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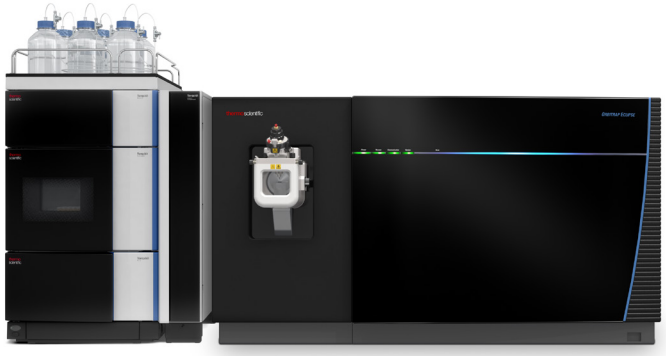
Go beyond in biopharmaceutical characterization

Unprecedented insights into the most advanced biotherapeutics are revealed by utilizing the pioneering technology and software developments incorporated into the Thermo Scientific™ Orbitrap Eclipse™ Tribrid™ mass spectrometer.

Abstract

The pioneering technology and software developments incorporated into the Orbitrap Eclipse Tribrid mass spectrometer enables superior analytical performance across all biopharmaceutical characterization approaches. Innovative developments of ion-ion reactions and high mass range MS^n capabilities can be combined with multiple dissociation modes to enable easy-to-use workflows for more comprehensive characterization. Biopharma discovery and development scientists can now achieve new levels of structural insight into therapeutic proteins; revealing ultra-low-level modifications and site-specific critical quality attribute (CQA) determination — delivering more confidence when progressing candidates throughout the development pipeline, whilst ensuring drug efficacy and patient safety.



Introduction

In the pursuit of efficacious treatments for many of today's most devastating diseases, biotherapeutics are becoming increasingly more complex. Advanced biologics such as antibody drug conjugates, fusion proteins, and monoclonal antibody mixtures are exhibiting improved pharmacokinetics and pharmacodynamics with exceptional specificity, and as such represent rapidly growing classes of biotherapeutics. As the molecular complexity of biotherapeutics increases, the need for innovative approaches to facilitate protein characterization becomes increasingly valuable to assess the candidate's quality. In addition, regulatory agencies continue to evaluate requirements to ensure the safety of drugs requiring drug manufacturers to develop evermore sensitive and precise assays early in the drug development pipeline.

Thermo Fisher Scientific has recently addressed this requirement by increasing the performance, expanding the capabilities, and enhancing the usability of the proven and trusted Thermo Scientific™ Tribrid™ mass spectrometer series.

Historically, increasing innovation in instrument methods was directly proportional to the complexity of operating the mass spectrometer to successfully execute the experimental goals. The increased experimental complexity therefore could hinder companies or organizations of all sizes to maintain efficiency to meet both the level of characterization per candidate and throughput. In addition, the output from the characterization stage must be transferable to downstream applications. Seamless transitions require common mass spectral interfaces to utilize instrument method settings identified during the characterization to be exported to secondary mass spectral applications whether on the same instrument or an alternative platform.

The following paper outlines the Orbitrap Eclipse Tribrid mass spectrometer and its performance benefits for biotherapeutic characterization studies. This system offers significant hardware and software innovations that not only improve user experience but boost operational simplicity, performance, and versatility.

Building upon robust mass accuracy and efficient desolvation, which have become the hallmarks of Thermo Scientific™ Orbitrap™ mass analzyer technology for intact mass analysis, the revolutionary Tribrid architecture has been optimized throughout (Figure 1). This instrument leverages the proven advantages of Tribrid mass spectrometers, utilizing a high-performance quadrupole ion selector, an ultra-high field Orbitrap mass analyzer, and a high sensitivity dual pressure linear ion trap mass analyzer. Innovative changes to hardware that allow for novel biopharmaceutical experimental capabilities are:

- Enhanced ion optics and QR5 segmented quadrupole mass filter for increased transmission efficiency for small and large molecules
- The option for extended mass range detection up to *m/z* 8000 (High Mass Range or HMR^n). Capabilities for native/denatured protein(s) and top-down structural analyses
- The option for variable pressure settings in the ion routing multipole (IRM) for increased trapping efficiencies for larger proteins and protein complexes
- Enhanced vacuum chamber in the C-trap and Orbitrap mass analyzer region to maintain better vacuum under higher IRM gas pressures resulting in greater signal-to-noise (S/N) measurements and resolution for larger molecules
- Optional proton transfer charge reduction (PTCR) to simplify MS and higher order MS^n spectra for improved intact protein and top-down data generation and interpretation
- Modified dual-pressure linear ion trap with extended high-pressure cell for greater ion-ion reaction efficiency

Charge reduction for simplifying complex mass spectra

Comprehensive characterization of advanced biologics presents significant challenges due to microheterogeneity. Current biologics are large proteins with multiple sites of posttranslational modifications, specifically consensus N-linked glycosylation sites resulting in multiple proteoforms that may have small mass differences. Simultaneous mass spectral analysis of proteoforms can result in significant charge state overlap decreasing the effectiveness of resulting mass spectral data to discern differentiating features between each proteoform. Utilizing enhanced vacuum technology and the PTCR option, the Orbitrap Eclipse Tribrid mass spectrometer addresses these challenges by increasing the spatial resolution of MS and MS/MS spectra to facilitate confident deconvolution to unravel glycoform heterogeneity and successfully characterize even the most heterogeneous biopharmaceutical molecules.

