Comparison of Collisional and Electron-Based Dissociation Modes for Middle-Down Analysis of Multiply Glycosylated Peptides
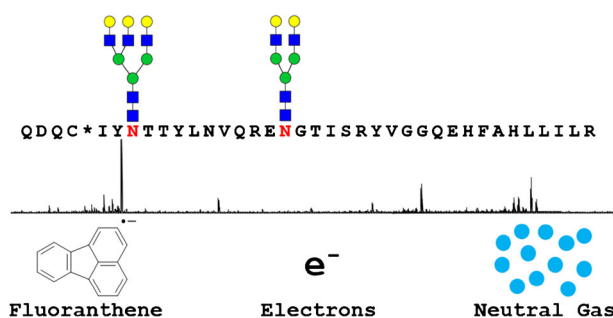
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Abstract. Analysis of singly glycosylated peptides has evolved to a point where large-scale LC-MS analyses can be performed at almost the same scale as proteomics experiments. While collisionally activated dissociation (CAD) remains the mainstay of bottom-up analyses, it performs poorly for the middle-down analysis of multiply glycosylated peptides. With improvements in instrumentation, electron-activated dissociation (ExD) modes are becoming increasingly prevalent for proteomics experiments and for the analysis of fragile modifications such as glycosylation. While these methods have been applied for glycopeptide analysis in isolated studies, an organized effort to compare their efficiencies, particularly for analysis of multiply glycosylated peptides (termed here middle-down glycoproteomics), has not been made. We therefore compared the performance of different ExD modes for middle-down glycopeptide analyses. We identified key features among the different dissociation modes and show that increased electron energy and supplemental activation provide the most useful data for middle-down glycopeptide analysis.

Keywords: Tandem MS, Glycoproteomics, Middle-down, Electron-activated dissociation, hotECD, EThcD, FTICR-MS

QDQC*IY**N**TTYLNVQRE**N**GTISRYVGGQEHFAHLLILR

Fluoranthene   Electrons   Neutral Gas

Introduction

Electron-based activation of glycans and glycoconjugates yields informative product ions that provide information critical to confident assignment of detailed structures and site-specific post-translational modifications [1–5]; however, the application of these methods to biological samples has been limited by instrument availability, sensitivity, and speed. Instrument and workflow development need to go hand-in-hand for efficient application and dissemination of these methods. Here, we developed and tested approaches for standardization of sample preparation and ExD (electron-activated

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dissociation) analysis of multiply glycosylated peptides (referred to here as middle-down glycopeptides) that are not characterized efficiently using collisional dissociation methods. These efforts identified key analytical features for these biopolymers using electron-based versus collisional dissociation modes.

Use of beam-type CAD (collisionally activated dissociation) for glycopeptide analysis requires high collision energies to allow fragmentation of the peptide backbone. A particular problem with such use of CAD is the low abundances of product ions from peptide backbone fragmentation because the relatively fragile glycosidic bonds fragment easily. The glycan composition can be inferred from the total mass of the precursor, once the peptide backbone has been identified, but it may not always be possible to identify the exact site of modification if peptide backbone fragments with attached saccharide units are not observed. This loss of the fragile saccharide moiety poses a greater issue when analyzing multiply glycosylated peptides, whereby the loss of saccharide from peptide backbone fragments prevents assignment of site-specific glycan compositions. This limits the extent to which CAD-based methods can be applied to analysis of heavily glycosylated peptides such as mucin-type O-glycosylated peptides, which contain stretches of serine and threonine residues with multiple glycans attached, or peptides with more than one N-glycosylation sequon. In some cases, it is possible to separate the multiple sites of glycosylation using a combination of proteolytic enzymes; however, the usefulness of CAD to analysis of multiply glycosylated peptides is limited.

We have previously developed analytical and bioinformatics workflows that combine proteomics, glycomics, and glycoproteomics information from bottom-up experiments to enable efficient and comprehensive analysis of the glycoproteomes [6–8]. When using a bottom-up method, it is possible to determine the range of glycoforms present at each site but not to assign the glycan combinations that exist simultaneously on different sites of the protein. In order to determine the precise glycosylation structures of the functional proteoforms(s) of a glycoprotein, it is necessary to develop methods for analysis of large and multiply glycosylated peptides; however, this is challenging because the addition of glycans to proteins multiplies the total number of molecular forms many-fold due to inherent glycosylation heterogeneity. In addition, the number of protein-glycan combinations multiplies with an increase in the number of glycosylation sites. Thus, for large glycoproteins with multiple glycosylation sites, only analysis of peptides with 2–3 glycosylation sites and masses in the range 8000 to 12,000 Da (defined here as middle-down analysis) is feasible [6–10].

The challenge to analysis of multiply glycosylated peptides increases with size and number of glycosylation sites. Methods for middle-down glycoprotein analysis are not yet mature and a detailed comparison of ExD-based methods [9, 11–20] combined with high-resolution and high-sensitivity MS has not appeared. Multiply O-glycosylated synthetic peptides smaller than 7 kDa have been analyzed using ExD by direct infusion electrospray [2, 21]. Generally, increased m/z and number of glycosylation sites results in inefficient dissociation using ExD. The authors show that this limitation can be overcome to a degree by increasing the electron energy in ECD to achieve hECD (hot electron capture dissociation). While their results indicate promise for use of these methods for glycopeptide analysis, a direct comparison of dissociation techniques for multiply glycosylated peptides with molecular weights exceeding 5–7 kDa is lacking.

The paucity of high-resolution and high-mass-accuracy ExD data on multiply glycosylated peptides has also limited the development of efficient bioinformatics methodologies for automated data analysis. In this work, we evaluated the performance of different fragmentation modes for analysis of bottom-up and middle-down glycopeptides from glycoprotein standards including human transferrin and human α1-acid glycoprotein (AGP). We made comparisons of different dissociation modes systematically transitioning from bottom-up to middle-down glycopeptide analyses, with particular focus on glycan heterogeneity, dissociation efficiency, charge-state dependence, and compatibility with online separation. The overall effort is geared at identifying and applying the best methods for middle-down analysis that will help develop an integrated workflow combining information from bottom-up and middle-down domains for the most comprehensive glycoproteomic analysis.

