Advances in Mass Spectrometry Based Glycoproteomics and Glycomics Workflows

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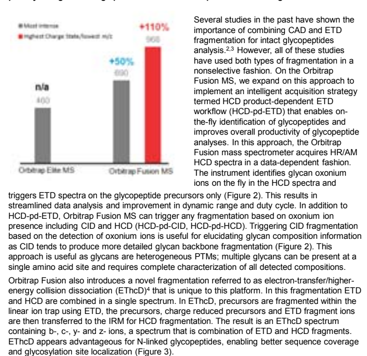
Glycoproteomics

Proteomics has benefited tremendously from collisional-activated dissociation (CAD) as this fragmentation technique generates abundant peptide bond cleavages resulting in large number of peptides identifications. However, CAD is not ideal for glycopeptides analysis as this fragmentation does not produce the desired peptide backbone cleavages for sequencing. Low energy CAD predominantly fragments the glycan on a glycopeptide rather than the peptide, generating spectra that are dominated by glycosidic bond cleavages rather than the desired peptide bond cleavages. Further complicating the issue is the cleavage of the peptide-glycan bond, resulting in the loss of information about glycosylation site. The increased collision energy on CAD can generate some peptide back bone fragmentation, but it can also generate mixed MS/MS spectrum where both glycan and peptide information are present making structural interpretation complicated. Regardless of whether high- or low-energy CAD is employed, fragmentation of the peptide-glycan bond still occurs limiting the ability to derive information about the site of glycosylation. To circumvent these issues, majority of researchers have abandoned the strategy of dealing with intact glycopeptides structures and trying to get complete information about the glycopeptide. Instead researchers are trying to obtain partial information, such as sequencing backbone of the peptide and identifying glycosylation sites. In this strategy, glycopeptides are treated with enzymes such as PNGase F/A resulting in the removal of glycans, the site of glycosylation asparagine is converted to aspartic acid during this process. By using high resolution mass spectrometers, researchers can identify the site of glycosylation due to the mass shift of 0.9840 Da. However, no information about the glycan structure is obtained due to the removal of the glycan prior to MS analysis. Additionally, the process of monitoring chemical deamidation for identifying glycopeptides leads to large number of false positives as studies have shown that chemical deamidation can occur during sample preparation and not due to the release of glycans. These issues highlight the importance of intact glycopeptide analyses and the search for alternative methods. Electron-transfer dissociation (ETD)[1] is a fragmentation techniques that is far better suited for glycopeptide analyses due to their nonergodic type of dissociation. ETD produces extensive fragmentation of the peptide backbone enabling sequencing of the peptide while preserving glycans on the peptide backbone. This allows for unambiguous assignment of the glycosylation sites. The Orbitrap Fusion mass spectrometer has multiple fragmentation techniques available such as CID, HCD and the much desired ETD for glycoproteomics. The ETD ion source used in Orbitrap Fusion MS is based on Townsend discharge ion source which generates a highly stable reagent ion flux with minimal user input for optimization and tuning as was required on previous ETD sources. Additionally, the Orbitrap Fusion has been implemented with intelligent, automated precursor ion sorting routines, reagent filtering using the quadrupole mass filter, and charge-state-specific calibration of ETD reaction times that maximize the quality of ETD spectra and increase the number of glycopeptides identified compared to previous generation mass spectrometers (Figure 1).

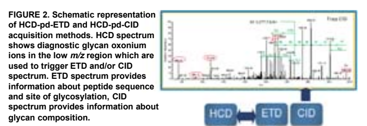
FIGURE 1. Comparison of Orbitrap Elite MS to Orbitrap Fusion MS for the identification of human serum glycopeptides. Orbitrap Elite MS selects precursors based on intensity while Orbitrap Fusion MS can acquire data with intelligent precursor selection giving priority to highest charge precursors which are optimal for ETD fragmentation.



Several studies in the past have shown the importance of combining CAD and ETD fragmentation for intact glycopeptides analysis.[2,3] However, all of these studies have used both types of fragmentation in a nonselective fashion. On the Orbitrap Fusion MS, we expand on this approach to implement an intelligent acquisition strategy termed HCD product-dependent ETD workflow (HCD-pd-ETD) that enables on-the-fly identification of glycopeptides and improves overall productivity of glycopeptide analyses. In this approach, the Orbitrap Fusion mass spectrometer acquires HR/AM HCD spectra in a data-dependent fashion. The instrument identifies glycan oxonium ions on the fly in the HCD spectra and triggers ETD spectra on the glycopeptide precursors only (Figure 2). This results in streamlined data analysis and improvement in dynamic range and duty cycle. In addition to HCD-pd-ETD, Orbitrap Fusion MS can trigger any fragmentation based on oxonium ion presence including CID and HCD (HCD-pd-CID, HCD-pd-HCD). Triggering CID fragmentation based on the detection of oxonium ions is useful for elucidating glycan composition information as CID tends to produce more detailed glycan backbone fragmentation (Figure 2). This approach is useful as glycans are heterogeneous PTMs; multiple glycans can be present at a single amino acid site and requires complete characterization of all detected compositions.

