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The benefits of hybrid fragmentation methods for glycoproteomics

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ABSTRACT

Glycosylation is an important and variable protein modification that can have a profound effect on the physiological characteristics of the substrate, warranting careful examination in applications ranging from the development and quality control of biopharmaceuticals to clinical glycoproteomics.

Glycoproteomics describes the mass spectrometric analysis of protein glycosylation in a site-specific manner, typically of proteolytically digested glycoprotein samples. This may be achieved by interpreting the mass (over charge) values of (glyco)peptides across a run of liquid chromatography coupled to mass spectrometry (LC-MS), and acquiring the fragmentation patterns of selected precursors to sequence the peptide and characterize the composition/structure of the glycan. It has become apparent, however, that most fragmentation mechanisms do not equivalently affect the glycan and peptide portion of a glycopeptide. For example, collision-induced dissociation (CID) and higher-energy collisional dissociation (HCD) primarily yield abundant B- and Y-ions from the glycan portion of a glycopeptide conjugate, whereas electron-transfer dissociation methods such as electron-capture dissociation (ECD) and electron-transfer dissociation (ETD) mainly affect the peptide backbone to yield c- and z-ions.

Hybrid fragmentation, i.e., the application of sequential or combinatorial fragmentation by orthogonal fragmentation strategies, has shown to greatly benefit the characterization of glycopeptides by combining the advantages of the individual methods. Examples of hybrid fragmentation methods include the sequential triggering of HCD and ETD on a closely situated precursor mass, using multiple steps of collision energy for the same precursor, and combining multiple methods on the same time/mass window. This is for instance the case with electron-transfer/collision-induced dissociation (ETciD) and electron transfer/higher-energy collisional dissociation (EThcD). Many modern-day mass spectrometers are capable of applying these fragmentation workflows, and the reported use of hybrid fragmentation for glycoproteomics is rapidly expanding.

This review will cover recent the developments and applications within the use of hybrid fragmentation for glycoproteomics. The work will be broadly centered on 1) energy-stepping in collisional activation, 2) sequential fragmentation, and 3) combinatorial fragmentation methods. We close by discussing remaining technical challenges, and outline possible future developments.

1. Introduction

Glycosylation is a highly abundant co- and post-translational protein modification (PTM), in which small or large carbohydrate moieties are covalently attached to specific sites on a protein [1]. Glycans are most frequently attached to proteins via *N*-glycosidic linkage to asparagines that are in an Asn-X-Ser/Thr motif (*N*-glycans), or via *O*-glycosidic linkage to serine or threonine without apparent sequence constraints (*O*-glycans). Other, less abundantly observed, forms of glycosylation can be found *C*-linked to tryptophan or *S*-linked to cysteine residues, and complex carbohydrates may also be found on other biomolecules such as lipids. Glycans themselves may differ from each other in terms of the number and types of monosaccharide moieties involved, as well as by the linkages between these. In all, this leads to large diversity in size and branching from one glycan to the next. There is no known production template for glycosylation (such as mRNA for proteins),

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0165-9936/

and thus the observed biological differences arise from complex remodeling by glycosyltransferases and glycosidases (in the endoplasmic reticulum, Golgi apparatus, or extracellularly). Consequentially, a single protein can exhibit many glycoforms, which may range from having sites occupied or not (macro-heterogeneity) to having qualitative differences in glycan structure (micro-heterogeneity) [1].

A multitude of biological functions have been attributed to protein glycosylation, including the management of folding and solubility, the protection of regions against proteases, and the direct interaction with carbohydrate-recognizing receptors (e.g., lectins) [1]. Because of its biological importance and responsiveness to changes in homeostasis, glycosylation is an important target for biomarker research, and it is a necessary aspect of the design and quality control of biopharmaceuticals [2]. Immunoglobulin G (IgG), for example, is majorly influenced in terms of plasma half-life, antibody-dependent cellular cytotoxicity (ADCC), and complement-dependent cytotoxicity (CDC), by specific glycoforms of the molecule [3]. All of this means that the study of protein glycosylation requires analysis tools that are of high sensitivity and throughput, and which must be capable of proving in-depth structural characterization of both the protein and glycan moieties.