Materials and Methods

Sample Preparation

For bottom-up glycopeptide analysis, human transferrin and human AGP (Sigma-Aldrich, St. Louis, MO) were denatured by heating at 90°C for 30 min, in the presence of 2,2,2–trifluoroethanol. Samples were reduced with dithiothreitol (DTT), alkylated using iodoacetamide (IAM), and digested with Trypsin Gold (Promega Corp., Madison, WI) in the presence of 100 mM ammonium bicarbonate (Sigma-Aldrich, St. Louis, MO) as buffer. The detailed digestion protocol has been described previously [6–8]. Glycopeptides were enriched using a ZIC-HILIC glycopeptide enrichment kit (EMD Millipore, Billerica, MA), as per the manufacturer's protocol. Samples were desalted, where necessary, using Pierce Pepclean C18 spin columns (Thermo Fisher Scientific, Pittsburgh, PA).

For middle-down glycopeptide analysis, AGP was denatured, reduced, alkylated, and digested using Asp N endoproteinase (Promega Corp., Madison, WI). We evaluated both endoproteinase Asp N and endoproteinase LysC for generation of middle-down glycopeptides from AGP. We used Asp N for these studies because it showed superior reproducibility of digestion. Middle-down glycopeptides were enriched by fractionation of the Asp N digest using a Superdex 75 (3.2/300) (GE Healthcare, Pittsburgh, PA) on a Beckman Gold HPLC system (Beckman Coulter, Inc., Indianapolis, IN). Ammonium formate (25 mm, pH 4.5) in 10% acetonitrile was used as mobile phase for separation at an isocratic flow rate of

50 μL/min. Fractions were collected manually based on UV absorbance at 230 nm and further desalted and fractionated using a Vydac C18 reversed-phase HPLC column (W.R. Grace & Co., Columbia, MD) on an Agilent 1200 series chromatograph (Agilent, Inc., Santa Clara, CA), fitted with an automated fraction collector.

Where indicated, for both bottom-up and middle-down analyses, the proteolytic digestion product mixtures were desialylated using α2-3,6,8 neuraminidase (New England Biolabs, Ipswich, MA), prior to LC-MS or nanoESI-MS to reduce glycoform heterogeneity.

Data Acquisition

Bottom-up glycopeptide samples were either directly infused for analysis using an Advion NanoMate™ alone or analyzed using online LC-MS at nanoliter flow rates on a Bruker solariX™ 12 T hybrid Qh-FTICR (Fourier transform ion cyclotron resonance) mass spectrometer mounted with an Advion NanoMate nano-ESI source (Advion Inc., Ithaca, NY). A Waters NanoAcquity™ nano-flow chromatograph (Waters Corp., Milford, MA) mounted with a Waters Xbridge™ reversed-phase column (150 μm × 100 mm) packed with 1.7 μm BEH C18 resin and a Waters trap column (180 μm × 20 mm) packed with 5 μm Symmetry™ C18 stationary phase, was used for online LC-MS. Bottom-up transferrin glycopeptides were also analyzed using CAD on an Agilent 6550 Q-TOF mass spectrometer using online HILIC enrichment combined with reversed-phase separation, as described previously [6].

Middle-down glycopeptide fractions from C18 LC separation were analyzed by nanoESI-MS using the Bruker solariX 12 T FTICR-MS and Advion NanoMate source described above. HCD and EThcD (higher-energy collisional dissociation and electron transfer dissociation supplemental collisional activation) [22–24] experiments were performed on ZIC-HILIC-enriched and unfractionated bottom-up and middle-down glycopeptide samples by LC-MS using an EASY-nLC chromatograph with an EASY-Spray C18 LC column on a Thermo Orbitrap Fusion™ instrument. The instrument was set to perform HCD-triggered EThcD, which allowed EThcD to be performed only on precursor ions that generated saccharide oxonium ions.

For LC-MS/MS data acquisition using the solariX, precursor ions of interest were isolated using a front-end quadrupole mass filter and accumulated in the collision cell for 100 to 1000 ms prior to MS/MS analyses. Hot electron capture dissociation (hECD) was performed by irradiating trapped ions in the ICR cell with 12–14 eV electrons, for up to 1 s. Cathode current was set at 1.5 A and the cathode bias was set between − 12 and − 14 V. Transients were summed to improve signal-to-noise ratio for middle-down experiments, as indicated in respective figure legends. LC-MS/MS experiments were performed by data-dependent precursor selection of the most-abundant parent ions or using a targeted inclusion list.

Data Analysis

Glycopeptide data analysis was performed either manually or in a semi-automated manner using Python scripts developed in-house, using the Pyteomics [25] and GlyPy libraries (https://pypi.python.org/pypi/glypy/0.0.5rc2) [26]. The protein sequence from Uniprot was digested with Asp N in silico using Pyteomics to generate a theoretical list of peptidoforms and combined with a list of human N-linked glycoforms from GlycomeDB [27, 28] using GlyPy's database module. Theoretical fragment ions were then generated for precursors that found a MS1 match. A 5 ppm mass error tolerance was used for matching and assignment of fragment ions. Bottom-up data were analyzed manually and ions were picked by visual inspection of the spectra. For middle-down data analysis, the FTICR-MS spectra were deconvoluted in the Bruker Compass Data analysis software (version 4.2), using the SNAP algorithm [29]. Fragment ion lists were matched against the deconvoluted/deisotoped peaklists (provided as part of supplement). Middle-down glycopeptide data from the Orbitrap were manually analyzed using the theoretical fragment lists.

Results and Discussion

Comparison of Dissociation Modes for Bottom-Up Glycopeptide Tandem MS

Before analyzing middle-down glycopeptide samples, we compared the efficacy of different dissociation modes, using bottom-up glycopeptides from widely used glycoprotein standards. To compare the characteristics of different dissociation modes, we acquired tandem MS of the same transferrin glycopeptide ([622]QQQHLFGSNVTDC*SGNFC*LFR[642]–Hex5 HexNAc4 NeuAc2) using CAD, ETD, and ECD, respectively. Figure S1 shows the tandem MS results using CAD. As expected [6], extensive dissociation of the glycan leads to formation of abundant oxonium ions. The intact peptide ion without any glycan attached and a glycan Y_1 ion are also observed, which confirm the intact peptide and glycan masses, respectively. A series of peptide backbone ions are also observed at low relative abundances. In addition to the bare peptide backbone ions, peptide fragment ions with an attached HexNAc are also observed. Together, these features allow unambiguous assignment of the peptide sequence and glycosylation site, as well as inference of the glycan composition from the residual precursor mass.