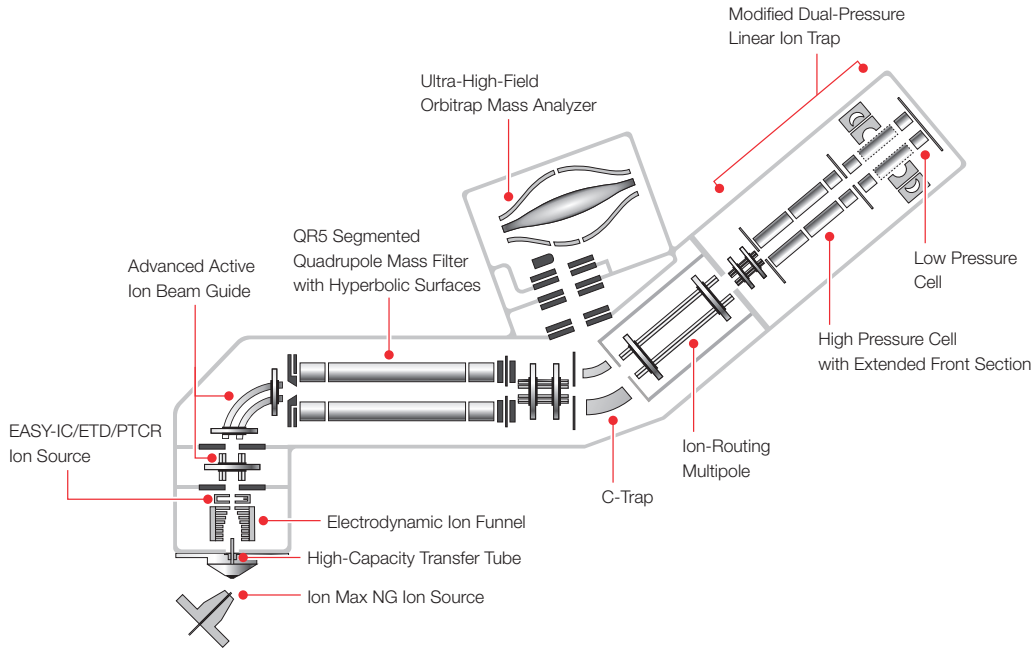


Figure 1. Orbitrap Eclipse Tribrid mass spectrometer schematic.

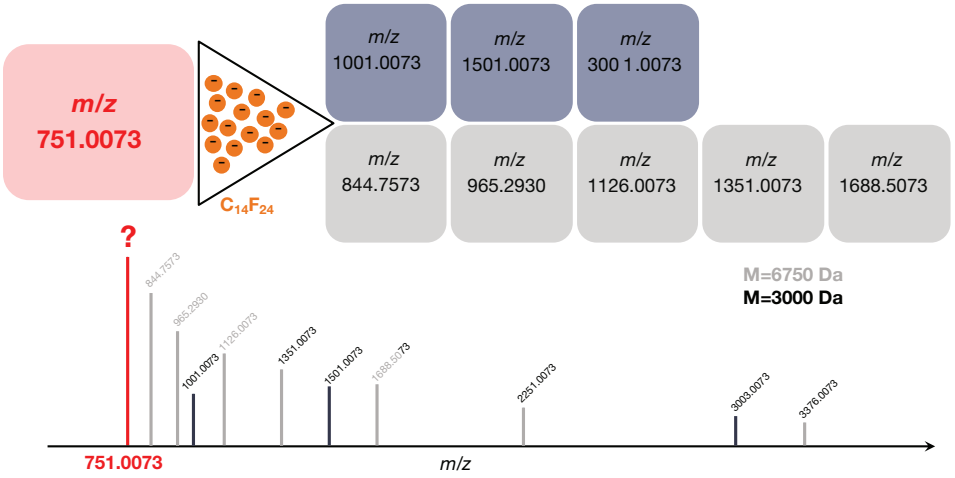


Figure 2. Charge reduction reveals charge state distributions of two different species.

Figure 2 illustrates how charge reduction can simplify spectra and ultimately lead to more comprehensive biopharma characterization by separating and therefore identifying isomers. A narrow precursor mass range is isolated either by the QR5 quadrupole mass filter or the linear ion trap (depending on the specific *m/z*), transferred to the extended high-pressure cell of the linear ion trap, and allowed to react with the singly charged anion based on the following reaction originally presented by McLuckey et al. [1]:

Reaction 1: $(M + nH)^{n+} + Y^{-} \rightarrow (M + (n-1)H)^{(n-1)+} + HY$

resulting in a charge reduced cation and a neutral. The charge reduced product ion can undergo further ion-ion reactions that produce a series of lower charge state product ions depending on the reaction time; PTCR becomes extremely beneficial for co-isolated multiply charged cations. As shown in Figure 2, all isolated precursors undergo ion-ion reactions and the reduction in charge state increases *m/z* separation as well as enables