Mass spectrometry (MS) is an attractive analytical method within the field of glycoproteomics, by virtue of its speed, sensitivity, and potential to characterize both glycan and protein sequences. Peptide sequencing by MS is well-established, and a typical workflow involves the digestion of a protein by trypsin and subsequent analysis of the tryptic peptides by MS coupled to liquid chromatography (LC-MS), followed by collision-induced dissociation (CID) or higher-energy collisional dissociation (HCD) [4]. MS is also particularly suitable for the sequencing of glycans released from the peptide backbone (chemically or using endoglycosidases like peptide-*N*-glycosidase F), whereby popular strategies involve matrix-assisted laser desorption/ionization (MALDI)-MS analysis or LC-MS with separation based on hydrophilic-interaction liquid chromatography (HILIC) or porous graphitic carbon (PGC) [2,5–7]. For the analysis of glycopeptides, however, these standard methods do not easily translate. Specific challenges include the sheer complexity of the glycoproteome, the ionization and separation characteristics of glycosylated peptides, and the notably different susceptibility of the glycan and peptide portion to distinct fragmentation techniques [8]. In all, this has prompted the development of new and specific analytical workflows for glycoproteomics, using new innovative MS fragmentation strategies.

A wide variety of MS-compatible peptide fragmentation strategies have been described, contemporary methods including collision-induced dissociation (CID, HCD), electron-driven dissociation (electron-capture dissociation, ECD; electron-transfer dissociation, ETD), and various forms of photodissociation (infrared photodissociation, IRPD; ultraviolet photodissociation, UVPD) [9,10]. For glycopeptides it has become apparent that CID/HCD preferentially cleaves glycosidic linkages (forming B- and Y-ions informative for the glycan composition), and depending on the collision energy may yield cross-ring fragmentation as well (A- and X-ions potentially informative for the glycan structure) [8]. Electron-driven dissociation, on the other hand, preferentially cleaves peptide bonds (for example forming c- and z-ions that provide information on the peptide sequence). Because of this differential susceptibility, MS glycoproteomics workflows have been applied that alternate CID and ETD in a single LC-MS run, sequentially informing on both aspects of the glycopeptide [6,10]. Recent developments have also given rise to methods which link these multiple fragmentation types, so-called hybrid fragmentation methods. These methods tend to generate more ions and ion-types, and have generally been of great benefit to structural characterization of the subjected analytes. For instance, dissociating a single precursor selection with first ETD then HCD (termed electron transfer/higher-energy collisional dissociation, EThcD) has shown highly instrumental in the field of phosphoproteomics, as it not only leads to the more reliable identification of peptides, but also to the more precise localization of phosphorylation sites [11–13]. As demonstrated further on, EThcD is rapidly being accepted within the field of glycoproteomics as well [6,10].

This review will focus on the ways in which the field of glycoproteomics has benefitted from the recent developments in hybrid fragmentation methodologies as depicted in Fig. 1, encompassing those methods that combine multiple types of fragmentation (dissociation mechanisms and/or energy levels) on the same set of precursor ions. In doing so, we will cover stepping-energy methods for compositional and structural characterization (e.g., stepped-energy CID and HCD), sequential fragmentation techniques with selected-ion triggering (e.g., CID- and HCD-product dependent-ETD, HCD-pd-ETD), and the fragmentation of a single selection of precursors with multiple consecutive fragmentation techniques (e.g., EThcD and electron-transfer/collision-induced dissociation, ETciD). For complementary recent reviews on related subjects, we direct the reader to the following in-depth studies on biopharmaceutical glycoprotein characterization (glycan, glycopeptide, glycoprotein) [14], large-scale and system-wide *N*-glycoproteomics [6,7], and (glyco)proteomics with ETD-based methodologies [10]. We have aimed to avoid overlap with these previous reports.