Orbitrap Fusion also introduces a novel fragmentation referred to as electron-transfer/higher-energy collision dissociation (EThcD)[4] that is unique to this platform. In this fragmentation ETD and HCD are combined in a single spectrum. In EThcD, precursors are fragmented within the linear ion trap using ETD, the precursors, charge reduced precursors and ETD fragment ions are then transferred to the IRM for HCD fragmentation. The result is an EThcD spectrum containing b-, c-, y- and z- ions, a spectrum that is combination of ETD and HCD fragments. EThcD appears advantageous for N-linked glycopeptides, enabling better sequence coverage and glycosylation site localization (Figure 3).

FIGURE 2. Schematic representation of HCD-pd-ETD and HCD-pd-CID acquisition methods. HCD spectrum shows diagnostic glycan oxonium ions in the low *m/z* region which are used to trigger ETD and/or CID spectrum. ETD spectrum provides information about peptide sequence and site of glycosylation, CID spectrum provides information about glycan composition.



Data courtesy of Sz-Wei Wu and Kay-Hooi Khoo, Academia Sinica

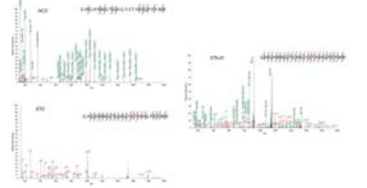
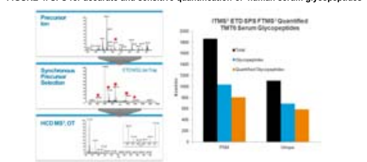


FIGURE 3. Comparison of HCD, ETD and EThcD spectra acquired on Orbitrap Fusion for a tryptically digested glycopeptide from Hemopexin protein.

To understand the functions of individual proteins and their place in complex biological systems, it is often necessary to measure changes in protein abundance relative to changes in the state of the system. Discovery-based relative quantification allows the determination of relative protein abundance changes across a set of samples simultaneously and without the requirement for prior knowledge of the proteins involved. Isobaric chemical tagging approach (TMT, iTRAQ) is popular for relative quantitation. In a single analysis, they can be used to identify and quantify relative changes in complex protein samples across multiple experimental conditions. Unfortunately, this approach in the past could not be applied to glycoproteomics. In the case of ETD, the reporter fragments are often uncharged and cannot be detected within the spectra. In HCD, generation of reporter ions is limited as primary fragmentation occurs for glycosidic not peptide bonds for glycopeptides. The implementation of synchronous precursor selection (SPS) exclusive to the Orbitrap Fusion MS overcomes these limitations.[5] In this approach, the parent ion is selected in MS^1, isolated in the quadrople and fragment by ETD in the ion trap. Upon fragmentation, multiple MS^2 fragment ions are selected and isolated using single trap fill and waveform (synchronous precursor selection). Up to 20 fragments can be isolated simultaneously. Selected MS^2 fragment ions are transferred back into the IRM and HCD fragmentation is performed. MS^3 fragments are detected in the Orbitrap for the most accurate MS^3 based quantitation. Using this approach, multiplexed quantification of glycopeptides in a variety of biological samples is possible and only possible on the Orbitrap Fusion (Figure 4).

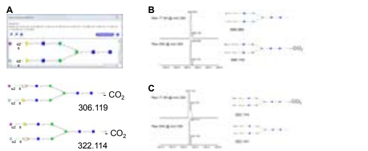
FIGURE 4. SPS for accurate and sensitive quantification of human serum glycopeptides



Glycomics

Glycan analysis requires characterization of the sugar sequence, branching, linkages between monosaccharide units, anomeric configuration, and the location of possible substituents. MS can provide information about glycan sequence, branching patterns, location of possible substituents and can be quantitative. One of the key mass spectrometer requirements for successful glycan analysis is the ability to generate useful fragment ions for structural elucidation. Unlike in proteomics, where peptide/protein identification can be performed from partial fragmentation of the peptide backbone owing to well defined protein databases and linearity of the peptide structure, glycan analysis requires as much fragmentation of the glycan backbone as possible. As monosaccharides can be linked to form larger structures in many branching patterns, this diversity in linkages often leads to many possible isomers which are structurally and biologically different. To fully characterize the glycan sample present, the MS must generate maximal backbone cleavages for structural elucidation. The Orbitrap Fusion has multiple fragmentation CID, HCD, and ETD. Each of these fragmentation techniques can be performed at any stage of MS^n, with detection of the fragment ions in either the ion trap or Orbitrap mass analyzer. Each of the fragmentation techniques provide unique advantages for glycan structural elucidation. Performing HCD fragmentation with high resolution and accurate mass (HR/AM) fragment ion detection in the Orbitrap mass analyzer on the Orbitrap Fusion allows for differentiation of near mass fragment ions which is required for accurate assignment of branching and linkages (Figure 5). Altering the HCD collision energy on the Orbitrap Fusion MS can provide different types of fragment ions within a glycan to allow further characterization. Lower collision energy produces primarily glycosidic fragments while higher collision energy produces cross ring and internal double cleavage for the determination of branching and linkage and for the resolution of isobaric structures. Use of step collision energy (SCE), where a given precursor is fragmented with multiple HCD collision energies and scanned out in a single mass spectrum, ensures maximal detection of all types of fragments at the fastest rate.

