

Confident O-glycosylation Site Identification for ENBREL (etanercept) Using the ECD Functionality of SELECT SERIES Cyclic IMS System

Samantha Ippoliti, Dale Cooper-Shepherd, Ying Qing Yu, James I. Langridge

Waters Corporation

This is an Application Brief and does not contain a detailed Experimental section.

Abstract

Characterization of glycosylation on the glycopeptide level can be challenging with traditional peptide mapping methods that employ reversed-phase liquid chromatography coupled with tandem mass spectrometry (RPLC-MS/MS or MS^E). These methods typically employ collision induced dissociation (CID) as the fragmentation technique, which has limitations for site specific analysis of labile modifications. The use of Electron Capture Dissociation (ECD) enables glycan moieties to be retained on the peptide backbone, and allow the site to be confidently assigned using the resulting fragment ions. In this study, we demonstrate the utility of ECD on the SELECT SERIES Cyclic IMS instrument for unambiguous assignment of glycosylation site locations for 11 O-glycopeptide species of ENBREL (etanercept).

Benefits

- ECD fragmentation generates critical site-specific O-glycosylation information
 - Improved sensitivity, resolution, and IMS capabilities of the SELECT SERIES Cyclic IMS instrument provide comprehensive analytical tools to unravel this complex set of biotherapeutic attributes
-

Introduction

Recombinant biotherapeutics produced in mammalian systems generally contain a variety of post-translational modifications (PTMs), one of the most complex being glycosylation. Glycosylation plays a critical role in stability, activity, and immunogenicity for biotherapeutics,¹ and must be clearly characterized and monitored. Unlike N-glycosylation, where there is a consensus sequence¹ for occupation of the asparagine site, any exposed serine or threonine site can potentially be O-glycosylated if presented to the glycosyltransferases and glycosidases in the proper 3D structural motif. This means they can be unpredictable and challenging to characterize without prior structural knowledge.

PTMs are analyzed at site level by peptide mapping techniques, typically by RPLC-MS/MS with CID fragmentation for peptide sequence confirmation via b and y ion formation. The shortcomings of CID are apparent with labile modifications such as glycosylation, which are prone to glycosidic bond elimination under these conditions to generate a truncated glycan chain but full peptide length glycopeptide. ECD, on the other hand, is an orthogonal fragmentation technique in which multiply charged peptide ions are passed through a confined low energy electron beam.² An energetic electron is transferred directly to the precursor ion, and the resulting free radical species can promote rapid peptide backbone fragmentation at the N-C α bond, generating c- and z-type ions.³ The SELECT SERIES Cyclic IMS instrument has the flexibility to install the ECD cell either pre- or post-IMS cell depending on experimental needs, as highlighted in Figure 1.

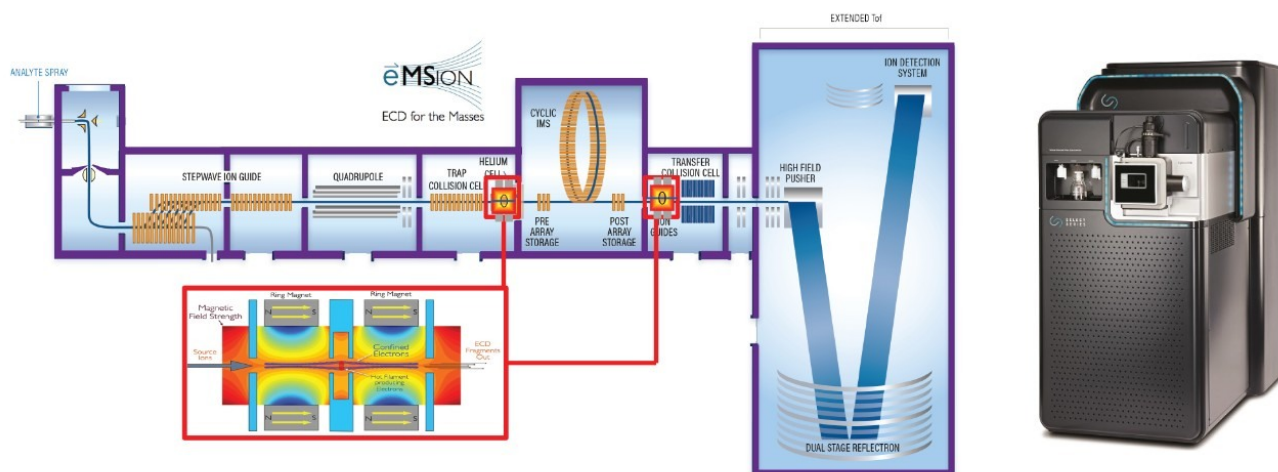


Figure 1. Instrument configuration of the SELECT SERIES Cyclic IMS with ECD installed either pre- or post-IMS.