2. Hybrid fragmentaton methods in glycoproteomics

2.1. Energy-stepping in collision-based dissociation

The most widely-used fragmentation method in proteomics is collision-induced dissociation. The term CID is usually reserved for resonance-excitation CID on ion trap-based instrument. HCD, on the other hand, is a higher energy variant of CID that is, for instance, available in modern Orbitrap-based instruments. CID and HCD generate primarily b- and y-ions from peptide precursor ions, as well as B- and Y-ions from glycans (affecting the labile glycosidic linkages between monosaccharides). While CID can reveal both sequence and branching information on the released glycan level, for glycoproteomics the technique is mostly limited to providing sequence information. The details of CID fragmentation on saccharide structures have been reviewed extensively by An and Lebrilla [9], and the use of CID and HCD on glycopeptides by Nilsson [15].

As different CID fragmentation energies give rise to different types of fragmentation, mass spectrometric workflows have been designed that make use of collision energy-stepping, i.e., the subsequent application of different collision energies on the same group of ions. Two strategies are commonly followed for this, namely, 1) the generation of large glycan fragments (B-ions) by mild fragmentation, which can then be targeted by further rounds of CID, or 2) the generation of Y-ion sequences by intermediate fragmentation followed by harsh fragmentation (on the Y_1 ion of *N*-glycopeptides or Y_0 ion of *O*-glycopeptides) to obtain (partial) peptide sequence information [16].

Until recently, multiple fragmentation rounds with the same or differential levels of collisional energy had to be carried out sequentially, resulting in undesired longer cycle times per analyte. However, current day mass analyzers allow for stepped fragmentation within one scan, yielding richer fragmentation spectra. This procedure has, for example, been applied by Stavenhagen et al. for the analysis of *N*- and *O*-glycosylation of the human C1-inhibitor protein [17]. Also HCD has recently been developed into a stepped fashion. Besides being installed on instruments with short cycle times and high mass accuracy, stepped-energy HCD yields