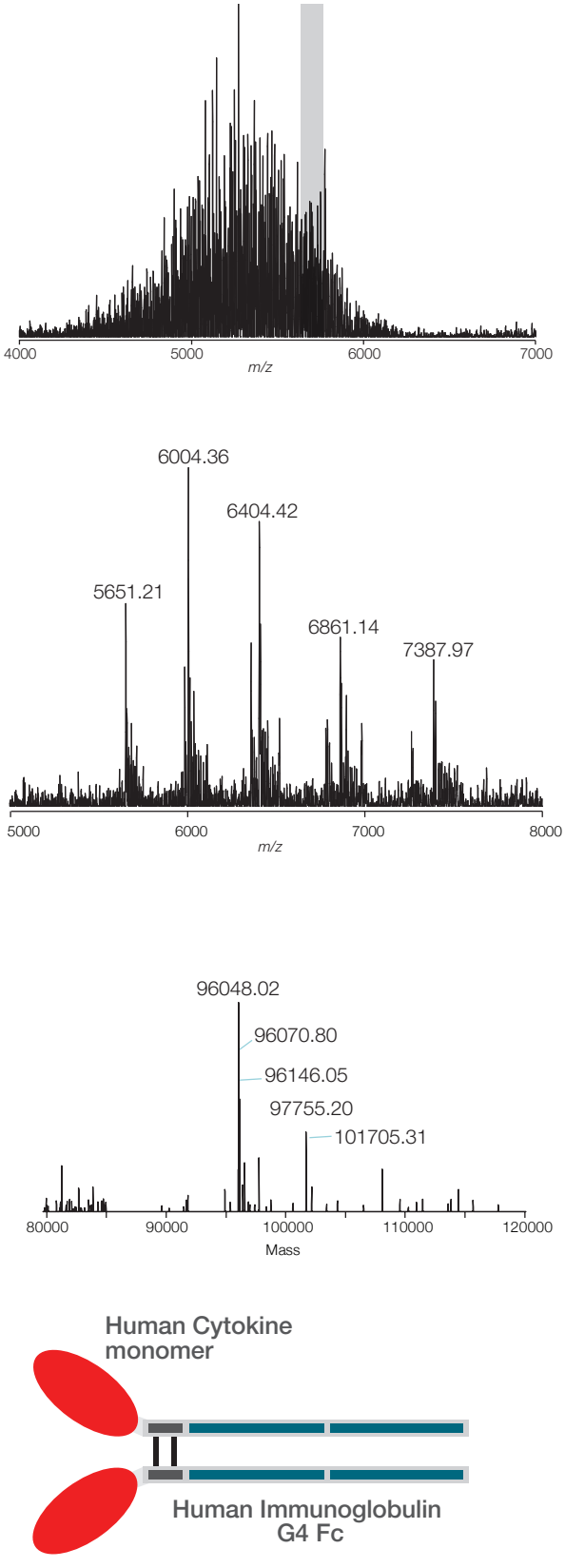


Figure 3. PTCR makes it possible to deconvolve a de-sialylated Cytokine-Fc fusion protein from the native MS analysis by first isolating a narrow precursor window (80 Da) in the linear ion trap and reacting all isolated precursors with the C14F24 anion for ca. 4 milliseconds. The resulting charge reduced product ions are detected in the Orbitrap mass analyzer, and deconvolved post-acquisition.

molecular weight determination by evaluating the newly formed charge state series. With this reduced spectral density, the data deconvolution software can confidently identify the two distinct species.

Implementing the strategy described above enables automated processing of complex biotherapeutics. Figure 3 shows the HMR^n, PTCR method on a Cytokine-Fc fusion protein. The instrument method editor template enables ease of use for complex biotherapeutic characterization where very heterogenous and isomers are common.

Shown at the top of Figure 3 is the native MS^1 spectra of a de-sialylated Cytokine-Fc fusion protein. This spectrum is uninterpretable due to its complexity. The molecule under investigation has 3 N-linked glycosylation sites per arm region (in red) which significantly contributes to its heterogeneity. Although native MS analysis does reduce the number of charge states, it is not sufficient to successfully deconvolve the MS^1 spectrum to determine the intact molecular weights for the detected proteoforms. However, combining native MS and high-mass precursor isolation with PTCR results in a tandem mass spectrum that shows significant reduction in spectra complexity enabling accurate automated data deconvolution. The Orbitrap Eclipse Tribrid MS can be set up to automatically acquire a series of tandem MS events stepping across 80-Da ranges in a single experiment; further identifying more proteoforms, which is unique to any current commercial mass spectrometer.

PTCR can be applied at any level

Proton transfer charge reduction reactions are used to simplify complex mass spectra, and with the Orbitrap Eclipse Tribrid mass spectrometer, can be implemented on precursors or any stage of product ions. Performing top-down or middle-down sequencing of large molecules can generate extremely complex product ion spectra due to the high charge state of isolated precursors which, following activation by any of the methods available on the Orbitrap Eclipse Tribrid mass spectrometer, can result in a large number of product ions with an extremely wide range of molecular weights. As the size of the product ions increase, so too the resulting charge state that can overlap with smaller product ions reducing the identification of overlapped product ion isotopes. Performing proton transfer reactions following MS/MS enables greater detection and characterization of the resulting product ions.

