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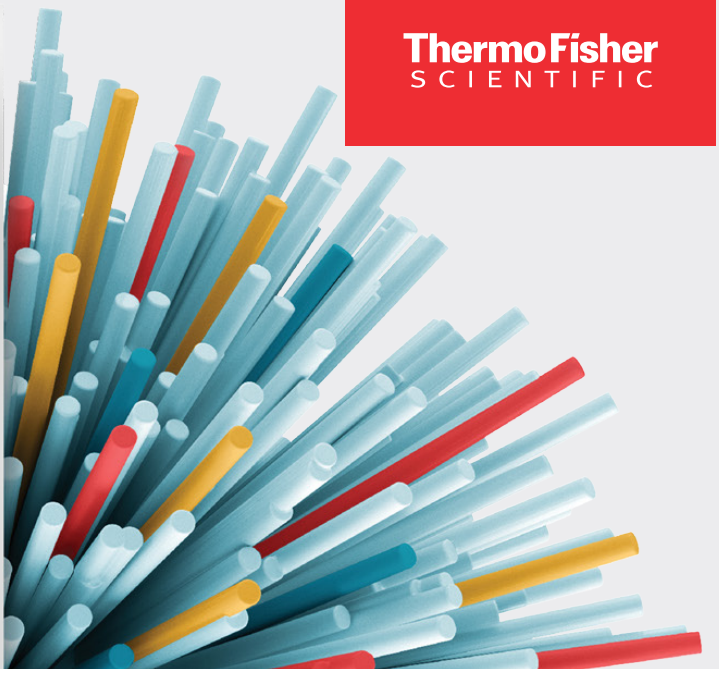

Mass spectrometry

Resolve complexity, achieve macromolecular clarity with charge-detection-enabled Orbitrap mass analyzer technology

What scientific trends are driving the need for charge-detection mass spectrometry (CDMS)?

Biological molecules of interest to the life sciences, particularly biopharmaceuticals, are becoming much larger and more complex (Figure 1). The extent of their increasing size, complexity, and heterogeneity have begun to exceed the limits of conventional ensemble-based mass spectrometry (MS) characterization by producing spectra that are refractory to deconvolution, resulting in measurements that remain in the mass-to-charge (*m/z*) domain.

The MS measurement challenges created by advances in biology are illustrated in Figure 2. MS measurement of less heterogeneous, relatively low-molecular-weight biomolecules, such as carbonic anhydrase, can have mass spectral isotopic spacing from high-resolution experiments that allow deconvolution to produce the charge state, which allows for mass determination. For relatively high-molecular-weight molecules of moderate heterogeneity, such as β-Galactosidase, charge state separation allows deconvolution and mass determination. However, for biological molecules of higher molecular weight and heterogeneity like viral particles, neither isotopic spacing nor resolved charge states are produced by conventional MS measurements, making the data uninterpretable.

Recently, characterization of these biomolecules of enormous size and complexity has become possible with the introduction of Orbitrap™ mass analyzer based charge-detection mass spectrometry (CDMS), an approach in which the mass (*m*) of each individual ion is directly determined from simultaneous measurements of both its mass-to-charge ratio (*m/z*) and charge (*z*), overcoming the limitations of deconvolution that relies on either resolution of isotopic spacing or charge-state resolution with conventional ensemble MS measurements.

Thermo Scientific™ Direct Mass Technology™ mode enables a Thermo Scientific™ Orbitrap™ mass analyzer to carry out CDMS that simultaneously measures multiple individual ions that provide high-resolution mass information for biomolecules and biotherapeutics too complex to resolve using other technologies. As a result, small changes in these molecules, for example post-translational modifications, can be revealed in exquisite detail.

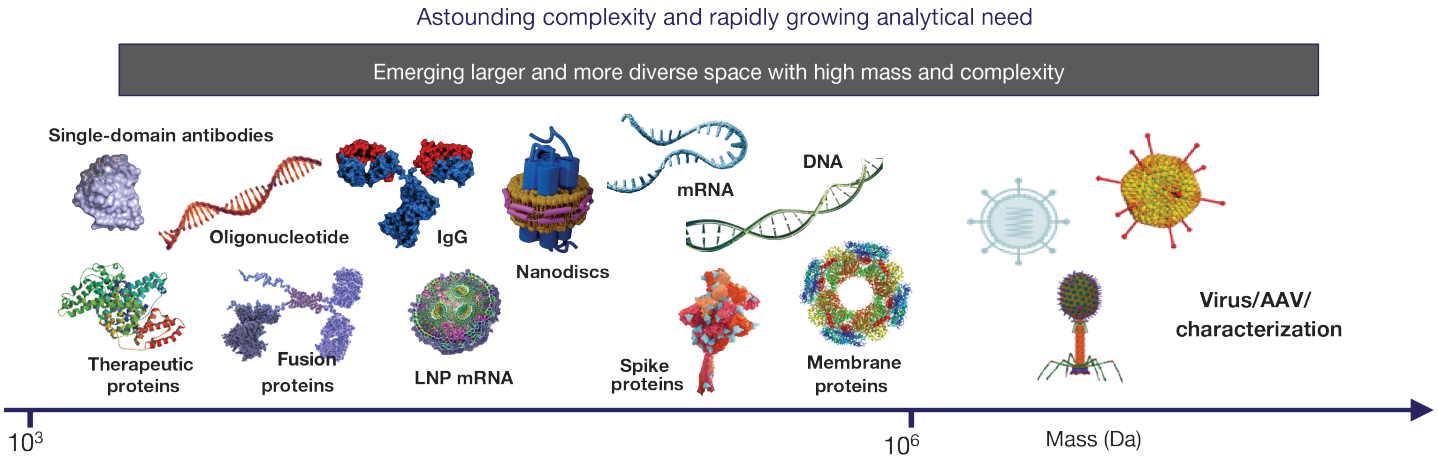

Figure 1. Increasing size and complexity of biological molecules requiring characterization