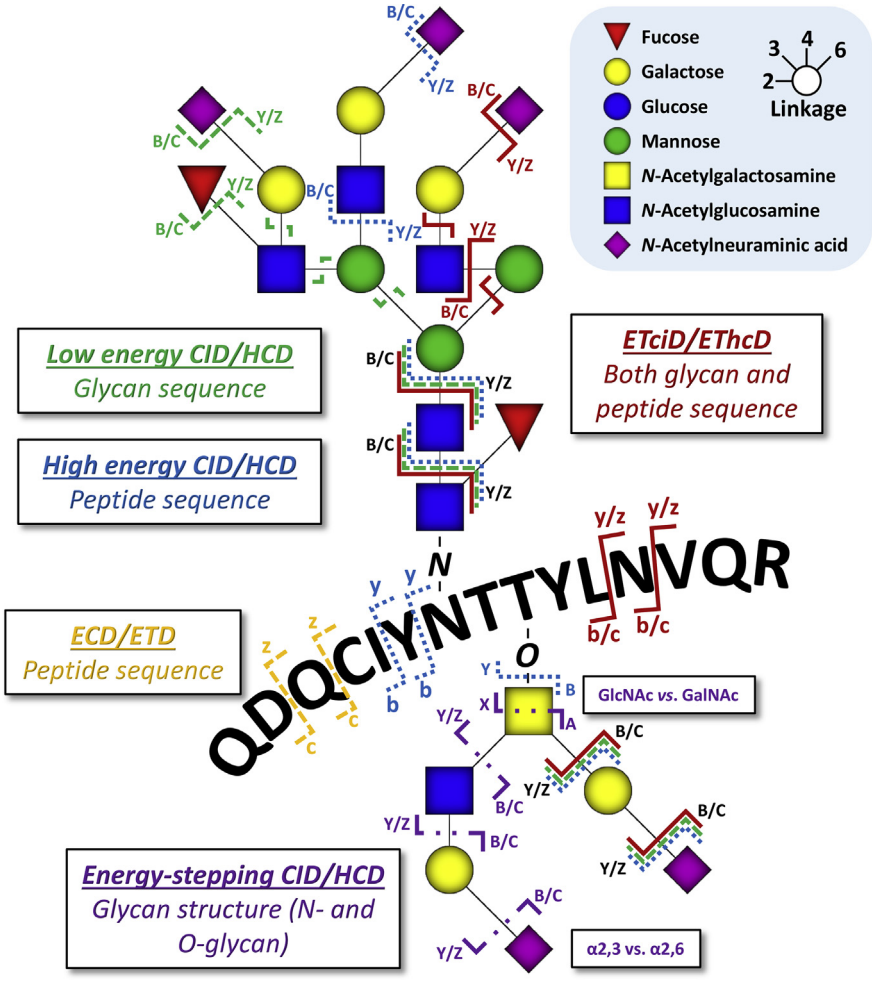

Fig. 1. Overview of contemporary glycopeptide fragmentation methods and their preferred sites of action. The displayed peptide sequence matches that of a tryptic *N*-glycopeptide from alpha-1-acid glycoprotein, whereas the *O*-glycan has been added for illustrative purposes. Locations of fragmentation are exemplary, and dissociation of glycosidic linkages and peptide bonds can also occur elsewhere on the molecule. The actual observed fragments will depend highly on both the glycan and the peptide in question and the particular energy deposited in the precursor ions.

several advantages over its CID counterpart. First of all, a single fragmentation cycle often results in not just glycan fragments but also some peptide backbone fragments, and secondly, the technique results in the detection of more cross-ring fragment ions. These specific ions can aid in the identification of linkages and isomers. For example, Halim et al. demonstrated the distinction between *O*-GlcNAc and *O*-GalNAc, otherwise isomeric structures, on basis of cross-ring fragment ions (the ratio of signals at *m/z* 126.06, 138.06, 144.07 and 168.07) [18], and sialic acid linkages appear discernible by the ratio of fragment ions as well [19].

In addition to the increased depth of information, instrumentation nowadays also allows for more complex sample analysis. For example, Yang et al. performed stepped HCD analysis (using 20, 30 and 40% normalized collision energy, NCE) on HILIC-enriched glycopeptides from human serum [20]. The single spectrum resulting from one stepped HCD cycle contains several B- and Y-ions (including Y_1 for *N*- and Y_0 for *O*-glycopeptides), as well as several peptide backbone b- and y-ions with and without (parts of) the attached glycan. From serum, 221 N-glycopeptides were detected, in total covering 65 sites of glycosylation. Another example of stepped HCD analysis (15, 25, 35% NCE) was provided by Bollineni et al., who combined the mass spectrometric approach with zwitterionic HILIC (ZIC-HILIC) and TiO_2 enrichment to detect sialylated tryptic glycopeptides from serum [21]. This resulted in the identification of 970 unique glycosylation sites on 257 glycoproteins, totaling 3447 non-redundant glycopeptide variants.

2.2. Product-ion triggered fragmentation strategies

Although HCD-MS^2 approaches often already allow for the correct identification of (particularly *N*-) glycopeptides, the determination of the precise (*O*-)glycan site and composition is highly dependent on both ETD- and CID-type fragmentation [22]. Ideally, one would like to combine multiple fragmentation strategies on the same glycopeptide precursor ions, but not many mass spectrometers have this capacity. Ion trap and quadrupole equipped instrumentation is well-suited for CID, whereas ETD is more common on quadrupole-time-of-flight (Q-TOF) and Orbitrap-based instruments, and ECD on Fourier-transform ion-cyclotron resonance (FTICR)-MS instruments. One way to circumvent the lack of multiple fragmentation modes on the same machine is to split the sample as close to the actual measurements as possible, and measure them on different systems, followed by a data analysis approach in which the complementary datasets are combined [23,24]. However, in this review we will not further cover this approach, but instead focus on recent developments allowing the use of different/combined methodologies on a single instrument.