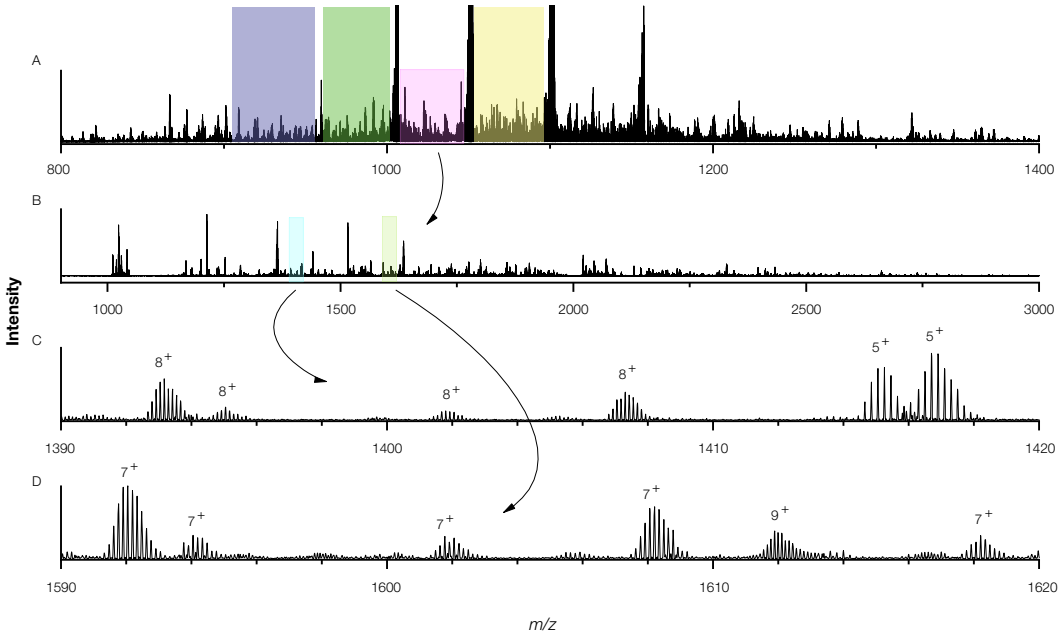


Figure 4. PTCR simplifies complex spectra: Middle-down analysis of NIST mAb.

Figure 4 shows an example of the automated data-dependent acquisition for middle-down sequencing of the Fd subunit of the NIST mAb standard. The automated data-dependent acquisition method first isolates a single charge state, performs ETD fragmentation, processes the MS/MS spectrum real time to determine the MS/MS product ion mass ranges selected for a series of subsequent MS^3 experiments consisting of MS/MS product ion isolation and ion-ion reactions.

Figure 4A shows the low-intensity MS/MS product ion spectrum consisting of the more intense unreacted precursor, and a series of charge-reduced precursors as well as many ETD product ions.

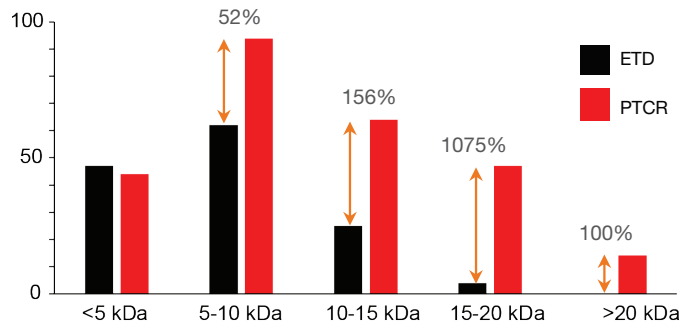


Figure 5. Increased ETD product ions identification as a function of molecular weight with the addition of PTCR.

The set of ETD product ions at any *m/z* range are incredibly dense and not ideal for comprehensive data interpretation or deconvolution. The automated data-dependent MS^3 method interprets the resulting MS/MS spectrum using intelligent algorithms to identify the precursor ions represented by the unreacted 23+ ion and the series of charge reduced product ions. This then establishes the MS/MS product ion mass range isolated and reacted with anions for PTCR, as shown by the region highlighted in red. Figure 4B shows the resulting MS^3 product ion spectrum consisting of the unreacted ETD MS/MS product ions originally isolated (centered around *m/z* 1050) and all charge reduced MS^3 product ions extending out to almost *m/z* 3000. The two MS^3 mass regions highlighted in Figure 4B are expanded in Figures 4C and 4D respectively, demonstrating the simplicity of the product distribution following PTCR.

Simplifying the ETD MS/MS product ion spectrum enhances the automated data processing. By adding sequential PTCR to the four MS/MS product ion mass ranges, automated data processing identified many more ETD product ions resulting in greater sequence coverage and more importantly, in confidence. Figure 5 shows a histogram comparing the number of ETD product ions identified as a function of molecular weight. Introducing PTCR decreases the product ion spectral complexity resulting in greater sequencing capabilities of the automated data processing routine, specifically for higher molecular weight product ions as compared against the