An ETD spectrum for the same precursor, generated on the FTICR instrument, is shown in Fig. S2. ETD generated few, if any, peptide backbone product ions for this precursor, while some glycan fragmentation was observed, likely resulting from vibrational excitation that occurred during ion transfer. The abundant charge-reduced species observed in the ETD spectrum indicates occurrence of ETnoD (electron transfer with no dissociation), where an electron is transferred to the precursor leading to charge reduction but fragments are not generated—or do not separate from one another [30, 31].

By comparison, ECD of the same precursor ion generated extensive glycopeptide fragmentation on the FTICR-MS (Fig. 1). We observed abundant c- and z-type peptide backbone ions with the intact glycan attached in the range m/z 1600–2400. In the lower m/z range, peptide backbone fragments not containing the glycosylation site were observed. A few b/y-type peptide ions and some peaks resulting from glycan fragmentation (oxonium ions and saccharide losses from the precursor) were also observed, indicating additional energy deposition. The presence of b-ions in ECD has been previously reported and discussed by Cooper [32].

Although the drastic difference between the ECD and ETD spectra of this glycopeptide may seem surprising given the common misconception that ECD and ETD are essentially the same processes with the only difference being the source of electrons, sufficient differences exist between these two methods, such as the lower energy deposition and collisionally cooling in ETD. Further, McLuckey and coworkers reported that the electron transfer probability is influenced by the electron affinity and the Franck-Condon factors associated with the anion radical and its associated neutral [31]. Simons and coworkers suggested that electron attachment to a Coulomb-stabilized C=O π* orbital can compete favorably with electron transfer to a charged site, and the probability of these

competing electron transfer processes depends on the location of their crossing points and the strength of coupling between electronic states [33]. The electron donor must get close enough to the potential attachment site before electron transfer can take place, and the size of the ETD reagent (comparing to electrons) may prevent access to certain fragmentation pathways, while favoring other dissociation channels, such as hydrogen loss. Finally, dissimilar fragmentation behaviors have also been observed in ECD and ETD of metal-cationized peptides [34].

While ECD of the 4+ precursor of the transferrin glycopeptide generated abundant peptide backbone product ions (Fig. 1), a 3+ precursor of the same glycopeptide did not yield useful tandem MS with ETD or ECD (Fig. S3), indicating presence of electron capture with no dissociation (ECnoD), described in Fig. S4. The poor ETD and ECD performance for the lower charge-state precursor suggested charge-state dependence of these processes. By contrast, hECD yielded abundant glycopeptide fragments for the same 3+ precursor (Fig. 2). As expected, in addition to c- and z-type ions, hECD also produced a series of b- and y-type fragment ions, indicating an increase in vibrational excitation during hECD.

While LC-coupled hECD has been reported with a quadrupole ion trap [2], we demonstrated LC-hECD with FTICR-MS
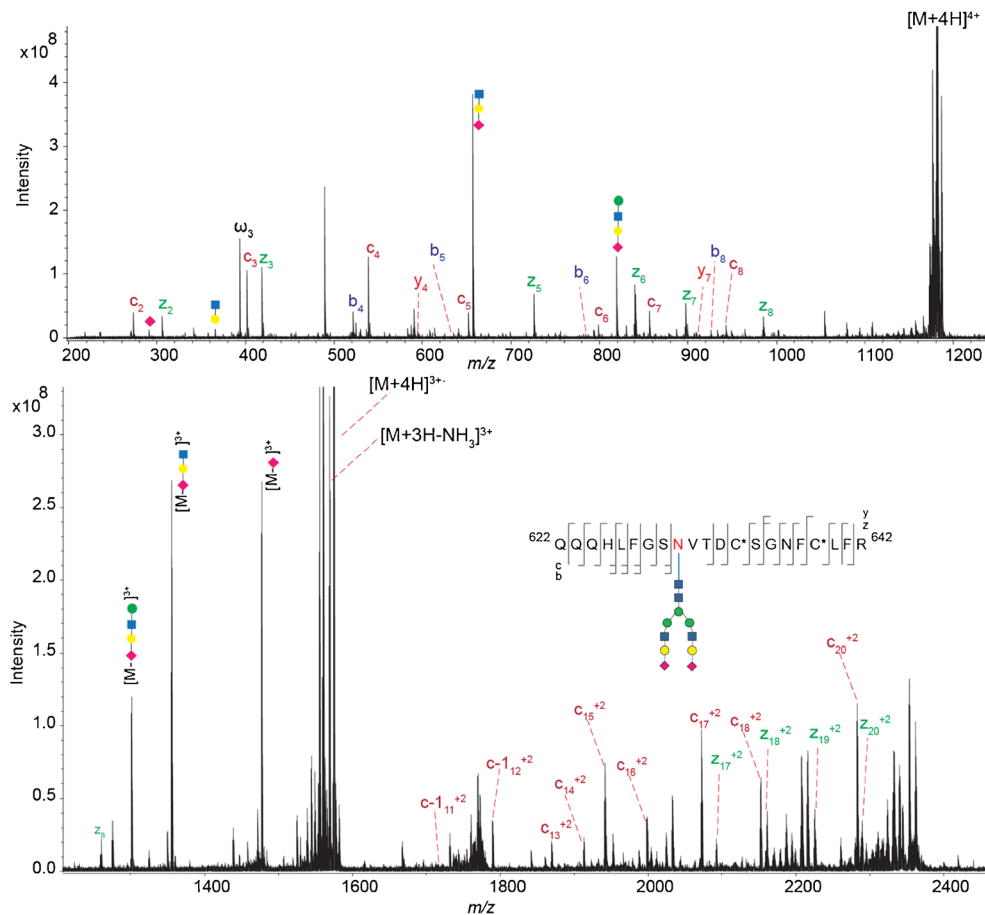
Figure 1. ECD (1.7 eV) tandem MS of a transferrin glycopeptide ([M + 4H]$^{4+}$, m/z 1180.7328) on FTICR-MS. ECD irradiation time was 0.1 s and 40 transitions were summed in this spectrum. C^* carbamidomethyl cysteine
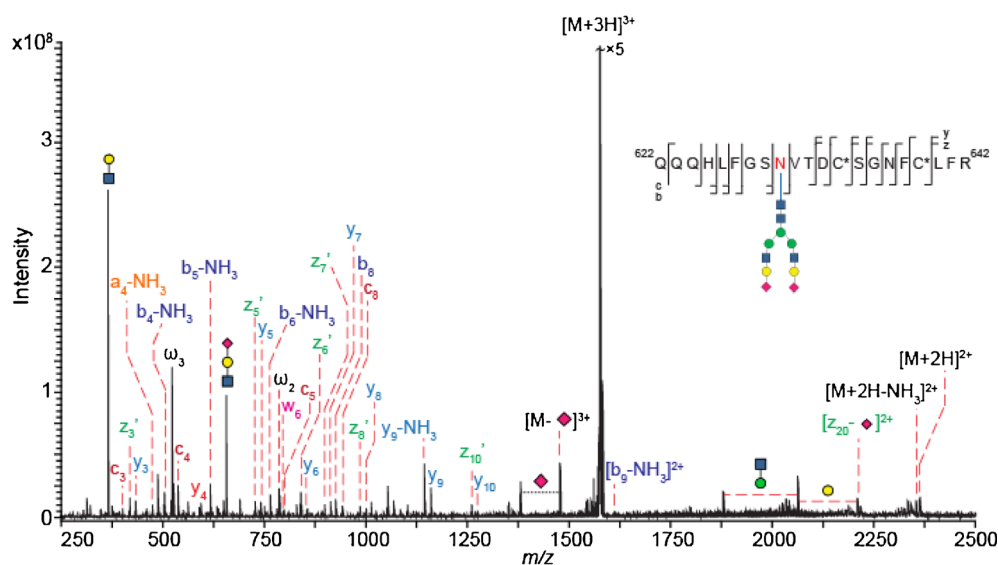
Figure 2. The hECD (14 eV) tandem mass spectrum of a transferrin glycopeptide ($[M + 3H]^{3+}$, *m/z* 1573.9704) acquired on FTICR-MS. ECD irradiation time was 1 s and 16 transients were summed for this spectrum. *C** carbamidomethyl cysteine

