL/min was employed for analyzing simple peptide solutions and complex tryptic digests, respectively. Mobile phases A and B consist of 0.1% formic acid (FA) in water and 0.1% FA in acetonitrile, respectively.

Mass spectrometry: Peptide mapping analysis of NISTmAb and etanercept digests were performed using a data-dependent EAD method on a ZenoTOF 7600 system (SCIEX). Selected peptides were targeted in EAD MRM^{HR} experiments by varying the KE (1–20 eV).

Data processing: The interpretation of the peptide mapping data of NISTmAb and etanercept, from which N- and O-linked glycopeptides were identified and chosen for EAD MRM^{HR}, was performed using the Biologics Explorer software (SCIEX). The EAD MRM^{HR} data of targeted peptides were analyzed using the Explorer module and the BioToolKit add-on in SCIEX OS software.

ZenoTOF 7600 system EAD cell

EAD for characterization of protein therapeutics

Peptide type	ECD (1 eV)	Hot ECD (7 eV)	EIEIO (>9 eV)
Common, multiply charged	+++	+++	+
Singly charged	-	++	+++
Amino acid isomers	-	+++	++
Glycosylated	+++	+++	+
Sulfated, low charge	-	+	+++

Figure 1. In-depth characterization of peptides using EAD. A platform EAD method using an electron KE = 7 eV via hot electron capture dissociation (ECD) mechanism is the best option for characterization of a wide range of peptides in a single injection. The low-energy EAD (KE = 1 eV) via classic ECD mechanism is suitable for characterization of common peptides and glycopeptides, while EAD with KE > 9 eV via electron-impact excitation of ions from organics (EIEIO) is particularly powerful for the analysis of singly charged and sulfated peptides.

RESULTS

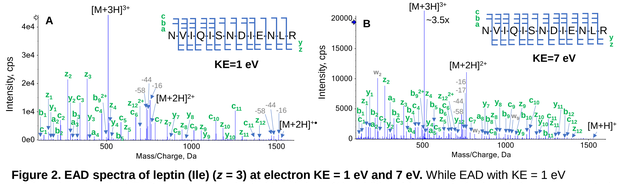

Figure 2. EAD spectra of leptin (Ile) (*z* = 3) at electron KE = 1 eV and 7 eV. While EAD with KE = 1 eV produced an excellent EAD spectrum dominated by *c/z* ions (A), additional fragments (*a*, *b* and *y*) were generated at KE = 7 eV, enabling more in-depth characterization of the peptide.

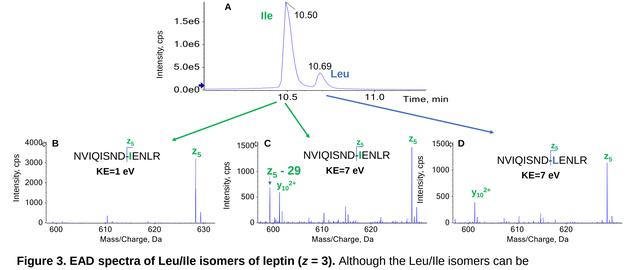

Figure 3. EAD spectra of Leu/Ile isomers of leptin (*z* = 3). Although the Leu/Ile isomers can be chromatographically separated (A), the differentiation of these isomers is not possible using MS1 or CID-derived MS/MS data. EAD generated a signature ion (z_5 - 29) for the Ile isomer at KE = 7 eV (C), but not at KE = 1eV (B). By contrast, this fragment was not detected for the Leu isomer (D) at KE = 7 eV, as expected.