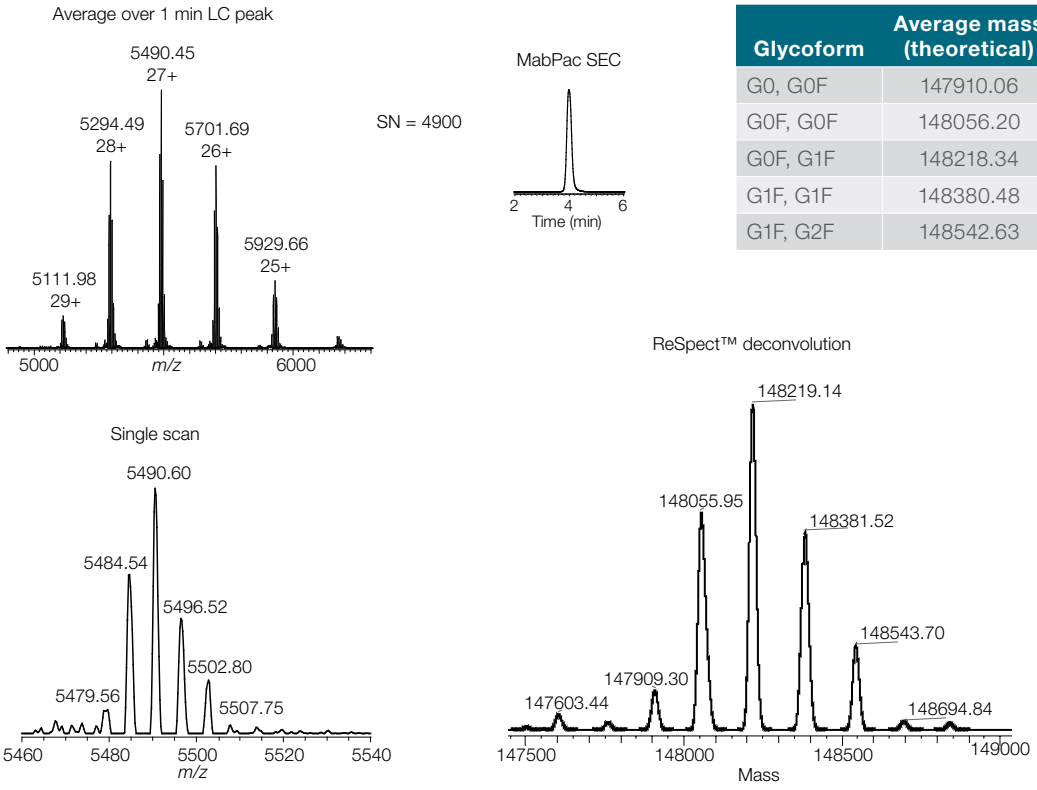
same experiment performed without PTCR. The greatest increase is observed for product ions with molecular weights in excess of 10 kDa. The increased detection of large product ions increased sequence coverage by 10% (52% vs 63%) but more importantly, significantly increased the number of complementary product ions from 12 to 54, enabling much greater confidence in sequence assignments as well as clippings, SNP and PTM locations while maintaining equivalent data acquisition cycle time.

Native top-down structural characterization of mAbs

Preserving the native state of a biotherapeutic molecule during analysis has many benefits, most notably reducing sample preparation related artefacts, preserving non-covalent interactions, and obtaining analytical information on the molecule as close to its biological state as possible. Ionization under native MS conditions generally reduces the number of basic residues available for protonation, resulting in lower charge state envelope measured at higher *m/z* values. Detection at the higher *m/z* values can increase the measured *m/z* spacing between proteoforms reducing complexity and enhancing automated spectral deconvolution to more accurately determine the number of proteoforms and accurate molecular weights.

Structural characterization of biotherapeutics in their native state can be challenging. Optimal mass spectral conditions require “soft” ionization and transfer from the ion source to the mass analyzer. In addition, the mass spectrometer must be able to measure the high-mass charge state envelop, isolate one or more charge states depending on the background complexity, and perform robust tandem mass spectral analysis resulting in high sequence coverage, SNP location, and/or PTM characterization (e.g. identification and location of modification sites).

The Orbitrap Eclipse Tribrid mass spectrometer is currently the most complete mass spectrometer for native MS biotherapeutic characterization. The Orbitrap Eclipse Tribrid mass spectrometer maintains an efficient ion source, enhanced quadrupole mass filter with increased transmission efficiency, the ability to modulate the neutral gas pressure in the ion routing multiple, enhanced vacuum technology to maintain high vacuum in the C-trap and Orbitrap mass analyzer region, high mass range detection, and precursor isolation capabilities up to *m/z* 8000. In addition, the Orbitrap Eclipse Tribrid MS offers multiple methods of fragmentation that can be performed at any MS^n stage, including HCD/CID, ETD, ETciD, EThcD,



Glycoform	Average mass (theoretical)	Average mass (measured)	Delta mass (ppm)
G0, G0F	147910.06	147909.30	5.1
G0F, G0F	148056.20	148055.95	1.7
G0F, G1F	148218.34	148219.14	5.4
G1F, G1F	148380.48	148381.52	7.0
G1F, G2F	148542.63	148543.70	7.2

Figure 6. Native MS analysis of trastuzumab using the MAbPac SEC-1 Size Exclusion Chromatography HPLC Column. The inset shows the chromatographic peak, and the full scan MS has been averaged across the elution profile. The spectrum below shows the expanded view for the 27+ charge state measured in one spectrum to be compared to the deconvoluted mass spectrum to the right. The corresponding table lists the glycoforms differentiated each proteoform and the resulting mass measurement accuracy.