could provide good sequence coverage for the peptide backbone cleavage and included fragments containing intact glycans that allowed determination of the glycoform present at each occupied site. Figure 3 shows the LC-hECD results for enriched glycopeptides from a transferrin tryptic digest. Comprehensive glycopeptide backbone coverage was generated by hECD in a single tandem MS scan, where the amino acid sequence of the peptide backbone was covered by both c/z- and b/y-type ions. In addition, we observed many secondary fragments. While the increased electron energy caused some glycan dissociation, we observed many peptide backbone fragments with intact glycan attached, which provides much more useful tandem MS compared to a collisional dissociation spectrum where the glycan moiety is lost from peptide backbone fragments. The higher mass accuracy and resolving power will also be useful in assigning MS and tandem MS when analyzing complex biological samples, where unexpected modifications and low abundance or overlapping ion clusters may be present.

The hECD technique can be performed on FTICR-MS instruments to improve fragmentation of glycopeptides, when ECD or ETD fragmentation is not efficient. However, FTICR-MS instruments suffer from lack of sensitivity and speed, prohibiting their use in large-scale glycoproteomics studies, with the possible exception of high-field instruments. The most successful implementation of ExD for large-scale studies has been demonstrated using hybrid Orbitrap instruments capable of performing ETD [23, 35–38]. Many groups have demonstrated further improvement in the efficiency of ETD fragmentation on Orbitrap instruments by use of supplemental collisional activation [23, 24, 35, 36].

We evaluated higher-energy collisional dissociation (HCD) (Fig. S5) and EThcD (Fig. S6) of an AGP glycopeptide using a Thermo Orbitrap Fusion instrument. As expected, collisional dissociation of the precursor by HCD generated primarily peptide backbone fragments while the glycan dissociated to

mono-, di-, and tri-saccharide oxonium ions. In addition to bare peptide backbone ions, y-type ions with an attached HexNAc (y_n/Y_1) were observed. Also, an intact peptide ion with a HexNAc (glycan Y_1 ion) was seen in the spectrum. EThcD is performed by first allowing the precursor ions to react with the electron donating radical anion, which leads to charge reduction and formation of a c/z ion pair that may still be held together by non-covalent interactions. Subsequent collisional activation of the charge-reduced species results in detection of peptide backbone product ions. The ETD reaction takes place in the LTQ linear ion trap, after which these ions are transferred to the HCD cell, where they are subjected to collisional activation that facilitates efficient separation of the complementary peptide backbone fragments by disrupting non-covalent interactions, while keeping the glycan largely intact. As seen in Fig. S6, EThcD of an AGP glycopeptide generated extensive coverage with c-type ions and a few b-ions. The z_7 ion was observed with the intact glycan attached. We also observed Y_1 and Y_2 ions, saccharide losses from the precursor, and oxonium ions; however, their abundances were much lower relative to the HCD spectra. Supplemental activation using collisional and photoactivation methods generally decreases the charge-state dependency of ETD and ECD and improves detection of product ions for both bottom-up and top-down analyses [36–38]. For proteins, it also causes unfolding and makes more of the backbone accessible to ExD cleavage.