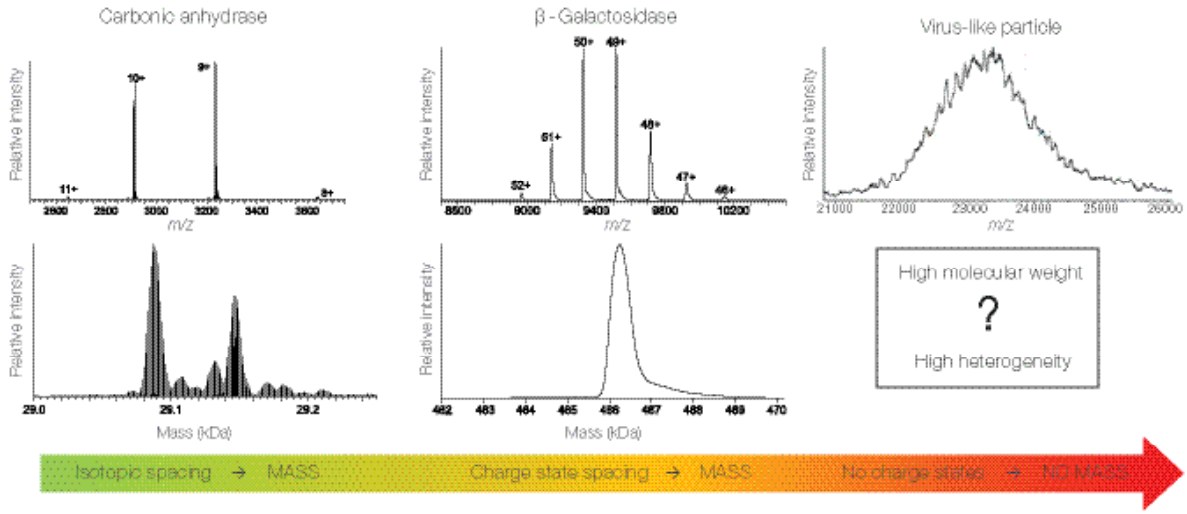

Figure 2. Advances in biology are challenging conventional MS approaches. Figure courtesy of Neil Kelleher, Northwestern Proteomics, Northwestern University.

What is the main difference between Direct Mass Technology mode and conventional ensemble MS operating modes?

Conventional ensemble MS technologies detect ionized molecules according to *m/z*, generating complex spectra obtained from thousands of ions. To determine the mass of these *m/z* signals, analysts rely on data-processing algorithms that convert *m/z* measurements to mass in a process called deconvolution. Deconvolution algorithms determine the charge, *z*, and then calculate the mass, *m*, from the *m/z* data in spectra. Deconvolution algorithms determine z from the spacing of *m/z* values in the mass spectrum; either the distance between resolved adjacent charge states or isotopes. The *m/z* value is then used to calculate m after *z* is determined. However, if the charge states or isotopes are not resolved, *z*, and subsequently *m*, cannot be determined and deconvolution algorithms fail to convert the *m/z* measurement into the mass domain (Figure 3).

To overcome the challenges of unresolved charge states and isotopes that are present in complex heterogeneous mixtures and large complexes, a Thermo Scientific™ Q Exactive™ UHMR Hybrid Quadruple-Orbitrap™ mass spectrometer equipped with Direct Mass Technology mode measures both *m/z* and *z* simultaneously for a relatively small number (approximately 25–1,000) of individual ions. Like traditional Orbitrap data acquisition, the *m/z* of the analyte ions are determined from their differing axial frequencies along the central electrode of the Orbitrap mass analyzer. Instead of inferring *z* through deconvolution of the *m/z* signal, it is directly measured. This is done by simultaneously measuring the induced charge on the outer electrodes over time for each of the hundreds of ions in the Orbitrap mass analyzer to produce Selective Temporal Overview of Resonant Ions (STORI) data (Figure 4).[1] The slope of the STORI plot for an individual ion is proportional to its charge, enabling the direct measurement of *z*. Thus, measurement of both *m/z* and *z* for each individual ion enables direct determination of m (*m/z* x *z* = *m*), omitting the traditional need for deconvolution that relies on resolved charge states or isotopes.