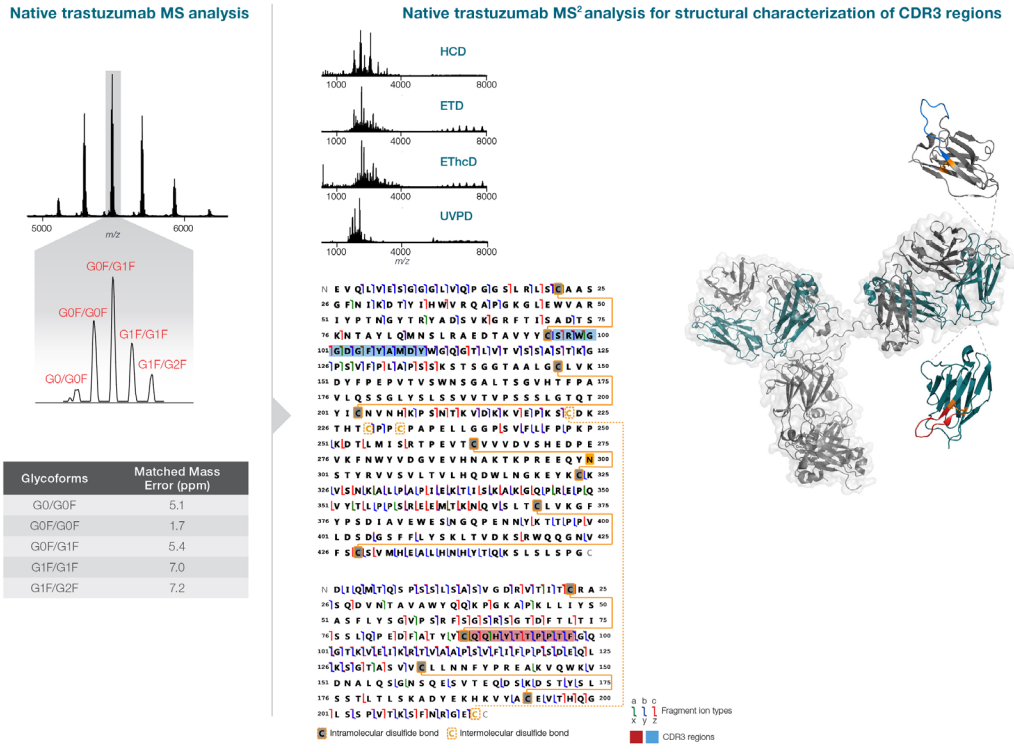
and UVPD. The combination of fragmentation methods has been demonstrated to generate complementary product ions that significantly increase biotherapeutic characterization.

An example of the entire workflow is presented for trastuzumab. Figure 6 shows the initial LC-MS analysis under native MS conditions. The sample was analyzed on an LC timescale by being injecting onto a Thermo Scientific™ MabPac™ SEC-1 Size Exclusion Chromatography HPLC column, washed, and quickly eluted into the ion source for native MS.

Figure 6 shows the mass spectrum generated for the native MS analysis of trastuzumab averaged across a 1 min LC peak. In addition, a mass spectrum resulting from a single scan is shown displaying the measured glycoform distribution which matches that of the deconvoluted mass spectrum data processed using the Thermo Scientific™ BioPharma Finder™ software using the ReSpect algorithm. The ReSpect algorithm deconvolves isotopically unresolved (or unseparated) mass spectra—that is, spectra in which it is not possible to distinguish the separate peaks for different isotopic compositions of the same component. In addition, the five major glycoforms are all well resolved and measured with high mass accuracy.

Native MS analysis can be further supplemented with various top down approaches to achieve more comprehensive characterization. Figure 7 shows native top-down analysis of trastuzumab via direct infusion employing multiple fragmentation modes, a single charge state was isolated and fragmented using ETD, EThcD, UVPD and HCD.

By combining fragmentation modes, we achieve 43% sequence coverage but more importantly, this coverage is achieved on the molecule in its native state with both covalent and non-covalent bonds attached. Depth of coverage can be further increase by supplementing the method with the analysis of the denatured molecule. In this case, trastuzumab is reduced prior to top-down analysis using the multiple fragmentation approaches. The efficiency of data acquisition on the Orbitrap Eclipse Tribrid MS enables fast chromatography to maximize throughput. LC-MS methods integrate fast chromatography on the Thermo Scientific™ Vanquish™ Horizon UHPLC system and are introduced into the Orbitrap Eclipse Tribrid mass spectrometer. Data acquisition methods utilize intelligent MS to acquire high-quality MS and MS^n using any of the above mentioned dissociation methods in combination with PTCR.



Glycoforms	Matched Mass Error (ppm)
G0/G0F	5.1
G0F/G0F	1.7
G0F/G1F	5.4
G1F/G1F	7.0
G1F/G2F	7.2