FIGURE 5. A) A glycan that has α2-6 linked NeuAc and α2-6 linked NeuGc. α2-6 linked NeuAc produces diagnostic fragment ion $^{0,4}A_2 - CO_2$ at *m/z* 306.119 while α2-6 linked NeuGc produces diagnostic fragment ion $^{0,4}A_2 - CO_2$ at *m/z* 322.114. These can be used as signatures to figure out the type of sialic acid linkages that are occurring.[6] B) Zoomed in region of the MS/MS spectrum acquired under different resolutions where the diagnostic ions appear. Here, α2-6 linked NeuAc produces diagnostic fragment ion $^{0,2}A_4 - CO_2$ at *m/z* 306.119. But from the same glycan, the loss of NeuGc results in an ion 306.083. In the zoomed region for MS/MS spectra, the 17.5 K, resolution does not fully resolve these two ions, while 30 K does. C) For the same glycan, NeuGc produces diagnostic fragment ion $^{0,2}A4 - CO_2$ at *m/z* 322.114, while the Z_1 ion (loss of GlcNAc-2-AB) produces fragment at 322.141. Again it takes a resolution of 30 K to fully resolve the ions.



Detailed glycan structural elucidation can be performed by combining permethylation with CID MS^n. This enables identification of branching, linkages and resolution of isobaric structures which are otherwise indistinguishable in MS^2 spectra. Traditionally, MS^n has been restricted to CID in linear ion trap mass spectrometers. In glycan analysis, CID fragmentation produces fewer fragment ions requiring multiple stages of fragmentation (MS^6, MS^7…) for structural elucidation. The primary advantage of HCD fragmentation is the production of glycosidic, cross-ring, and internal double cleavage ions at the MS^2 level, where branching, linkage and resolution of isobaric structures are derived from the latter two types of ions (Figure 6). The availability of HCD within the Orbitrap Fusion MS for fragmentation at MS^3 and higher levels enables comprehensive glycan structural elucidation at much lower MS^n stages. Both CID and HCD fragmentation can be used at any level of MS^n uniquely on the Orbitrap Fusion MS. Though glycan analysis can be done by direct infusion into the mass spectrometer, separation prior to MS analysis can provide benefits. Primarily, separation can reduce sample complexity, minimize ion suppression, increase dynamic range and provide separation of structural isomers. In the past, due to insufficient LC separation, chromatography profiles contained only a small number of separated peaks, where each peak (abundant) contained many glycans (contribution from multiple isomers). As the separated peaks were very abundant most commercial mass spectrometers were fast enough to obtain MS/MS spectra on a LC time scale, however, their coverage and separation were low. With the introduction of new mixed mode columns that have the ability to separate structural isomers, thereby increasing the number of separated peaks, the speed of the mass spectrometer on an LC-timescale becomes essential. Additionally, the range of peak abundance varies across the structural isomers introducing a wide dynamic range for detection for these glycans. The mass spectrometers must generate good quality MS/MS data for high and low abundance peaks at a scan rate amenable to LC separations. Instruments that are fast, sensitive, and have wide dynamic range are essential for glycomics. With the Orbitrap Fusion MS, ions are first collected in the Ion Routing Multipole (IRM) before being shuttled to the mass analyzer of choice (Orbitrap or dual cell linear ion trap). During mass analysis, the next ion of interest is accumulated in the IRM increasing the productivity of the system and ultimately enabling faster scan rate which is amenable to the LC separation described here (18 Hz MS/MS at 15,000 resolving power at m/z 200). Additionally, the new functionality allows for sensitive detection of HCD fragments in the Orbitrap or ion trap over wide mass and dynamic ranges, providing essential information for structural elucidation of glycans. Figure 7 shows high quality MS/MS spectra generated for both abundant and low abundant glycans. Due to the Orbitrap Fusion mass spectrometer's unique configuration, the Orbitrap mass analysis can be extensively parallelized with operation of the mass selecting quadrupole, the IRM (used for both accumulating ions and for HCD fragmentation) and the ion trap. This allows for maximal concurrent ion manipulations and mass analyses, increasing both scan rates and duty cycle of the instrument. Having fast HR/AM full scans along with fast MS/MS detection are essential for achieving enough scans across the LC peak widths for full scan quantitation and MS/MS for confirmation of identity. Figure 8 shows the separation and identification of 2 glycan structural isomers. The Orbitrap Fusion MS is fast enough to maintain more than enough full scans (>16 scans) across the peak for precursor quantitation, thereby, ensuring that both qualitative and quantitative information can be obtained within a single analysis