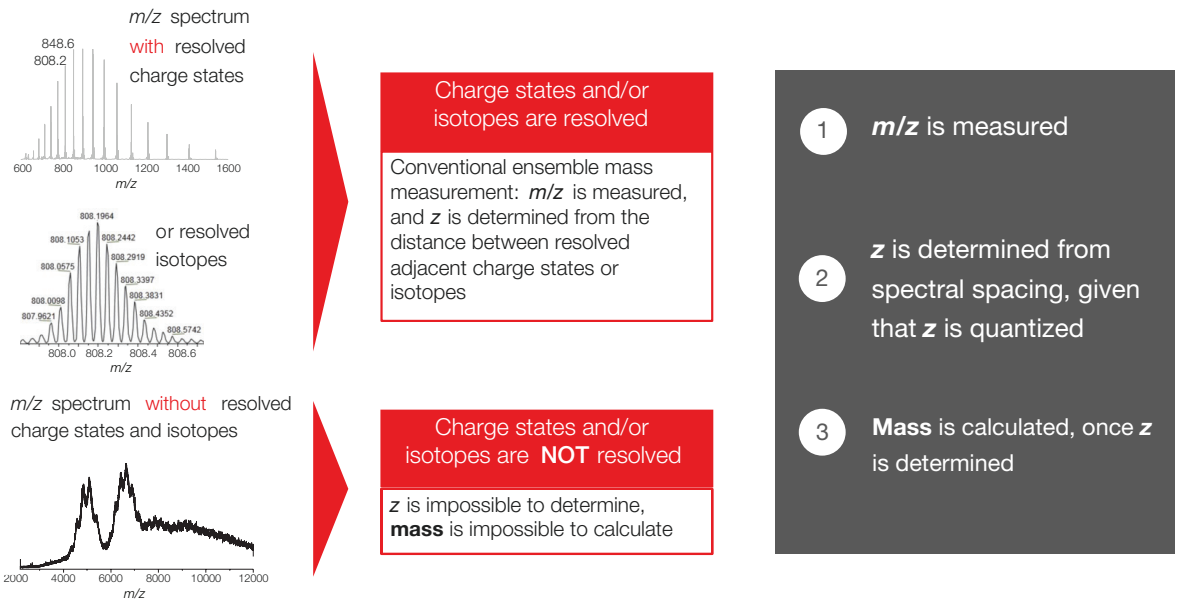

Figure 3. Deconvolution of *m/z* spectrum with resolved charge states or isotopes yields a mass-to-charge spectrum with traditional ensemble measurements. However, without resolved charge states or isotopes, it is impossible to determine the charge, *z*, and, therefore impossible to calculate the mass.

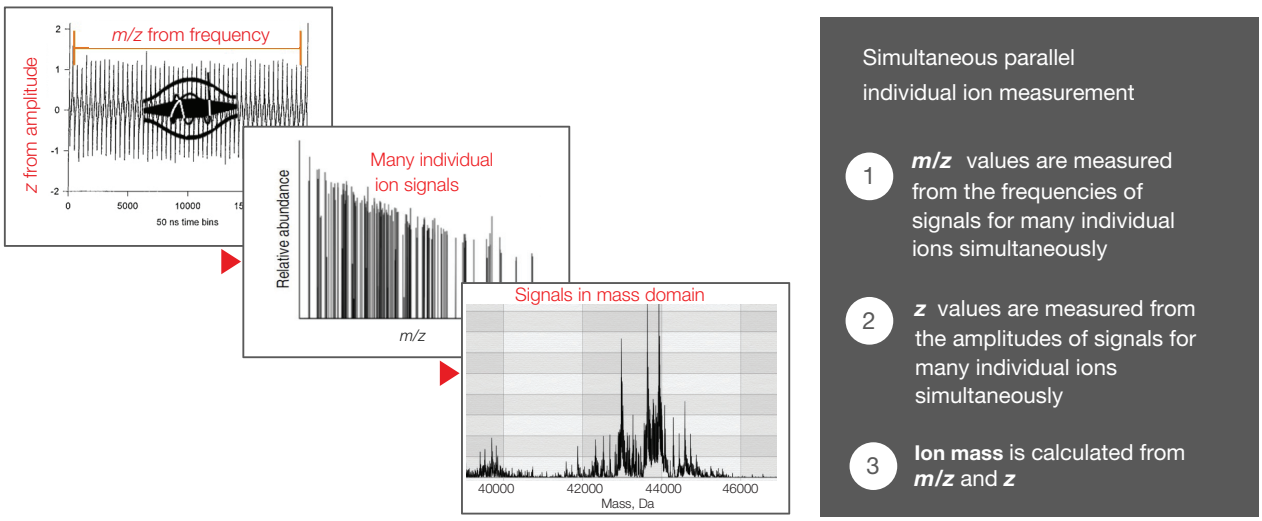

Figure 4. Charge detection mass spectrometry with Direct Mass Technology mode simultaneously measures *m/z* and *z* to enable calculation of individual ion masses without the need for deconvolution via resolved isotopes or charge states.

What is the Direct Mass Technology mode data acquisition and processing workflow?

The Direct Mass Technology mode workflow for acquiring, processing, and visualizing data is carried out by the instrument control software in the steps shown in Figure 5. The workflow, developed and described by Kafader, et al.,[1,2] adds extra dimensionality to Orbitrap measurements with parallel individual ion measurements that produce high-resolution results directly in the mass domain.

Step 1. The Orbitrap mass analyzer collects the abundance and frequency (*m/z*) of hundreds of individual ions in parallel, for each spectrum.

Step 2. The integrated signal for each ion as a function of acquisition time is plotted using the process developed and described by Kafader, et al.[2] called the Selective Temporal Overview of Resonant Ions (STORI). STORI plots sum the amplitude read-back present in a time-domain signal at a specific frequency corresponding to a single ion peak in the *m/z* spectrum. Thus, STORI plots provide the framework to accurately analyze low-level time-domain signals of individual ions. STORI plots also allow for correction of intermittent signals, the differentiation of single and multiple ions at the same frequency, and the association of signals that spontaneously change frequency. The slope of the STORI plot of an individual ion is proportional to its *z*. To determine *z*, the slope of the STORI plot for each individual ion is compared to a charge calibration curve.