From our evaluation of the different dissociation modes for bottom-up glycopeptide fragmentation, CAD-based fragmentation was the most efficient in terms of speed and sensitivity. However, when considering the level and quality of information generated, CAD did not produce any peptide backbone fragments retaining the intact glycan and would be incapable of clearly defining the glycosylation site with multiple glycans attached to the same peptide. EThcD and hECD generated abundant peptide fragments, while keeping the glycan moiety
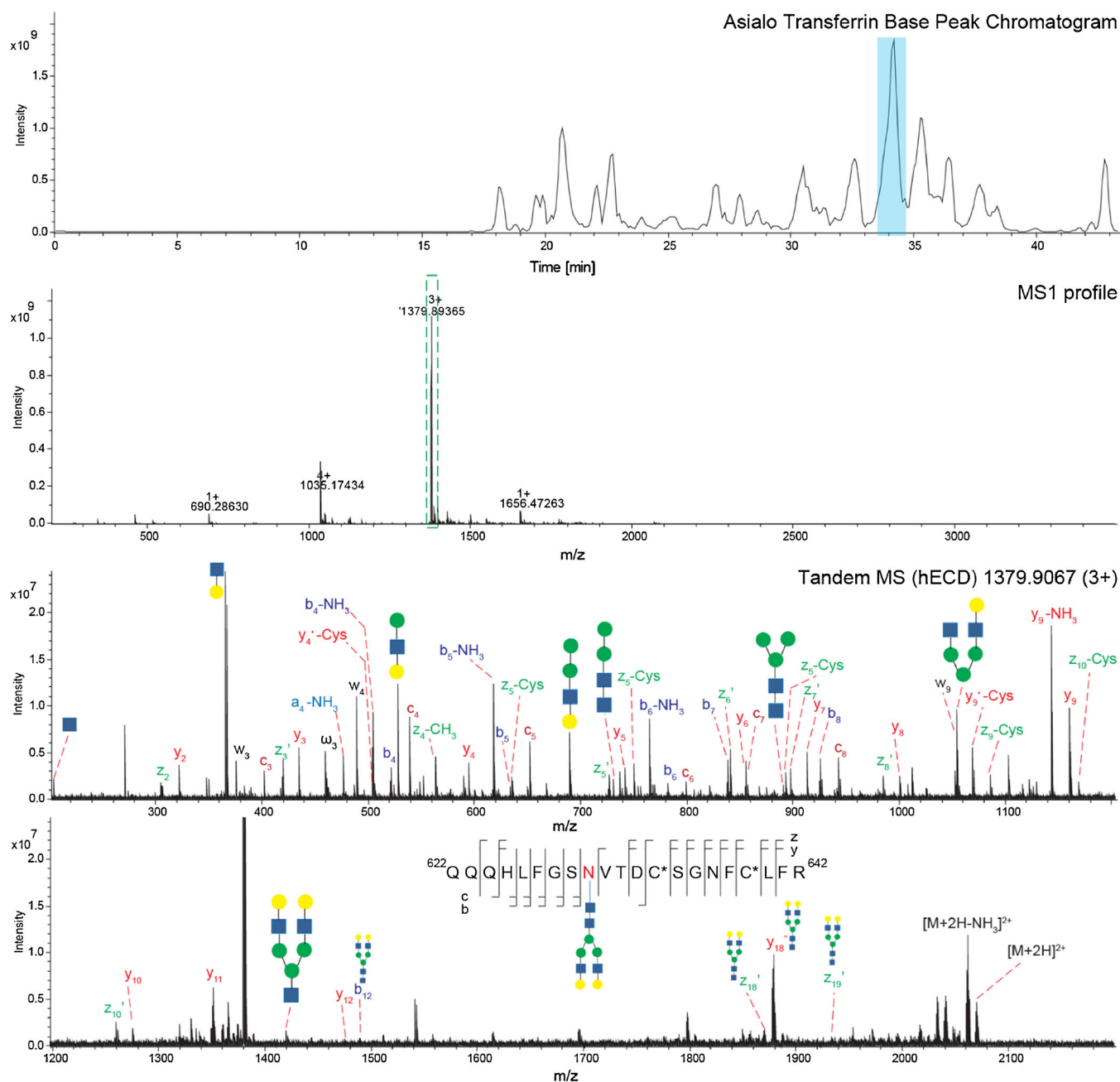
Figure 3. LC-hECD (14 eV) analysis of enriched tryptic glycopeptides from human transferrin on FTICR-MS. Tandem MS for m/z 1379.9067, $[M + 3H]^{3+}$, is shown. –Cys indicates loss of the Cys side chain. ECD irradiation time was 1 s and a single transient was acquired for this spectrum. C^* carbamidomethyl cysteine

intact during the fragmentation process, thus indicating potential for application in the analysis of middle-down glycopeptides with multiple glycosylation sites.

Tandem MS of Multiply N-Glycosylated Peptides

For middle-down method development, we selected the human AGP, a protein which we and others have characterized in detail using bottom-up mass spectral methods [6, 8, 39, 40]. AGP has five N-glycosylation sites, each with a variety of complex-type N-glycans, and occurs as a mixture of two

protein isoforms (hAGP1 and hAGP2) [8, 41]. We performed in silico digestion of AGP with different enzymes and found that digestion of AGP using endoproteinase Asp N generates multiple peptides that contain two N-linked glycosylation sequons. Therefore, we selected the Asp N digests of AGP for development and evaluation of methods for middle-down glycopeptide analysis. We reduced glycan heterogeneity by trimming the glycans from the non-reducing end, where necessary. We used α2-3,6,8 neuraminidase to remove NeuAc residues from the AGP Asp N digests. The resulting mixtures contained unglycosylated peptides and glycopeptides with 1, 2,

or 3 glycosylation sites. To resolve multiply glycosylated peptides from those with fewer glycosylation sites, we used a size exclusion chromatography fractionation step, as shown in Fig. 4. SEC provided more specificity in enrichment of glycopeptides with two glycosylation sites than did HILIC SPE techniques, such as those used for bottom-up sample preparation [20, 42, 43]. MALDI-MS of SEC fractions showed presence or absence of middle-down glycopeptides (Fig. S7).

SEC fractions containing two glycosylation sites were further fractionated using an offline C18 analytical column and the glycopeptide fractions were analyzed by FTICR-MS, using static nano-electrospray. We compared the different ExD modes available on FTICR-MS for middle-down glycopeptide analysis. Similar to bottom-up glycopeptides, ETD of middle-down glycopeptides only produced charge-reduced species, and while ECD produced a few fragments covering the ends of the peptide, no fragmentation between the glycosylation sites was observed (data not shown). Middle-down glycopeptides were then irradiated with higher-energy electrons to achieve hECD.