Most of these modern methodologies have been developed on Orbitrap-based mass spectrometers.

In particular, HCD allows fragment ion detection with great accuracy especially at low *m/z* values with only 100 ms fragmentation cycles. This capacity is used often to quickly scan for the presence of glycans by the detection of specific diagnostic fragment ions. These ions include the oxonium ions at *m/z* 204.09 (HexNAc), 366.14 (Hex-HexNAc) and (more rarely) 657.23 (Neu5Ac-Hex-HexNAc), or the HexNAc cross-ring fragment ions at *m/z* 126.06, 138.06, 144.07, 168.07, and 186.08 (Table 1) [25]. The ion at *m/z* 138.06 is frequently used in particular, as it is one of the most abundant fragment ions from specifically GlcNAc, a monosaccharide that is ubiquitous in *N*-glycans [15]. Generally, a combination of *m/z* 204.09 and one of the smaller fragment ions is used, although it has been shown that the presence of *m/z* 138.06 coincides in over 99% with the presence of *m/z* 204.09, and in over 93% of the spectra with *m/z* 366.14 [26]. These glycan fragment ions are then used to trigger an additional round of fragmentation, commonly ETD [25,27,28], although HCD-pd-CID has also been reported [26]. Using an Orbitrap Fusion, Wu et al. were able to perform a first scan with HCD, and then perform product-dependent CID and ETD at the same time, gaining the best of two methodologies [22]. Of note, in the case of HCD-pd-CID, HCD-MS^3 had to be applied to obtain sufficient information on the peptide backbone. On the other hand, increased certainty about the glycan portion can be obtained with CID. The clear advantage of this triggered approach is that the final dataset is quite concise, containing only extensive fragmentation of glycopeptides, which is beneficial as they are typically relatively rare and low abundant in complex protein digests.

While product-dependent triggering methods have shown great uses in the field of (glyco)proteomics, several challenges persist. First, MS acquisition strategies invoking neutral-loss-mediated ETD-based fragmentation typically require additional time to detect the relevant precursor *m/z* value and fragment in a second round of MS^2. However, the signal may not be present for the second ETD fragmentation cycle anymore, particularly for analytes that are of low abundance. Second, in case the precursor is detected again, the glycan may be of the same composition but of different structure. For instance, when separating glycopeptides by C18 chromatography, the retention time will mainly depend on the peptide portion of the conjugate, leading to the co-elution of different glycoforms for the same glycopeptide [29]. However, the separation depends also slightly on the glycan isomerism [29]. Hypothetically, this means that a glycopeptide carrying a diantennary *N*-glycan may have CID triggered (by HCD) on a species with an antennary fucose, but CID fragments acquired on a species with core fucosylation − a different glycan. Third, the size of the molecules limits the efficiency of fragmentation, and ultimately the sequence coverage of both the glycan and the peptide moieties. For these reasons, it may be beneficial to perform multiple fragmentation methods on the same precursor ions, as can be achieved by modern-day mass spectrometric instrumentation.

2.3. Combined fragmentation strategies

A range of methods have been reported that combine orthogonal types of fragmentation on the same selected precursor, mostly representing a combination of collisional dissociation (CID or HCD) and electron-driven dissociation (ECD or ETD) [10]. The concept was introduced by Swaney et al. with the application of resonance-excitation CID on precursors that were charge-reduced by ETD (ETciD), initially to increase the total yield of fragment ions, as this is typically low in ETD-only mode [30]. The concept was expanded upon by Liu et al., who performed high-energy CID on the precursors that were charge reduced [31]. Using a somewhat different strategy, Campbell et al. performed ETD on precursors and sought to employ CID on the unreacted population to capture the orthogonal information contained therein [32]. The Heck-group contributed to the field by the introduction of EThcD, i.e., the

Table 1
Accurate masses and identities of glycan fragment ions commonly used for product-dependent fragmentation triggering and/or targeted data analysis strategies.