In this study, we used ENBREL (etanercept) as a model for studying O-glycosylation site identification via LC-HDMS^E CID fragmentation or LC-HDMS (ECD MS) fragmentation mechanisms. ENBREL is a highly glycosylated dimeric fusion protein comprised of TNFR domains fused with a monoclonal antibody Fc domain via an extended O-glycosylated transitional domain. Previous studies⁴ have reported the presence of 3 N-glycosylation sites, and up to 13 O-glycosylation sites in this transitional region. Many of these O-glycosylation sites are very close in proximity to one another, making ECD fragmentation patterns critical to distinguish positional isomers.

Results and Discussion

A reduced and alkylated tryptic digest of ENBREL (etanercept) was analyzed by LC-MS using an ACQUITY UPLC I-Class System coupled to a SELECT SERIES Cyclic IMS instrument. Chromatographic separation of the digest was carried out on an ACQUITY Premier CSH C₁₈ Column (130 Å, 1.7 mm, 2.1 x 100 mm, p/n: [186009488 < https://www.waters.com/nextgen/global/shop/columns/186009488-acquity-premier-peptide-csh-c18-column-130a-17--m-21-x-100-mm-1-.html >](https://www.waters.com/nextgen/global/shop/columns/186009488-acquity-premier-peptide-csh-c18-column-130a-17--m-21-x-100-mm-1-.html)), maintained at 60 °C. Mobile phases A and B are 0.1% formic acid in water and 0.1% formic acid in acetonitrile respectively, with a linear gradient of 1–35 %B over 25 minutes. For HDMS^E, the SELECT SERIES Cyclic IMS was set to single pass IMS mode (the ion mobility settings were as follows: travelling wave height was ramped from 12 V to 26 V at a rate of 1.8 V/ms; inject time 10 ms; separate time 2 ms;

total cycle time 46 ms).

Figure 2 shows the BPI chromatogram for the LC-HDMS^E analysis of the ENBREL tryptic digest. 11 O-glycopeptide species were detected via MS1 and characteristic glycosidic fragmentation patterns were further investigated via targeted LC-HDMS/MS with ECD fragmentation (ECD cell was positioned post-cIMS). Manual analysis of the glycopeptide ECD fragments was performed to assess site occupancy for each of these species.

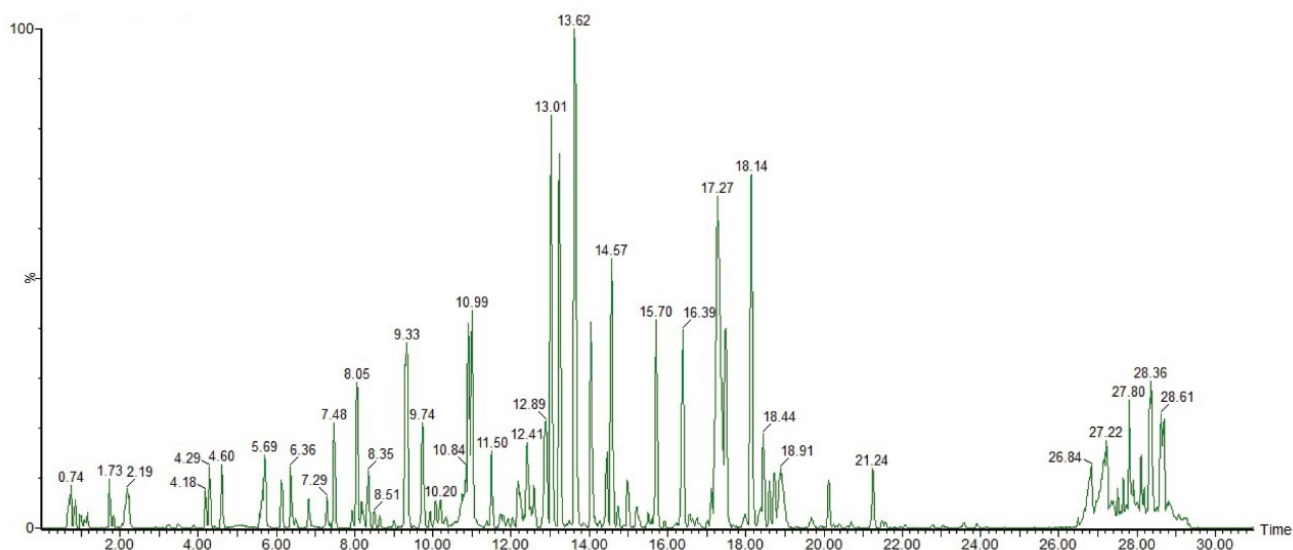


Figure 2. BPI chromatogram of tryptic digest of ENBREL (etanercept) analyzed via RPLC/HDMS^E (single pass IMS).