As shown in Fig. S8, comprehensive fragmentation of a middle-down glycopeptide from AGP was achieved using hECD. Predominantly c-type ions were seen in the hECD tandem mass spectra. Overall, better coverage was observed along the peptide portions not carrying glycosylation, while peptide backbone coverage between the two glycosylation sites was sparse. Nevertheless, a few c-type fragment ions carrying the intact glycan could be identified. Vibrational excitation also led to generation of three peptide b-ions that still had intact glycans attached to them. A few z-ions were also observed in the tandem mass spectra, complementing the information from the c-ion series. The example shown in Fig. S8 had the same glycan composition present at the two glycosylation sites.

For the glycopeptide whose middle-down hECD tandem mass spectrum is shown in Fig. 5, the glycan compositions identified at the two glycosylation sites were different. In this case, abundant c- and z-type ion series were observed, again covering the N- and C-termini of the peptide, along with y-type ions whose presence resulted from vibrational excitation. Coverage between the two glycosylation sites was again poor, with only one fragment ion each of c- and z-type identified. Interestingly, z_{21} ions corresponding to both a tri-antennary and a tetra-antennary glycoform were detected in the spectrum. This indicated the presence of glycoform positional isomers in the glycopeptide mixture. The abundance of the tri-antennary glycoform containing z_{21} ion was much lower than that for the tetra-antennary glycoform. For this minor component, the corresponding c_{15} ion with a tetra-antennary glycan, which would also be expected to have low abundance, was not detected. This represents another level of complexity in the analysis of multiply glycosylated N-glycopeptides, where the presence of positional isomers that cannot be resolved by liquid-phase separation methods leads to the generation of chimeric tandem MS. These results also demonstrate the value of high-resolution and high mass accuracy in FTICR-MS for the low-abundance fragment ions which could not have been confidently assigned if the isotopic peaks were not well-resolved or if the mass error were high. We were also able to confirm the presence of these glycoforms at the sites indicated in Fig. 5 using bottom-up glycopeptide analysis, as shown in Supplemental Figs. S9 and S10 and based on previously reported analyses of AGP [8, 39, 40, 44].

Our results from hECD fragmentation of middle-down glycopeptides with two glycosylation sites showed that, although coverage between the glycosylation sites still needed improvement, presence of as few as one or two fragment ions between
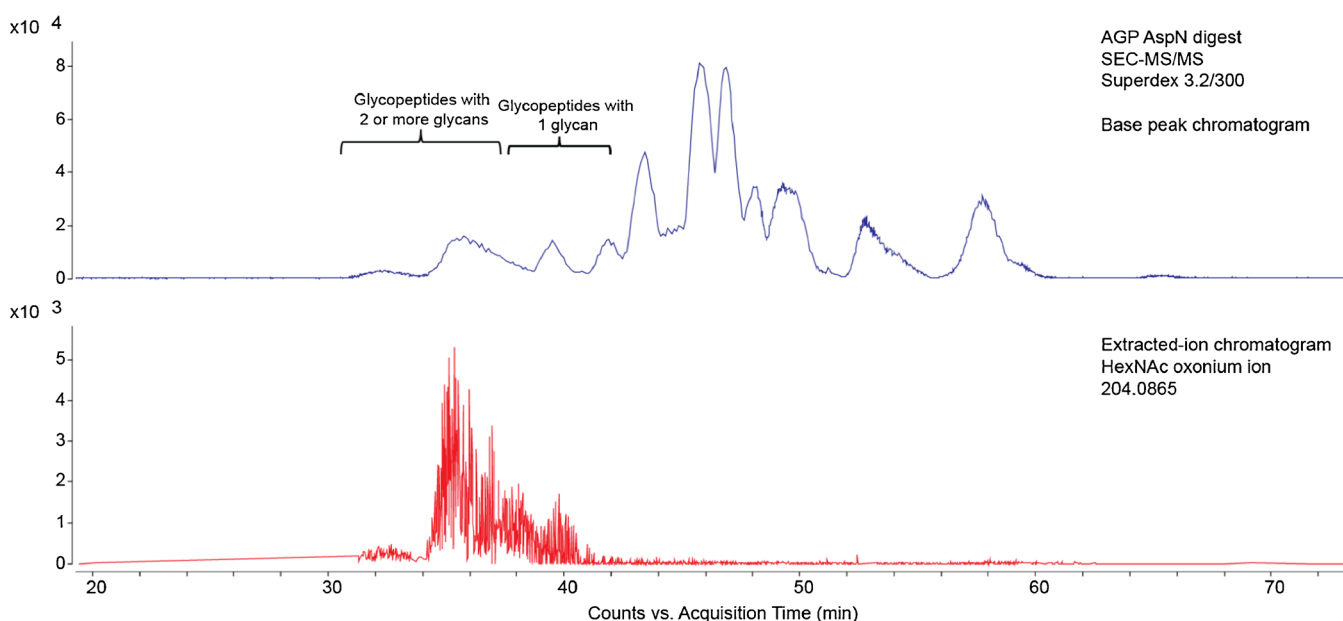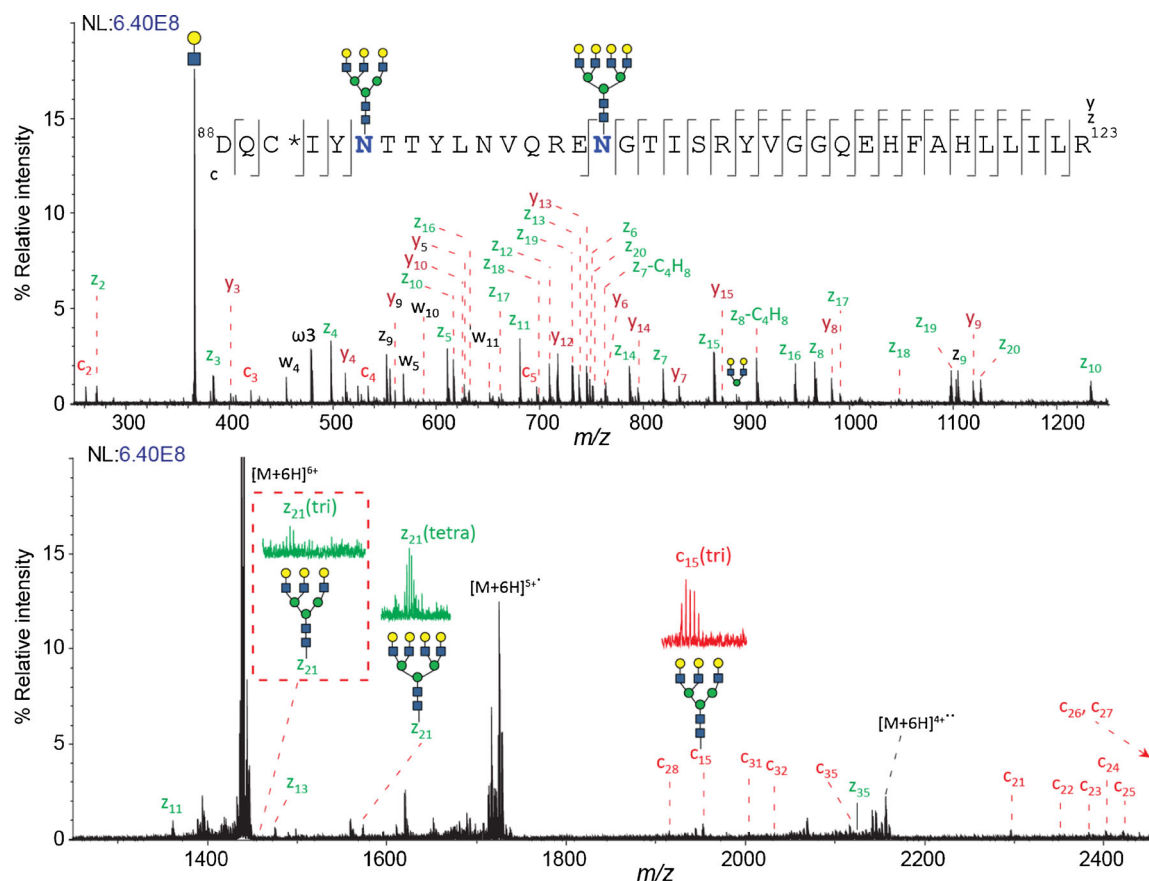