Figure 7. High Mass Range (HMR^n): Native top-down analysis of protein drugs

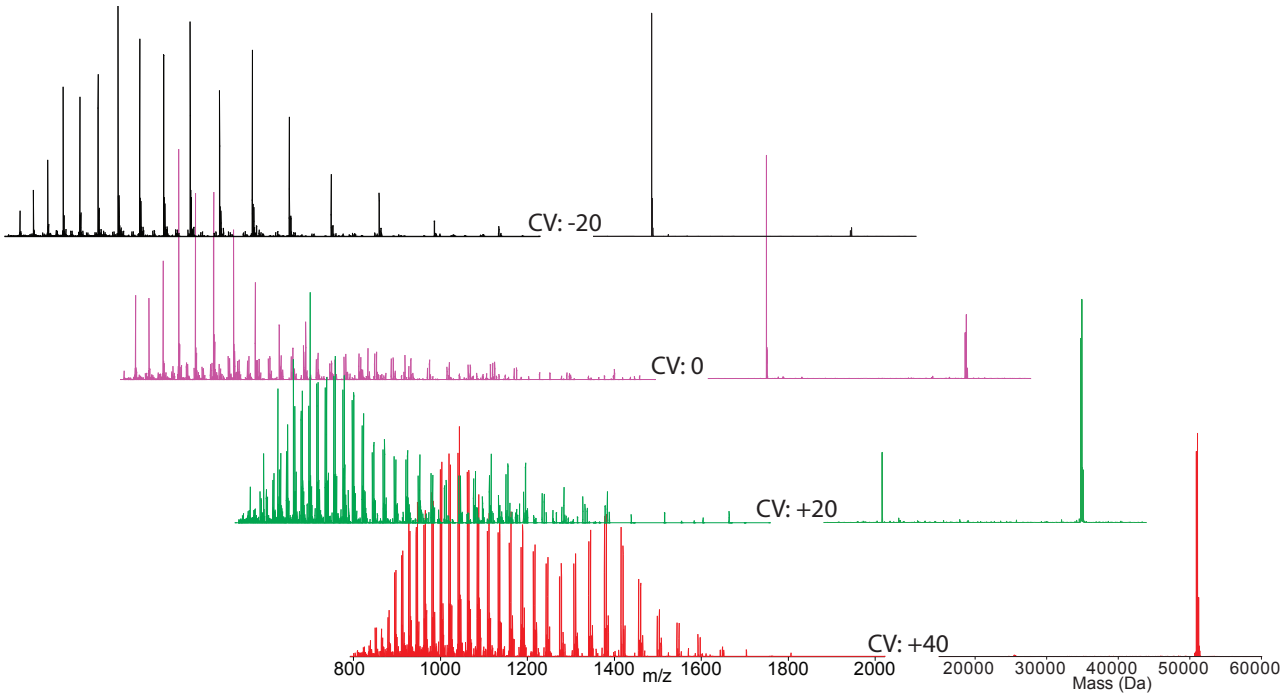


Figure 8. Rapid characterization of mAbs chains: FAIMS Pro interface dTD MS on NIST mAb by changing the compensation voltage and recording the subsequent full scan MS.

Incorporation of the FAIMS Pro interface for enhanced top-down characterization

Rapid and comprehensive protein characterization can be required to increase sample throughput. Despite sample preparation steps such as affinity capture and reduction and alkylation, the resulting protein mixtures can still present significant challenges to successful characterization using direct infusion or loop injection. Incorporating the Thermo Scientific™ FAIMS Pro™ interface on the Orbitrap Eclipse Tribrid mass spectrometer can enhance gas-phase selectivity prior to entering the mass spectrometer, enabling the Tribrid components to perform high-quality intact mass analysis, as well as top-down or middle-down sequencing using the multitude of dissociation options in conjunction with PTCR for unmatched characterization capabilities.

The FAIMS Pro interface has demonstrated orthogonal selectivity benefits for peptides using different compensation voltages to selectively transmit specific groups of peptides through the interface and into the mass spectrometer. Similar selectivity enhancements can be realized for intact proteins or protein subcomponents. By alternating compensation voltage (CV), light and heavy chains can be analyzed either simultaneously or separated in the gas phase and introduced sequentially into the MS, shown in Figure 8. The NIST mAb standard was reduced and alkylated prior to being directly infused into the Orbitrap Eclipse Tribrid mass spectrometer. The results show that changing the CV alters which precursors successfully pass through the FAIMS Pro interface for detection. Note the degree of selectivity using two different compensation voltages effectively separating the two chains.

The increased selectivity enables ion activation on either the entire charge state envelope or following precursor selectivity narrowing the targeted precursor charge states. Top-down or middle-down sequencing can be performed utilizing multiple fragmentation modes including ETD, EThcD, HCD and UVPD.