Monoisotopic mass[a]	Chemical formula	Glycan composition	Examples[b]			Remarks
			Triggering	Composition	Structure	
Hexose-derived ions						
85.0284[c]	$[C_4H_5O_2]^+$	Hex-$C_2H_6O_3$		[78]		Detected in case of terminal mannose (e.g. high-mannose/hybrid glycan) [78].
97.0284	$[C_5H_5O_2]^+$	Hex-CH_6O_3		[78]		
127.0390	$[C_6H_7O_3]^+$	Hex-$2H_2O$		[78]		
145.0495	$[C_6H_9O_4]^+$	Hex-H_2O		[78]		
163.0601	$[C_6H_{11}O_5]^+$	Hex		[78]		
N-Acetylhexosamine-derived ions						
84.0444	$[C_4H_6NO]^+$	HexNAc-$C_4H_8O_4$		[78]		*O*-GlcNAc: NCE 25−50% gives increasing *m/z* 138 over 126 and 186 [18].
126.0550	$[C_6H_8NO_2]^+$	HexNAc-$C_2H_6O_3$			[18,40,78,79]	
138.0550	$[C_7H_8NO_2]^+$	HexNAc-CH_6O_3	[22,25,26]		[18,40,78,79]	*O*-GalNAc: NCE ≥30% gives increasing *m/z* 144 over 126 or 186 [18].
144.0655	$[C_6H_{10}NO_3]^+$	HexNAc-$C_2H_4O_2$			[18,40,78,79]	
168.0655	$[C_8H_{10}NO_3]^+$	HexNAc-$2H_2O$			[18,40,78,79]	The presence of Gal or Neu5Ac extensions of the *O*-HexNAc does not alter the HexNAc fragmentation pattern.
186.0761	$[C_8H_{12}NO_4]^+$	HexNAc-H_2O		[18]		
204.0867	$[C_8H_{14}NO_5]^+$	HexNAc	[18,22,25,27,28,78]	[18,79]	[19]	
Sialic acid-derived ions						
274.0921	$[C_{11}H_{16}NO_7]^+$	Neu5Ac-H_2O	[25]	[18]	[19]	Neu5Ac: NCE ≥20% gives increasing *m/z* 274 and 292 over 204 and 366, strongly with α2,3-linkage, moderately with α2,6-linkage [19].
292.1027	$[C_{11}H_{18}NO_8]^+$	Neu5Ac	[25]	[18]	[19]	
290.0870	$[C_{11}H_{16}NO_8]^+$	Neu5Gc-H_2O				
308.0976	$[C_{11}H_{18}NO_9]^+$	Neu5Gc				
Di- and trisaccharide ions						
366.1395	$[C_{14}H_{24}NO_{10}]^+$	Hex-HexNAc	[18,22,25,78]		[19]	Common indicators for the presence of *N*-glycans (366 and 657), and branched *O*-glycans or LacdiNAc (407 and 569)
407.1660	$[C_{16}H_{27}N_2O_{10}]^+$	$HexNAc_2$		[79]		
569.2188	$[C_{22}H_{37}N_2O_{15}]^+$	Hex-$HexNAc_2$		[79]		
657.2349	$[C_{25}H_{41}N_2O_{18}]^+$	Neu5Ac-Hex-HexNAc	[25]	[19]		

[a] All species in the table are considered to be $[M + H]^+$.
[b] These references are not intended to be fully comprehensive, but provide examples that are described in this review.
[c] Monoisotopic masses are calculated using atomic masses obtained from CIAAW. Atomic weights of the elements 2017. Available online at www.ciaaw.org ($^{12}C = 12.0000$, $^{1}H = 1.0078$, $^{14}N = 14.0031$, $^{16}O = 15.9949$).

application of HCD on all ions resulting from the ETD process, subjecting both the charge-reduced and unaffected precursor ions to collisional activation in the HCD cell [11]. By this last method, a significantly larger fraction of the ions is used to generate fragment ions, leading to considerably more and more intense signals (and fragmentation mechanisms) that can be used for analyte interpretation and spectral matching. Further developments in this area include activated-ion ETD (AI-ETD), in which precursor ions are irradiated by infrared photons prior to ETD, AI-ETD$^+$, in which said ions are also post-activated by infrared multiphoton activation, and ET-UVPD, which refers to ETD with supplemental UV photoactivation [10].