Figure 4. SEC-MS/MS (CAD) of human AGP digested with Asp N, showing separation of glycopeptides and peptides based on size and number of glycans attached

Figure 5. Tandem MS (hECD at 14 eV) of an AGP (Asp N) glycopeptide with two glycosylation sites on FTICR-MS (m/z: 1437.4571, [M + 6H]$^{6+}$). ECD irradiation time was 0.1 s and 100 transients were summed for this spectrum. C^* carbamidomethyl cysteine

the glycosylation sequons is sufficient to discriminate the exact glycan compositions simultaneously occupying the glycopeptide. Data acquisition on the FTICR-MS for middle-down glycopeptide tandem MS was performed using static nano-ESI and has not yet been coupled to online LC-separations due to the need for signal averaging to achieve sufficient S/N ratio. The speed and sensitivity of this instrument did not allow for online LC-MS/MS of the middle-down samples. Middle-down glycopeptides were analyzed using LC-MS/MS on the Orbitrap Fusion instrument that offers much higher speed and sensitivity.

Since the hybrid Orbitrap instruments had shown promising results for online LC-MS/MS of bottom-up glycopeptides using EThcD, we next evaluated the performance of this method for middle-down glycopeptides. HILIC SPE-enriched glycopeptides were subjected to online C18 LC-MS on an Orbitrap Fusion instrument. Similar to bottom-up glycopeptide separation, middle-down glycopeptide resolution on the reversed-phase column was peptide-centric, leading to clustering of different glycoforms for the same glycopeptide in the LC-elution profile. An example of an MS1 spectrum showing a distribution of co-eluting middle-down glycopeptide glycoforms is shown in Fig. S11. The higher speed and sensitivity of Orbitrap-MS made data-dependent LC-tandem MS more feasible on this instrument, compared to FTICR-MS.

While the Orbitrap-MS was able to sample many more precursors for tandem MS during a data-dependent acquisition experiment, only a few middle-down glycopeptides generated fragments suitable for resolving glycosylation site microheterogeneity. As shown in Fig. 6, the terminal regions of the peptide backbone were well covered in the tandem MS but coverage for fragment ions carrying the glycan moieties was again sparse, similar to hECD results. A z-ion series was predominant for this glycopeptide, with only two ions covering the N-terminal region of the peptide. Supplemental collisional activation also generated y-type fragment ions and glycan oxonium ions (Fig. 6). The two z-type ions with intact glycan attached, occurring in the region between the two glycosylation sites, provided adequate information for assigning the glycan compositions at these two sites. A HCD tandem MS of the same precursor generated primarily saccharide losses, with minimal peptide backbone coverage, as shown in Fig. S12.