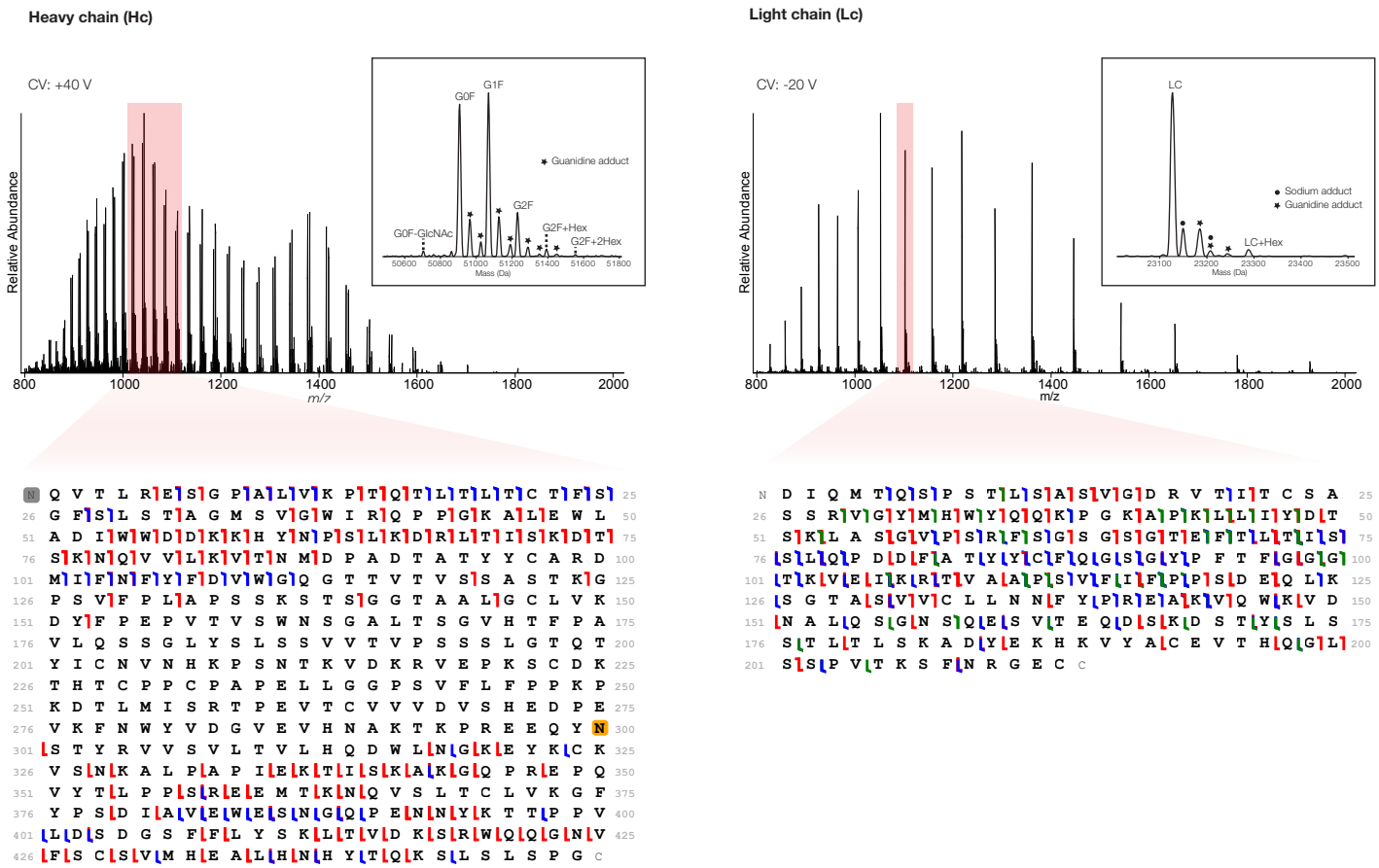


Figure 9. Rapid characterization of mAb chains: FAIMS Pro interface dTD MS on NIST mAb.

Figure 9 shows the subunit characterization via direct infusion analysis using the FAIMS Pro interface on the Orbitrap Eclipse Tribrid mass spectrometer. The full scan mass spectra measured at each CV setting were deconvoluted prior to analysis resulting in the molecular weight determination for the various glycoforms. In addition, tandem mass sequencing was done following precursor isolation of either a wide window as shown for the heavy chain, or selection of a single charge state as demonstrated for the light chain. The resulting data processing showed 65% sequence coverage for the light chain and 45% for the heavy chain and was achieved in less than five minutes. The demonstrated workflow can reduce the need for an LC system, or the resulting sample analysis can be automated using loop injection via the LC autosampler.

The Orbitrap Eclipse Tribrid mass spectrometer now enables scientists to go beyond their current capabilities and reach deeper insights into even their most complex biopharmaceutical candidates.

Cutting-edge capabilities facilitate protein structural insights in biopharmaceutical research and development

Biopharmaceutical research and development laboratories are tasked with in-depth biotherapeutic characterization, employing multiple orthogonal cutting-edge analytical workflows to accurately assess protein structural details. LC-MS-based characterization of therapeutic proteins is carried out to attain a detailed molecular knowledge of every residue and sidechain, as well as every intact protein isoform. The extreme microheterogeneity presented by advanced protein drug formats such as ADCs, bispecifics and fusion proteins is constantly pushing the boundaries of current technologies and requires a multitude of analytical approaches to be performed in parallel. It is not uncommon for a research team(s) to utilize peptide mapping for amino acid composition determination, top/middle-down sequencing to determine disulfide bridges and post-translational modifications, glycopeptide mapping with ETD tandem MS, and native intact mass analysis. Success is measured by reaching deeper more comprehensive levels of molecular characterization and ultimately ensuring the safety and efficacy of candidate drugs prior to further costly development and processing steps.

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Conclusions

To summarize, the Orbitrap Eclipse Tribrid mass spectrometer delivers:

1. Advanced performance through new and improved hardware and software features. See more than ever before.
2. Ability to overcome complex research challenges successfully with innovative instrument additions — new ionization, acquisition, and fragmentation modes enable greater structural insight. See new perspectives through unique acquisition modes.
3. Operational simplicity with predefined workflow templates, streamlined calibration, and intuitive instrument control software to easily perform complex experiments with intelligence-driven, comprehensive data acquisition and instrumental management. See more with ease.

References

1. McLuckey, S. A., Stephenson, J. L. Jr., Ion/Ion Chemistry of High-mass Multiply Charged Ions, Mass Spectrom. Reviews, 1998, 17, pp. 369-407

Find out more at **thermofisher.com/OrbitrapEclipse**

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