ECD is a rapid fragmentation technique which generates c and z peptide backbone ions that retain the glycan structures and provide site occupancy information. It is especially useful in distinguishing positional isomers for singly and multiply glycosylated peptides. In many cases, these isomers are separated chromatographically, yet without the ECD fragmentation for clarification, one would have no way of knowing which site(s) is modified.

One striking example (Figure 3) that shows ECD spectra for two isobaric T19 (SMAPGAVHLPQVSTR) + 2 HexNAc + 2 Hex + 1 NeuAc (m/z 890.7, 3+) glycopeptides. The fragment ions in the c series are identical for both isomers until c14 fragment, corresponding to position Ser₁₉₉. The c14 ion from the peak at RT 11.7 minutes, confirms the presence of 1 sialylated 'Core 1' O-glycan on Ser₁₉₉. The c15 ion from this peak contains this sialylated Core 1 O-glycan on Ser₁₉₉ and a non-sialylated O-glycan on position Thr₂₀₀. The z ions in this series support this

assignment, as z2 (corresponding to Thr₂₀₀) is present with the non-sialylated O-glycan, and z3 (containing both S₁₉₉ and Thr₂₀₀) is present with both the sialylated and non-sialylated O-glycans. For the second T19 peak at RT 11.9 minutes, the ECD spectra supports the opposite assignment — Ser₁₉₉ can be observed with the non-sialylated O-glycan and Thr₂₀₀ with the sialylated O-glycan.

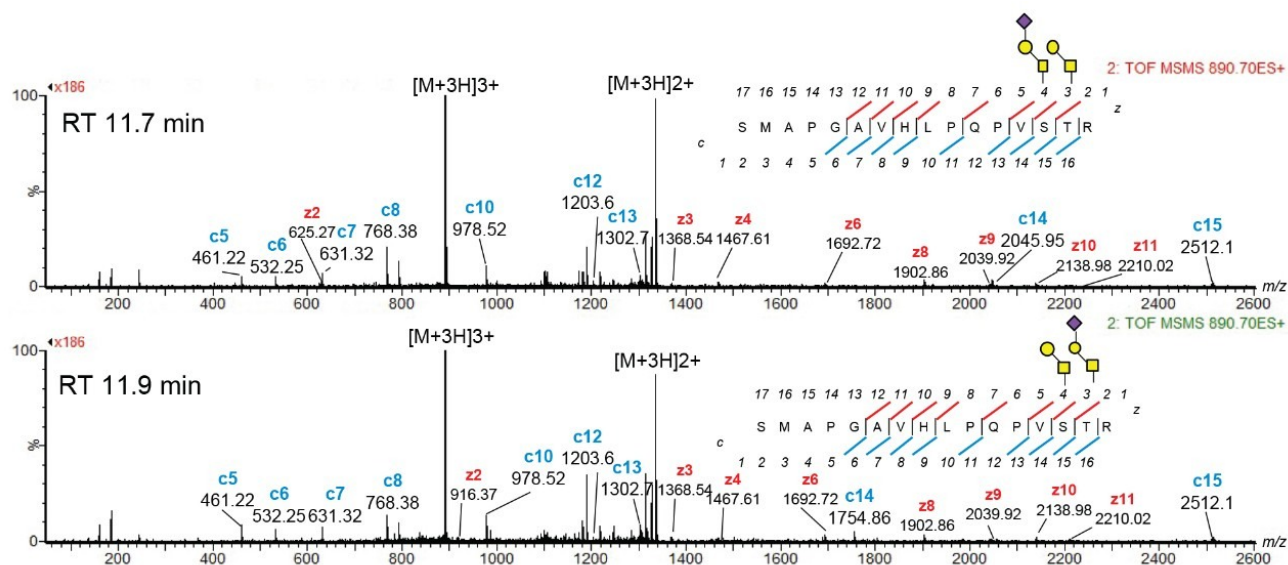


Figure 3. ECD MS spectra for LC-HDMS/MS peaks at RT 11.7 and 11.9 min (m/z 890.7, 3+). Selected m/z for T19 tryptic peptide (SMAPGAVHLPQPVSTR) with 2 HexNAc + 2 Hex + 1NeuAc. ECD fragmentation confirms locations and identities of two Core 1 O-glycans at positions 14 and 15.