Step 3. A high-resolution mass spectrum is generated from the mass of each individual ion using the *m/z* and *z* information generated by the STORI analysis and calibration.

What are the advantages of Direct Mass Technology mode?

Mass measurements now match the scale of biological molecules of interest

Direct Mass Technology mode provides direct *m/z* measurements up to 80,000 and direct *m* measurements in the megadalton range, increasing limits of the addressable molecule size up to tenfold. Using a CDMS-enabled Oribitrap instrument, McGee et al. have obtained isotopic resolution of protein complexes up to 466 kDa.[3] Accurate direct mass determination eliminates challenges of *m/z*-based deconvolution of large, complex analytes.

Enhanced resolution addresses increasingly complex analytes

Compared to traditional ensemble measurements at the same resolution settings, individual ion measurement provides as much as a twenty-fold increase in resolution for large and complex analytes (Figure 6). The most important reason for this effect, explained by Kafader, et al.,[4] is that Direct Mass Technology mode measures independent, non-adjacent isotope ions, separated in different acquisition events, allowing calculation of more accurate centroided mass values. Increased resolution enhances tolerance of complex analytes by transforming previously uninterpretable spectra into useful mass information. In addition, the notably clean baselines of the resulting spectra serve to extend the dynamic range for intact mass determination even when complexity is high.[4]

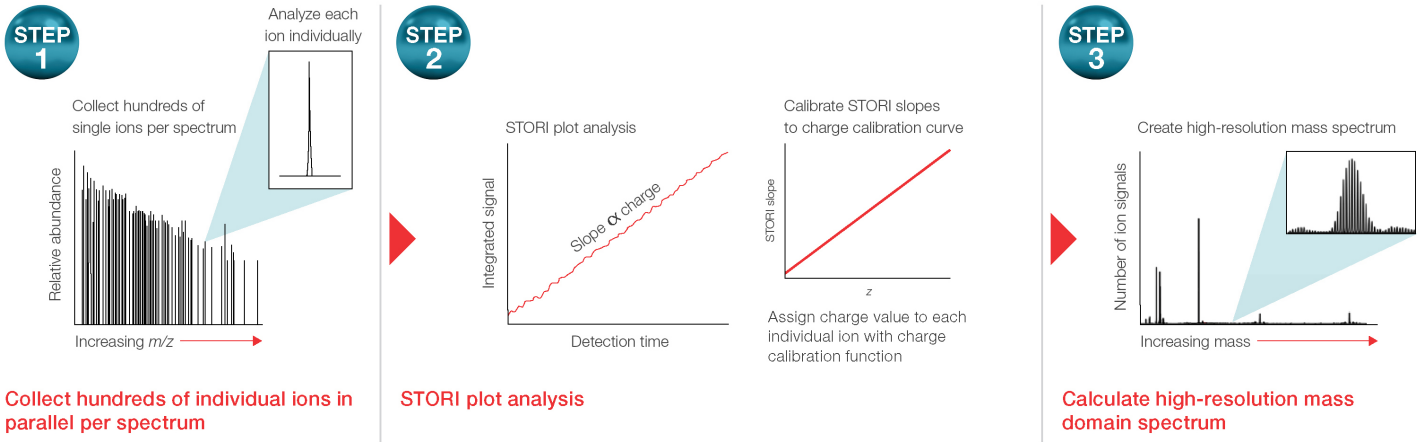

Figure 5. Direct Mass Technology mode data workflow

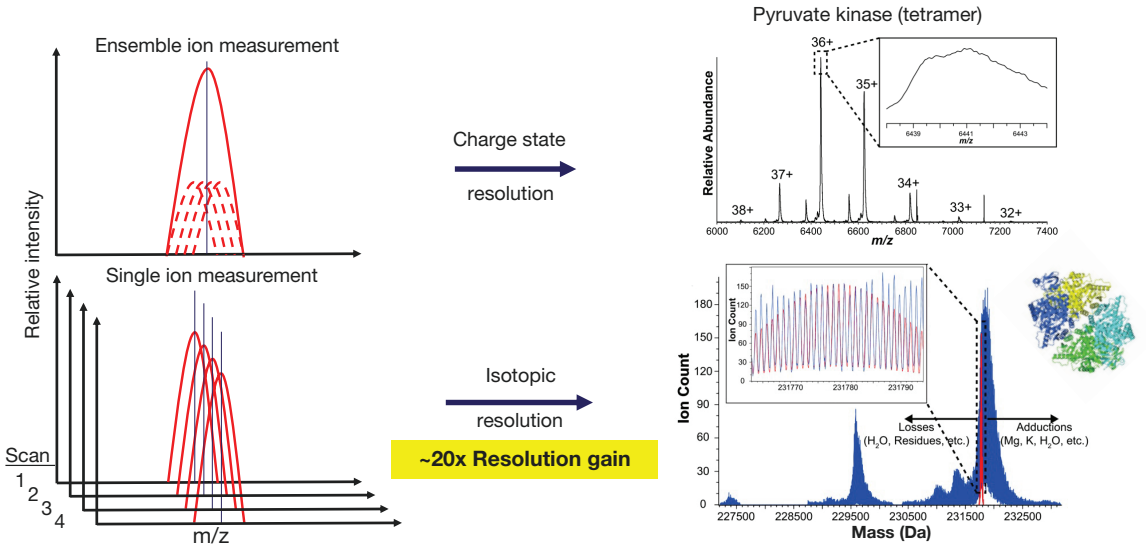

Figure 6. Ensemble measurement of overlapping isotope ions only allows for charge state resolution. Direct Mass Technology mode measures individual ions, allowing for calculation of individual centroided mass values.[4] (Reprinted with permission from "Measurement of individual ions sharply increases the resolution of Orbitrap mass spectra of proteins", © 2019, American Chemical Society.)