FIGURE 6. HCD MS^2 spectrum of permethylated glycan results in the production of cross-ring fragment ions and internal double-cleavage ions which, even with permethylation, can be lacking with low energy CID fragmentation.

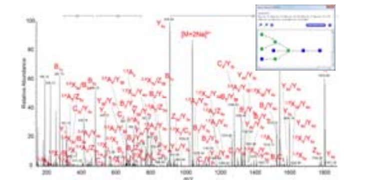


FIGURE 7. High quality HCD MS^2 data over wide dynamic range. MS spectrum was acquired at a resolution of 120,000 (at m/z 200) while MS^2 were acquired at a resolution of 30,000 (at *m/z* 200).

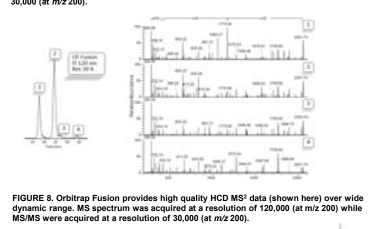
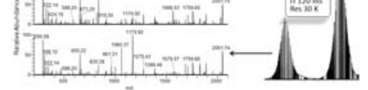
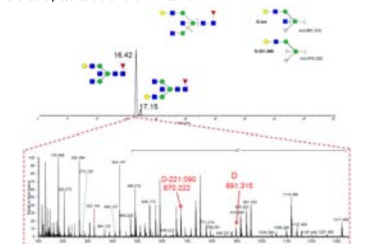


FIGURE 8. Orbitrap Fusion provides high quality HCD MS^2 data (shown here) over wide dynamic range. MS spectrum was acquired at a resolution of 120,000 (at m/z 200) while MS/MS were acquired at a resolution of 30,000 (at *m/z* 200).



The structural characterization of glycans is essential in the bio-therapeutics and bio-pharmaceutical industry. Glycans contribute to the efficacy and safety of protein based drugs, for example, recombinant proteins and monoclonal antibodies (mAbs) are often dependent on the structure and types of glycans attached to the proteins. The structures of glycans are quite diverse, complex and heterogeneous due to PTMs and physiological conditions. Minor changes in glycan structure (creation of structural isomers) can result in striking differences in biological function and clinical applications. The selection of appropriate stationary phases to separate these isomers along with mass spectrometers to generate useful fragments to help identify these resolved structures are crucial in glycan analysis. HILIC amide columns are particularly useful for the separation of N-linked glycans released from antibodies, for example mAbs, where the majority of glycans are neutral. The typical workflow here is to label the glycans with 2-aminobenzamide (2-AB) separate them with HILIC columns and identify with mass spectrometry. Additionally, selecting the right mode of ionization is important for this workflow as negative mode much more than positive mode will generate useful fragment ions which aids in identification of structural isomers. Unfortunately, not all commercial mass spectrometers can operate and produce useful fragmentation in the negative mode. Figure 9 shows the separation and identification of 2 glycan structural isomers released from human IgG. The HCD MS/MS spectrum generated in negative mode on Orbitrap Fusion was able to produce useful fragment ions that aided in the assignment of correct glycan structures to the resolved peaks.

FIGURE 9 A) Shows schematic representation of useful fragment ions needed to assign galatose to the correct antennae. B) HCD MS^2 spectrum acquired in negative mode that shows the presence of the D and D-221.090 ion.



Conclusion

The introduction of the Orbitrap Fusion MS provides a giant step forward for glycomics and glycoproteomics field. The innovative instrument design contains new functionalities, including a mass selecting quadrupole coupled to both a linear ion trap and Orbitrap mass analyzer with highly optimized parallel operation. The instrument allows for increased scan rate, full flexibility of CID, HCD and ETD dissociations at any stage of MSn analysis, ultra high field Orbitrap detection with advanced signal processing, front-end compact and robust ETD source and dedicated internal mass calibration. These functionalities combine to produce a significant performance improvement for standard glycomics and glycoproteomics experiments. Additionally, the unique tribrid architecture makes a wide array of novel acquisition experiments possible facilitating workflows that were previously inaccessible with previous generation platforms.

References

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