Conclusions and Future Perspectives

From our evaluation of methods for middle-down glycopeptide analysis, it is clear that front-end separation plays a crucial role in managing the extreme heterogeneity of glycoforms. Even with a combination of different enrichment, fractionation and online
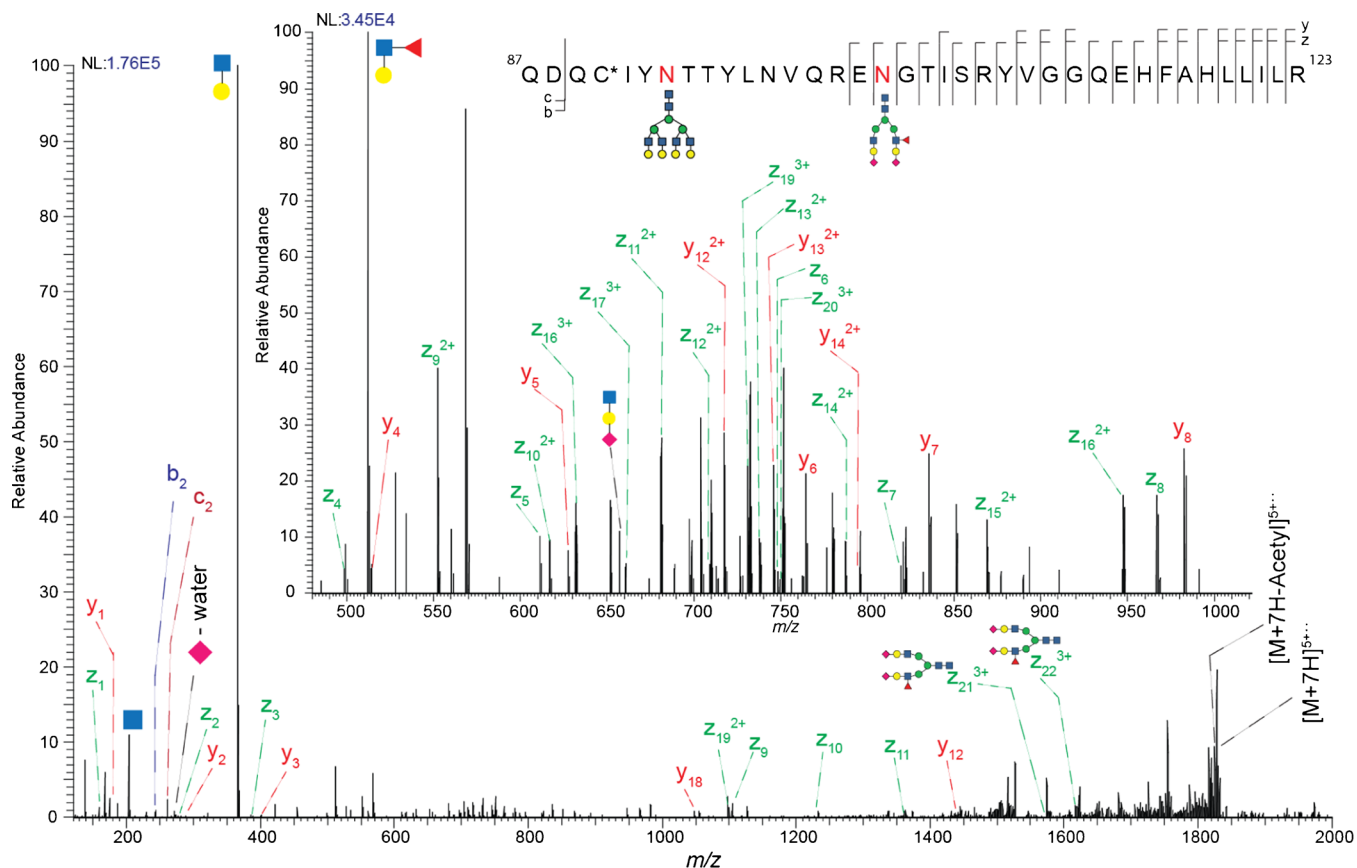
Figure 6. Tandem MS (EThcD) of an AGP middle-down glycopeptide on an Orbitrap Fusion instrument (precursor m/z: 1302.4178, $[M + 7H]^{7+}$). ETD reaction time was 8.75 m sec and HCD normalized collision energy was set at 20 for this scan. C^* carbamidomethyl cysteine

separation methods, the glycopeptide complexity extended far beyond what is typically seen for bottom-up glycopeptides. Another level of complexity comes from the presence of glycoform combinations that are isobaric due to the presence of glycosylation-site positional isomers. Such isobaric compositions cannot be resolved using liquid-phase separation methods and generate chimeric tandem mass spectra due to co-isolation in the mass spectrometer. Gas-phase separation methods such as ion mobility might be useful in separating such molecules prior to fragmentation. Thus, successful middle-down glycopeptide analysis will require a combination of analytical tools.

Further development of analytical methods is necessary to enable routine acquisition of high-quality middle-down glycopeptide tandem MS. While hECD and EThcD have shown promising results in the analysis of multiply glycosylated peptides, the sensitivity and speed of these methods remains adequate only for abundant glycopeptides.

Several recent studies have underlined the value of combining collisional and photoactivation modes with electron-based activation methods [35, 45–49] Zhang and Reilly have demonstrated the use of photodissociation for simultaneous analysis of peptide sequence and glycan structure from glycopeptides [50]. Coon and coworkers have recently reported the successful application of AI-ETD for large-scale glycopeptide analysis [51]. The Brodbelt group is actively exploring the use

of UVPD for glycopeptide analysis. They showed that negative mode UVPD generated more extensive fragmentation for both the peptide and the glycan compared to positive mode UVPD or CAD in positive or negative modes. More recently, they have presented the negative mode UVPD results on acidic glycopeptides from kappa Casein glycoprotein and an *Acinetobacter baumannii* Ompa/MotB glycoprotein [48]. In both cases, UVPD produced abundant a- and x-type peptide backbone fragments that retained the labile glycan modifications. In addition, useful b/y- and c/z-type glycan fragments were observed, along with some cross-ring fragments that helped sequence the glycan attached.

At the same time, electron-based activation modes are now being implemented on faster, more sensitive instruments [52–54]. Ion mobility-mass spectrometry is now being used for efficient online separation of glycopeptides [55, 56]. Glaskin et al. have also reported the incorporation of an ECD cell into an ion mobility-QTOF MS and its use for oligosaccharide analysis [57, 58]. Such a combination of ion mobility and tandem MS can be extremely useful for separation and identification of isomeric species. Marshall and colleagues have recently reported successful top- and middle-down characterization of monoclonal antibodies, using a 21 T FTICR-MS [59]. This entailed ETD MS/MS analysis of large protein subunits comprising the antibody light and heavy chains. The antibody

heavy chain contains a single *N*-glycosylation site, which adds to the complexity of analysis. It is clear that technology development will play a key role in advancing top- and middle-down workflows and dissemination of these methods in the glyco-analytics community.

Our study provides a systematic comparison of methods for middle-down glycopeptide analysis, whereby we first evaluated methods for bottom-up analysis and then used the knowledge from bottom-up analyses to tackle middle-down method development and data analysis. The data discussed here were analyzed using a combination of manual interpretation and prototype data analysis tools written using Pyteomics [25] and GlyPy Python libraries [26]. It is clear from these analyses that middle-down glycopeptide analyses alone are not sufficient for efficient bioinformatics and defining glycosylation site relationships would require a combination of bottom-up and middle-down data sets. We have previously presented such a workflow for bottom-up glycoproteomics and the bottom-up data would help drive analysis of the middle-down data [7, 8]. The ideal informatics workflow would integrate the different data streams for efficient and comprehensive data analysis.

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