Requires less sample or less-concentrated samples

Due to the low ion counts required to perform individual ion measurements, Direct Mass Technology mode offers remarkable sensitivity. It is now possible to collect substantially more meaningful data from significantly less (up to 500 times less) concentrated samples, allowing hundreds-of-fold dilution and single-run characterization to conserve precious sample.

How is Orbitrap mass analyzer technology uniquely suited to CDMS?

Ion transfer and utilization throughout an Orbitrap instrument are highly efficient, enhancing sensitivity such that individual ions can be detected. Most fundamentally though, the Orbitrap mass analyzer itself can trap and separate small numbers of ions and has been shown to be sensitive enough to detect single multiply charged ions.[4-6] Additionally, the Orbitrap mass analyzer design enables reliable assignment of the integer charge states that are needed for CDMS.[1] As a result, the *m/z* and z of thousands of single ions can be measured directly in minutes, yielding the mass information needed to accelerate characterization of large biomolecules.

Orbitrap mass analyzer technology provides additional advantages for CDMS experiments. In particular, Thermo Scientific™ Hybrid Quadrupole-Orbitrap™ mass spectrometer technology equipped with ultra-high mass range (UHMR) provides a fit-for-purpose combination of unprecedented ultra-high *m/z* range of up to 80,000, in-source trapping, high sensitivity, high resolution, and MS^2 capability, which can all be utilized for Direct Mass Technology mode experiments.

The intrinsic trapping capability of the Orbitrap mass analyzer allows ion source processes to be decoupled from downstream mass analysis, enhancing experimental performance and flexibility. The in-source trapping capability of the Thermo Scientific™ Q Exactive™ UHMR Hybrid Quadrupole-Orbitrap™ mass spectrometer allows users to optimize desolvation of large ions, while also allowing application of energy to dissociate ions by in-source collision-induced dissociation (CID) or higher-energy collisional dissociation (HCD) prior to mass analysis. By increasing the in-source trapping energy and applying high mass quadrupole isolation of up to *m/z* 25,000, the user can release protein subunits for top-down sequencing by HCD. Alternatively, with gentle activation, the user can retain membrane proteins bound to multiple ligands allowing for whole-complex analysis.

To attain the STORI plots for charge (*z*) determination, ions must remain in orbit for long periods of time. The vacuum manifold has been optimized by creating differential vacuum regions and precise pressure regulation to allow the single ions to stay in orbit for higher resolutions (200,000 resolving power at *m/z* 400). Users can control the pressure setting that is needed for optimal transmission of their molecules of interest using the pressure control in the HCD cell. Lower pressures reduce collisions between neutral gas molecules and analytes of interest. The ions must survive a certain amount of time for the STORI plot analysis. Higher vacuum pressure allows for larger macromolecules to better disperse their kinetic energy by collisions with neutral background gases before being packeted for injection into the Orbitrap analyzer. By utilizing the pressure regulation in the HCD cell, we can achieve better ion retention and sensitivity for all scan types, but especially for large, complex analyses in which Orbitrap-based CDMS is utilized.

How is Direct Mass Technology mode implemented on an Orbitrap mass spectrometer?

Direct Mass Technology mode provides a complete workflow for acquiring, processing, and analyzing CDMS data collected using an Orbitrap instrument. An addition to the standard instrument control software, Direct Mass Technology mode can be toggled on or off, allowing the Orbitrap instrument to seamlessly operate in either CDMS or standard mode, expanding experimental flexibility. When turned on, Direct Mass Technology mode embeds CDMS data for STORI analysis in the acquired raw (.raw) file automatically and allows the user to utilize an automated ion population control procedure called automatic ion control (AIC) to maximize throughput and signal quality for parallel individual ion measurements.

STORIboard software, developed by Thermo Fisher Scientific in collaboration with Proteinaceous, Inc., is used to process and visualize CDMS data collected by Direct Mass Technology mode in an easy-to-use interface. The software's capabilities include charge calibration curve creation via an optimized charge calibration algorithm, STORI plot analysis, assignment of charge values to individual ions using the charge calibration, and generation of high-resolution centroided mass spectra. CDMS data visualization, interpretation, and method optimization features include heat map visualization of STORI plot slopes to differentiate analytes with the same *m/z* but different charge states, and mirror plots for spectral comparisons. The software also provides multiple processing templates for different types of analytes, customization of those templates that can be saved, easy import of multiple file types, and storage, as well as management of charge calibration files. One or many data files can be processed using a user-selected processing template and calibration file, and data can be filtered using multiple customizable parameters. An overview of STORIboard software capabilities is presented in Figure 7.