The ECD spectra for the remaining 9 O-glycopeptide species were examined in a similar manner, and results for O-glycan site occupancy are shown in Table 1. The T1 peptide (LPAQVAFTPYAPEPGSTCR) was found to contain either singly or doubly sialylated O-glycans at position Thr₈. In addition to the two O-glycovariants described previously for T19 peptide, an additional five glycoforms were detected. The species at RT 10.9 minutes and 12.2 minutes corresponds to Ser₁₉₉ with 1 HexNAc, and Thr₂₀₀ with either 1 HexNAc + 1 Hex or 1 HexNAc + 1 Hex + 1 NeuAc, respectively. T19 was also detected in singly O-glycosylated form, with either Ser₁₉₉ or Thr₂₀₀ occupied by 1 HexNAc + 1 Hex + 1 NeuAc. The last T19 glycoform is doubly occupied, Ser₁₉₉ and Thr₂₀₀ each occupied by 1 HexNAc + 1 Hex + 1NeuAc. A final example is for T22-23 (THTCPPCAPELLGGPSVFLFPPKPK), one of the tryptic peptides in the hinge region of this fusion protein. The ECD data confirms a single O-glycosylation site, Thr₂₄₅, with either singly or doubly sialylated core 1 O-glycan present.

RT (min)	Peptide	O-glycan(s)	Position(s)
14.7	T1: LPAQVAF ₈ PYAPEPGS ₁₆ T ₁₇ CR		T8
15.3			T8
10.9	T19: S ₁₈₆ MAPGAVHLPQPVS ₁₉₉ T ₂₀₀ R	and	S ₁₉₉ and T ₂₀₀
11.7		and	S ₁₉₉ and T ₂₀₀
11.9		and	S ₁₉₉ and T ₂₀₀
12.2		and	S ₁₉₉ and T ₂₀₀
12.3			T ₂₀₀
12.6			S ₁₉₉
12.8		and	S ₁₉₉ and T ₂₀₀
18.3		T22-23: T ₂₄₃ HT ₂₄₅ CPPCPAPELLGGPS ₂₅₉ VFLFPPKPK	
19.6			T ₂₄₅

Table 1. List of ENBREL (etanercept) O-glycopeptides and glycosylation sites identified by ECD.

Conclusion

ECD is a powerful and orthogonal fragmentation technique complementary to CID, as it generally retains labile modifications, such as glycosylation, to enable preferred fragmentation of the peptide backbone. This allows for more confident characterization of glycosylation sites and unambiguous occupancy assignments where multiple sites are occupied on a single glycopeptide. ENBREL (etanercept) is one such protein that contains multiple O-glycosylation sites located close to one another, for which we have demonstrated successful characterization by ECD on the Cyclic IMS platform.

ENBREL is a trademark of IMMUNEX Corporation. Cyclic, SELECT SERIES, and ACQUITY are trademarks of Waters Technologies Corporation.

References

1. Houel S, Hilliard M, Yu YQ, McLoughlin N, Martin SM, Rudd PM, Williams JP, Chen W. N- and O-glycosylation Analysis of Etanercept Using Liquid Chromatography and Quadrupole Time-of-Flight Mass Spectrometry Equipped with Electron-Transfer Dissociation Functionality. *Anal Chem*. 2014. 86, 576–584.
2. Voinov, VG; Deinzer, ML; Barofsky, DF. Electron Capture Dissociation in a Linear Radiofrequency-Free Magnetic Cell. *Rapid Commun. Mass Spectrom*. 2008. 22, 3087–3088.
3. Bakhtiar R, Guan Z. Electron Capture Dissociation Mass Spectrometry in Characterization of Post-translational Modifications. *Biochem and Biophys Res Comm*. 2005, 334(1), 1–8.
4. Mulagapati S; Koppolu V, Raju T.S. Decoding of O-Linked Glycosylation by Mass Spectrometry. *Biochem*. 2017 56 (9), 1218–1226.

Featured Products

[ACQUITY UPLC I-Class PLUS System <https://www.waters.com/134613317>](https://www.waters.com/134613317)

[SELECT SERIES Cyclic IMS <https://www.waters.com/waters/nav.htm?cid=135021297>](https://www.waters.com/waters/nav.htm?cid=135021297)

[MassLynx MS Software <https://www.waters.com/513662>](https://www.waters.com/513662)

720007458, December 2021

© 2022 Waters Corporation. All Rights Reserved.