Several studies have made use of the above-mentioned combined fragmentation strategies to characterize *N*- and *O*-glycopeptides of biological samples. A recent large-scale application of EThcD was reported by Yu et al., in a substantial work on the characterization of the human plasma glycoproteome and glycopeptides derived from rat arteries [33]. From plasma, they were able to detect 331 unique *N*-glycopeptides across 66 N-glycosylation sites, and from rat carotids over 2000 glycopeptides were identified, and fragmentation by EThcD was compared to that with HCD (Fig. 2). Using a similar strategy, Yu et al. also reported the analysis of a complex sample of *O*-glycopeptides, but only applied the fragmentation when oxonium ions were detected in a prior HCD fragmentation scheme, i.e., HCD-pd-EThcD, saving on time spent on ETD [34]. HCD-pd-EThcD was also recently used by Pap et al. to screen complex *N*- and *O*-glycan samples from urinary protein tryptic digests, with a focus on the data analysis aspects of the workflow [35].

O-Glycoproteomics proved a particularly useful approach for the analysis of immunoglobulin A nephropathy (IgAN), a pathology that is characterized by IgA1 polymerization associated with aberrant *O*-glycosylation [36]. Individual IgA1 hinge-region *O*-glycosylation sites could be studied using an AI-ECD fragmentation regimen for LC-FTICR-MS, which was compared by Takahashi et al. with ETD fragmentation. The method led to the successful determination of the sites that were deficient in galactosylation [37]. Recently, Zhang et al. studied the IgAN-patient whole serum *O*-glycopeptidome with HCD and EThcD fragmentation, which led to the detection of 499 non-redundant *O*-glycopeptides covering a total of 173 *O*-glycosylation sites [38]. Next to information on the IgA1 *O*-glycosylation, EThcD in particular allowed for the *O*-glycan characterization of 48 other serum proteins.

A further application in *O*-glycoproteomics, both EThcD and CID/HCD-triggered ETD assisted in the recognition and site-determination of *O*-GlcNAc residues on glycopeptides presented by class I human leukocyte antigen (HLA), as observed by both Malaker et al. and Marino et al. independently [39,40]. The latter study furthermore employed stepping-energy HCD to distinguish *O*-GlcNAc residues from *O*-GalNAc residues, allowing for an estimation of the relative occupancy by these HexNAcs on a site-to-site basis [40].

Acidic sugars typically have been more challenging to analyze with mass spectrometry than neutral sugars, in a large part due to the labile nature of sialic acids. On the other hand, acidic moieties do enable specific enrichment with stationary phase materials such as TiO_2, providing the opportunity to enrich for sialylated as well as phosphorylated peptides. As such, Glover et al. have reported immobilized metal affinity chromatography (IMAC) enrichment for combining phosphoproteomics, sialylated glycoproteomics, and phosphoglycoproteomics (which concerns glycans containing mannose-6-phosphate) [41]. Comparing EThcD to HCD-pd-EThcD (triggering on dehydrated Neu5Ac at *m/z* 274.09), the authors identified a higher number of sialylated peptides with the latter method. Čaval et al. have recently used HCD-pd-EThcD on Fe^{3+}-IMAC enriched glycopeptides, triggering on a particular phosphomannose marker ion (*m/z* 243.026) to specifically target the mannose-6-phosphorylation-containing peptides that are typically involved in lysosomal trafficking [80]. Kuo et al. used TiO_2 enrichment and a variety of MS^2 methods (CID, HCD, ETD and EThcD) to study not only sialylated peptides, but also sulfoglycopeptides [42].