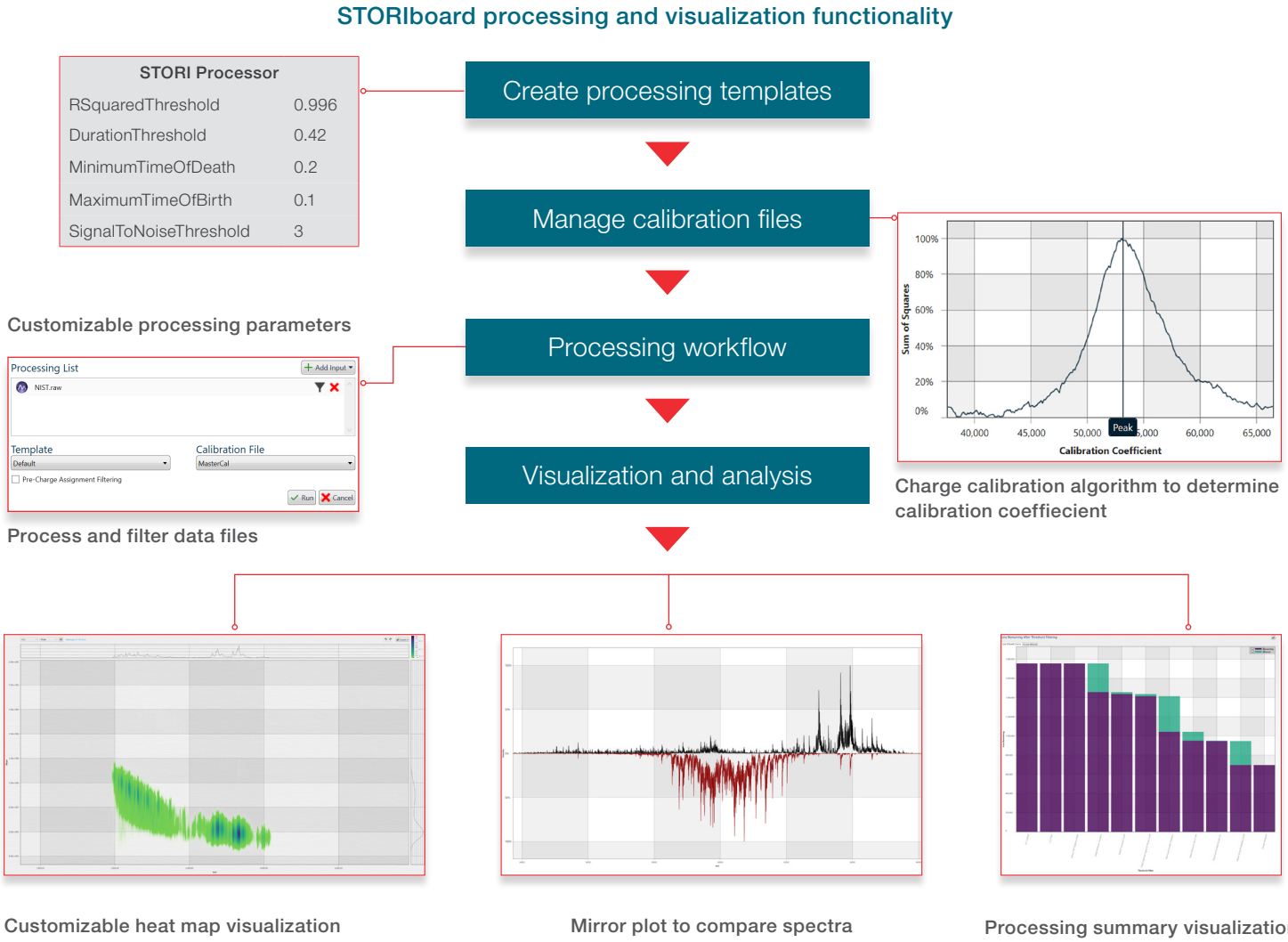

Figure 7. STORIboard software overview

What application challenges does Direct Mass Technology mode address?

A charge-detection-enabled Orbitrap mass spectrometer can unravel complexity and unlock new insights into proteoforms, biotherapeutics, and next-generation drug modalities. With traditional ensemble MS measurements of only *m/z*, the overlap in charge-state distributions caused by residual solvation, ionic adducts, and post-translational modifications results in a degree of complexity that often yields spectra that are intractable to deconvolution. Direct Mass Technology mode overcomes these challenges, enabling detailed characterization of membrane proteins, large noncovalent protein complexes, glycoproteins, viral and extremely heterogeneous protein- and non-protein-based DNA and RNA therapeutics, with enhanced resolution, sensitivity, and dynamic range.

Reveals proteoforms in unprecedented detail

Glycosylation of proteins is a key post-translational modification (PTM) involved in many physiological functions and in the progression of certain diseases. Due to heterogeneity, glycoprotein characterization using traditional MS methods has challenged researchers with extremely complex spectra. Additionally, glycoform detection by native MS has been limited by the ability to differentiate overlapping charge state envelopes within a complex sample, especially for low abundance species.

Direct Mass Technology mode unlocks new possibilities by adding previously unachievable resolution and dynamic range, enabling accurate, efficient, and detailed identification of glycoforms.

The complexity of glycoproteins can be clearly conceptualized using human fetuin-A (Figure 8), a heterodimeric protein composed of A and B chains connected by a disulfide bond weighing approximately 37 kDa without modifications. Increased levels of this protein have been linked to higher risk of cardiovascular disease and Type 2 diabetes,[7] making its precise characterization a pressing challenge. The heterogeneity of native fetuin-A is due to its two N-glycosylation sites, up to seven O-glycosylation sites, and seven phosphorylation sites. Six disulfide bonds and sequence variants further complicate analysis. Using the Q Exactive UHMR mass spectrometer powered by Direct Mass Technology mode, a more comprehensive glycoform profile for intact fetuin-A can now be obtained. As seen in Figure 8, we nearly double the number of detectable proteoforms, including higher molecular weight glycoforms otherwise completely uninterpretable by conventional MS analysis highlighted in the red boxes and labeled. Being able to precisely map the presence of these species may help researchers fully understand the role this glycoprotein plays in human disease.