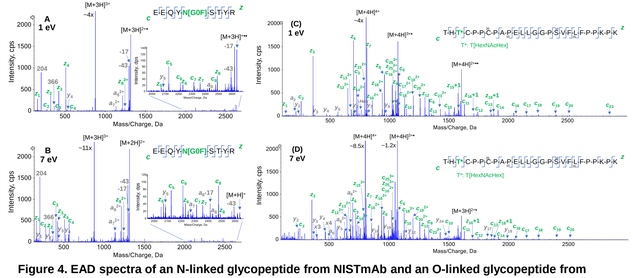

Figure 4. EAD spectra of an N-linked glycopeptide from NISTmAb and an O-linked glycopeptide from etanercept. Accurate localization of G0F in EEQYNSYR was achieved using KE = 1 eV (A) or 7 eV (B). Compared to KE = 1 eV, the EAD platform method with KE = 7 eV yielded more *y*-ions, a higher abundant oxonium ion at *m/z* 204 and a more even distribution of *c/z*-ions containing the glycosylation G0F. These results from the KE = 7 eV experiment facilitated glycopeptide identification and characterization. Similar observations were made for the O-linked glycopeptide from etanercept (C and D). The location of O-glycosylation (HexNAcHex) was confidently assigned to T3, instead of T1 or S17, based on the presence of abundant c_2 and c_3 ions.

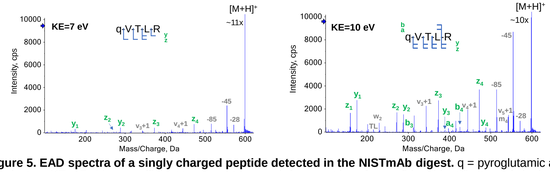

Figure 5. EAD spectra of a singly charged peptide detected in the NISTmAb digest. q = pyroglutamic acid. An electron KE = 7 eV was sufficient to generate enough fragment ions for confident identification of singly charged qVTLR (A). However, further increase of electron KE to 10 eV yielded the optimal outcome for this peptide in terms of the number and intensity of the fragment ions (B).

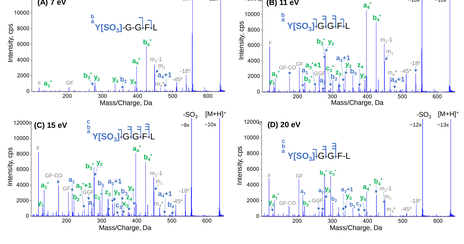

Figure 6. EAD spectra of singly charged LES at different KEs. KE = 15 eV (C) was found to be optimal for the generation of sulfate-containing *a/b* ions enabling confident localization of this labile modification. Lower or higher KEs (A, B, D) resulted in less abundant sulfate containing fragments. The fragments in blue contain the sulfate group while those labeled with * do not contain this labile modification.

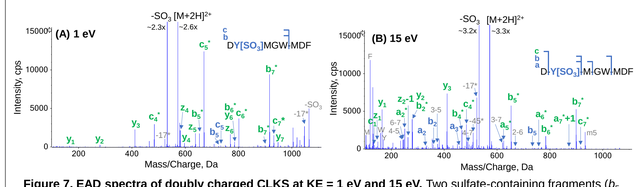

Figure 7. EAD spectra of doubly charged CLKS at KE = 1 eV and 15 eV. Two sulfate-containing fragments (b_5 and c_5) detected at KE = 1 eV (A) did not provide sufficient information to pinpoint the location of the sulfate group. By comparison, an electron KE of 15 eV (B) produced a non-sulfated c_1 and sulfated a_2/b_2-ions for accurate determination of the site of sulfation. The fragments highlighted in blue contain the sulfate group while those labeled with * do not contain this labile modification.

CONCLUSIONS

- Efficient and comprehensive characterization of a wide range of peptides in a single injection can be achieved reliably with the optimized EAD platform method using an electron KE = 7 eV
- Confident peptide identification, unambiguous differentiation of amino acid isomers, accurate localization of PTMs, and fragmentation of singly charged peptides with EAD enable an unprecedented level of information, not achievable with CID or traditional ExD techniques in a single injection
- Accurate localization of labile tyrosine sulfation on a singly and doubly charged peptide was achieved using EAD with tunable electron KE set to 15 eV
- The ability to fine-tune electron KE offers additional mechanisms of fragmentation (low-energy ECD or EIEIO) for full flexibility on the fragmentation of specific analytes
- The results described here demonstrated the power and potential of EAD for routine and in-depth characterization of protein therapeutics, including challenging molecules with fragile modifications that may be encountered in the characterization of new modalities with increasing complexity

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