Ultra-comprehensive antibody Fc-fusion protein characterization using a Tribrid Orbitrap mass spectrometer modified for extended mass range applications

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ABSTRACT

Purpose: To tackle the 'extreme' microheterogeneity presented by some protein drug formats, such as antibody Fc-fusion proteins, we demonstrated the feasibility of a workflow for monitoring protein isoform heterogeneity of etanercept as analyzed in the intact and subunit forms.

Methods: We analyzed etanercept, which is highly glycosylated and contains numerous O- and N-glycosylation sites, using Proton Transfer Charge Reduction (PTCR) at intact and subunit levels by native size exclusion chromatography (SEC)-MS on the Thermo Scientific™ Tribrid Orbitrap Eclipse mass spectrometer, equipped with PTCR and extended mass range detection and ion trap isolation.

INTRODUCTION

Mass spectrometry has emerged as the predominant analytical platform for characterizing therapeutic proteins. Etanercept is an extensively glycosylated therapeutic protein product involving the fusion of a human IgG1 Fc amino acid sequence to the TNF-α receptor (TNFR) sequence (**Figure 6**). We tested the performance characteristics of the Orbitrap Eclipse, as a single MS platform for characterization of etanercept.

MATERIALS AND METHODS

As shown in the workflow, the Fc and TNFR subunits were generated by using GENOVIS FabRICATOR-digested (**Figure 1**). Intact and subunit etanercept were analyzed by native SEC-MS, with and without prior removal of sialic acids and O- and N-glycans using SialEXO and OglyZOR from GENOVIS and PNGase F from New England Biolabs. Native SEC-MS was accomplished using a Vanquish UHPLC connected to the Eclipse. Data were analyzed using Thermo Scientific™ BioPharma Finder™ 3.1 software.

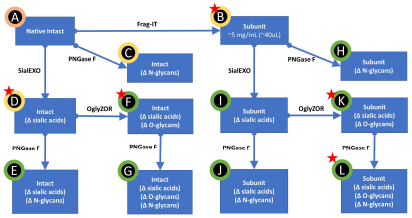

Figure 1. Schematic of 12-way intact sample prep workflow

CHARGE REDUCTION OF INTACT ISOFORM MIXTURES

Comprehensive biologic drug characterization involves the combined efforts of peptide mapping and intact mass analysis. Highly complex biologics require the use of advanced methods for comprehensive characterization. Electron transfer dissociation (ETD)-based tandem MS has been proven to be necessary for extensively glycosylated peptides [**Reference 1**]. For intact mass analysis, recently published strategies for handling extreme microheterogeneity posed by extensive glycosylation include native MS, limited enzymatic dissection, and innovative intact protein chromatography, to perform glycoform distribution measurements across multiple levels of heterogeneity (**References 2-3**).

Orbitrap Eclipse MS is a single MS solution for so-called 'ultra'-comprehensive characterization of highly complex biologic drugs which require both ETD and extended mass range for native MS and charge reduction (**Figure 2**).

Figure 2. Orbitrap Eclipse MS

To test the performance of ion trap isolation and PTCR for performing gas phase charge reduction of intact protein ions we focused on trastuzumab, an antibody drug sample which presents a simple mixture of intact protein isoforms. We repeated injections of trastuzumab using denaturing and native size exclusion chromatography (SEC)-MS (**Figure 3**). Ion trap isolation prior to PTRC concentrates ions and significantly boosts sensitivity (**Figure 4**). Isolation width regulates the availability of ion current and thus influences the extent of observed charge reduction of trastuzumab ions using native SEC-MS platform (**Figure 5**).

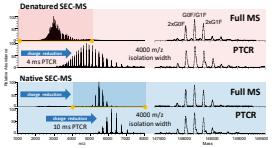

Figure 3. Full MS and PTCR spectra from denaturing and native SEC-MS experiments

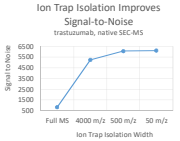

Figure 4. Ion trap isolation (SIM scan) allows ion current to be concentrated, which improves S/N.

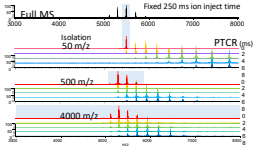

Figure 5. Isolation width and fixed ion inject time influences the extent of observed charge reduction of trastuzumab ions using native SEC-MS platform.

AUTOMATED PLATFORM FOR Fc-FUSION PROTEIN INTACT MASS ANALYSIS

We performed a battery of sample preparations for measuring glycoform heterogeneity present on intact etanercept as well as TNFR and Fc subunits. We determined the isoform composition of several multi-enzyme subunit preparations ("L" and "K") using SEC-MS and intact preparations ("F" and "D") using an automated form of nano-spray infusion (**Figures 6-8**).

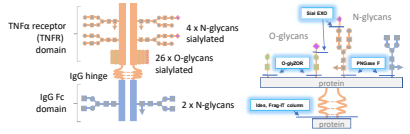

Figure 6. Etanercept structure and enzymatic tools for characterization

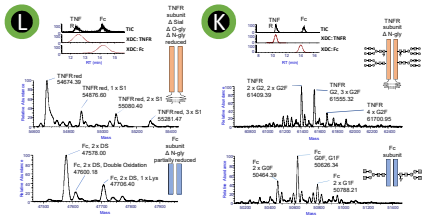

Figure 7. Deconvolution results from native SEC-PTCR-MS analyses

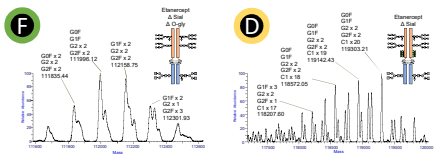

Figure 8. Deconvolution results from native SEC-PTCR-MS analyses

NATIVE SEC-PTCR-MS METHOD ALLOWS DIRECT MEASUREMENT OF TNFR

Owing to the extensive glycosylation present on the fully glycosylated TNFR subunit, we obtained only a partial deconvolution result using a full MS measurement; however, PTCR charge reduced spectra (15 ms) provided sufficient separation of charge states to produce an accurate deconvolution analysis (**Figure 9**). We confidently assigned all abundant isoforms based on highly accurate mass (< 50 ppm) with specific glycoform assignments based on evidence from our other sample preparations, consistent with other publications (**References 2-3**).

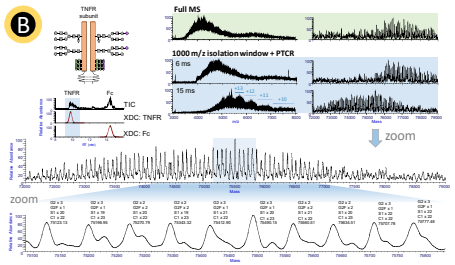

Figure 9. Full MS and PTCR spectra of the fully glycosylated TNFR subunit.

CONCLUSIONS

We demonstrate that native SEC-MS with PTCR charge reduction is a powerful, automatable, state-of-the-art approach for intact mass analysis of highly complex biologic drugs.

REFERENCES

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