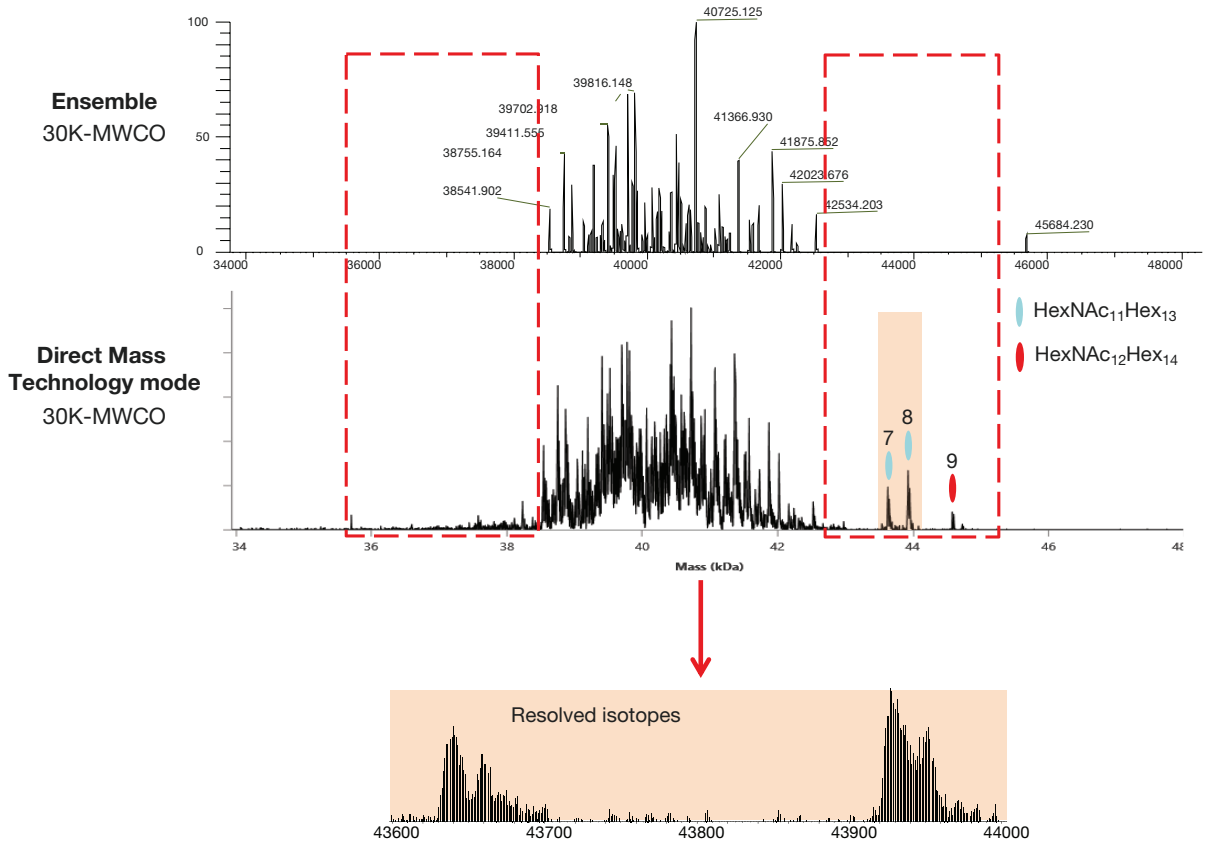

Figure 8. Deconvoluted spectra of human fetuin-A from traditional ensemble MS data (top) and from scans obtained using the new Direct Mass Technology mode (bottom). High molecular weight glycoforms only detected in Direct Mass Technology mode scans are highlighted in tan.

Recent publications have also described how a CDMS-enabled Orbitrap mass spectrometer can be applied to protein characterization to substantially improve resolution of proteoforms. For example, Kafader et al. described the use of CDMS to reveal various modifications of transferrin (~80 kDa) and to identify possible water-loss products of NIST-antibody (Ab).[4] Multiple theoretical isotope distributions can be superimposed on the experimental spectrum to facilitate identification of adducts and degradation products.

Kafader et al. also described CDMS-enabled Orbitrap mass-spectrometer-based characterization of highly complex mixtures of proteoforms and their complexes under both denatured and native conditions, revealing details not observable by conventional MS.[1] To explore the limits of Orbitrap-based CDMS, high-complexity samples including the entire <30 kDa range of the human proteome, obtained from HEK 293T cells fractionated by gel-eluted liquid fraction entrapment electrophoresis (GELFrEE), were analyzed using both traditional MS and CDMS. Although no protein masses were determined using traditional MS, CDMS detected over 500 proteoform masses that were assigned using intact-mass tagging by referencing liquid chromatography–tandem mass spectrometry (LC–MS/MS) top-down proteomics data available in the Human Proteoform Atlas.

In another publication, Kafader et al. described Orbitrap-based CDMS analysis of a mixture of heavy and light chains produced by the disulfide reduction of an IgG_1.[8] CDMS spectra provided sensitive adduct identification that was not possible using ensemble measurements. Various forms of the heavy- and light-chain species, including guanidine adducts and glycosylation, were distinguished using CDMS. The authors noted that the CDMS results simplified data analysis, even for non-experts.

Increases sequence coverage for top-down protein characterization

Though CDMS has mainly been applied to the determination of the masses of intact molecules, Kafader et al. extended the technique to top-down MS^2-level proteoform characterization using what the authors termed "individual ion MS/MS analysis."[9] Because Direct Mass Technology mode is a software add-on for the Q Exactive UHMR Orbitrap mass spectrometer, all of its features, including dissociation techniques such as HCD and in-source CID, can be deployed during CDMS analysis. The authors isolated multiple ions by *m/z* using the quadrupole, fragmented them in the HCD cell, measured the individual fragment ion signals in the Orbitrap mass analyzer to accurately determine their charge, and then created a mass spectrum for each fragment. In this manner, the CDMS-enabled Orbitrap mass spectrometer identified low-abundance fragment ions containing many hundreds of residues that were undetectable by traditional MS, doubling the sequence coverage for triosephosphate isomerase when combining the results of the CDMS data with traditional ensemble data. The authors determined that addition of CDMS to MS/MS analysis substantially improved high-mass fragment ion detection and provided information complementary to conventional top-down results, thus allowing more complete proteoform characterization.

Ultrasensitive analysis of heterogeneous protein assemblies

Wörner et al. demonstrated the value of single-particle CDMS-enabled Orbitrap mass-spectrometer-based analysis of megadalton biomolecular assemblies ranging from 150 kDa to 9.4 MDa.[10] The authors were able to resolve mixed ion populations to determine the masses of individual ions. This enabled ultrasensitive mass analysis of heterogeneous protein assemblies such as immunoglobulin oligomers, ribosomes, protein nanocontainers, and genome-packed adeno-associated viruses. The exact mass of an intact macromolecular complex can be used to infer the subunit composition and the stoichiometry, post-translational modifications and ligands bound to the complex.

Rapid characterization of virus-like particles and DNA/RNA-based therapeutics

Kafader et al. explored CDMS-enabled Orbitrap mass spectrometer analysis of wild-type (WT) and mutant (MINI) versions of virus-like particles (VLPs) that were engineered from viral capsids carrying varying amounts of DNA and mRNA cargo.[1,8] Although the structure of the VLPs is understood, characterization of cargo loading and stoichiometry of therapeutic proteins or RNA would be highly valuable. CDMS data for both WT and MINI VLPs were compared with conventionally acquired MS data. Presumably due to the heterogeneity of the varying lengths of DNA and mRNA, MS analysis did not reveal resolved states, and thus no mass information, for either type of VLP. In contrast, mass distributions of 3190 ± 38 kDa and 990 ± 16 kDa were obtained for the WT and MINI VLPs using CDMS. The authors determined that CDMS is applicable to VLP engineering and could ultimately be used to characterize other DNA/RNA-based therapeutic systems including Qβ virions, adeno-associated virus (AAV), and hepatitis B.

AAVs are important gene therapy vectors with several approved clinical applications and many more progressing through clinical trials.[11] Genome packaging is a key step in AVV bioprocessing that must be monitored to ensure the proper delivery of transgenes to produce safe and effective therapeutics. However, only a fraction of the total AAV particles generated by a production system contain the desired genome. Current methods to monitor genome packaging provide limited sensitivity, are labor intensive, and struggle to distinguish between the intended packaging and unwanted side-products. Analytical sensitivity is

of particular concern because most laboratory-scale preparations of recombinant AAVs (rAAVs) yield no more than a few milliliters of purified samples (equivalent to 0.1–10 mL at about 10^{13} viral capsids per milliliter).

The need for a robust analytical technique that can distinguish accurate and comprehensive molecular weight (MW) profiles, empty/full ratios of transgenes, and charge state distributions between the different biologics is imperative for our ability to characterize and regulate these AAV-based biologics. Since AAVs tend to be above 1 MDa in mass with over 100 charges, the ability of CDMS to convert complex and high charge species into the mass domain is uniquely suited for this challenge. Mietzsch, et al. explain why using Direct Mass Technology mode and a newly developed standard AAV1-VP3-only capsid complex allows for more accurate determination of biologics for regulatory bodies and in the development of new drugs.[10,11]

In a recent publication, Ryan et al. demonstrated how STORI-based processing of CDMS data provides comprehensive information on AAV samples. Improved peak shape and mass resolution enhanced the ability of the method to detect and resolve empty and filled AAV capsids in the same sample with 3-15 min acquisition times. In addition, the study demonstrated the ability to discern between partial and over filled capsids, indicative of the method's suitability for rapidly monitoring the integrity and amount of genome-packed AAV particles.

The use of the above described standard,[11] the sensitivity, accuracy, and resolution of CDMS-enabled Orbitrap mass spectra demonstrated on several AAV serotypes,[12] show how Direct Mass Technology mode on the Q Exactive UHMR platform allows scientists and regulators to gain vital insights and information on their biotherapeutic samples.

Summary

Direct Mass Technology mode using Orbitrap mass analyzer technology on the Q Exactive UHMR platform enables scientists to carry out CDMS with parallel individual ion measurements that provide high-resolution mass information for protein complexes and biotherapeutics that are too complex to characterize using other technologies. The applications using this technology are varied and include, but are not limited to, the characterization of membrane proteins, large non-covalent protein complexes, glycoproteins, viral and extremely heterogeneous protein- and nonprotein-based DNA and RNA therapeutics, and even top-down protein fragmentation spectra with enhanced resolution, sensitivity, and dynamic range. The ability to gain comprehensive MW profiles and charge distribution of these species using CDMS-enabled Orbitrap mass spectrometry begets a future in which biotherapeutics are truly and completely characterized.

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