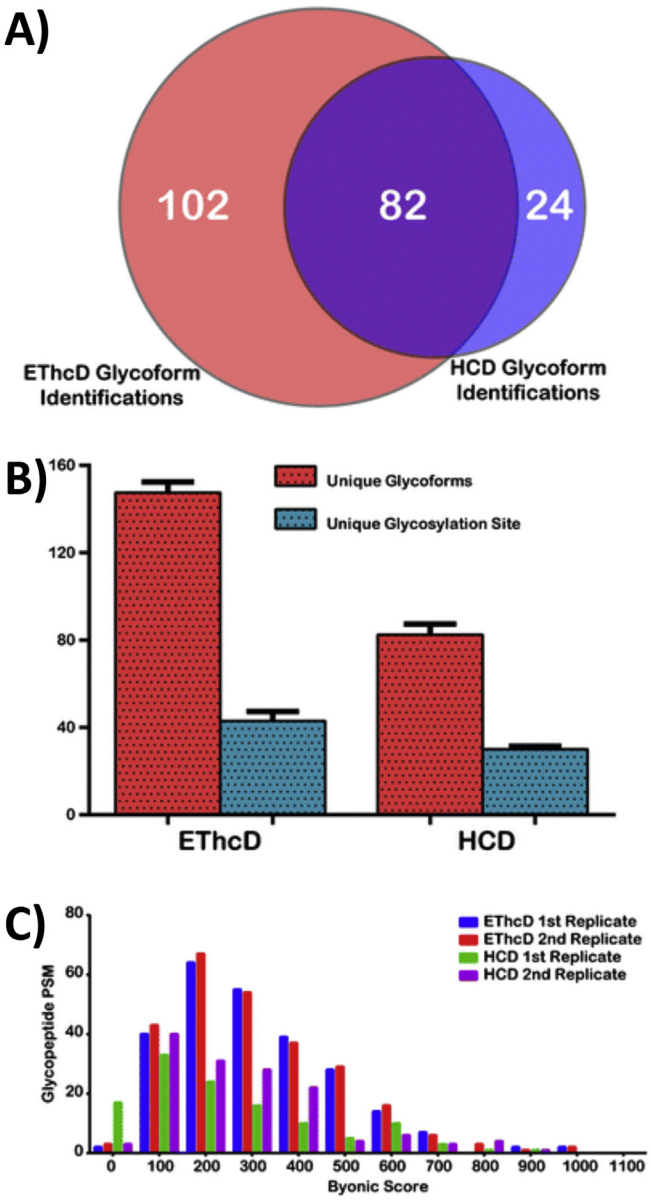

Fig. 2. Comparison of EThcD and HCD for the identification of tryptic glycopeptides from human serum. A) and B) show the number of glycopeptides and sites of glycosylation as identified by the used Byonic software suite (Protein Metrics Inc.). C) The distribution of Byonic scores of the identified glycopeptide spectral matches. Reprinted with permission from Ref. [33], © American Society for Mass spectrometry 2017.

One particular strength of EThcD is the applicability to 'middle-down' proteomics, i.e., the study of large peptides and glycopeptides such as typically generated by proteases other than trypsin, or the study of peptides that are multiply glycosylated. Giansanti et al. explored workflows for using several of these alternative proteases (i.e., chymotrypsin, LysC, LysN, AspN, GlyC, and ArgC) in phosphoproteomics [43]. Through this and other studies, it has become apparent that EThcD outperforms ETD and HCD especially for larger middle-sized (glyco)peptides [44,45].

Combining middle-down proteomics with native MS proved to be an helpful approach for the deep characterization of proteoforms, as demonstrated on fetuin, erythropoietin and several complement proteins [46–49]. In this approach, site-specific glycosylation analysis could be performed on the digested proteins (using tryspin, GluC and AspN) by middle-down MS using HCD and EThcD, characterizing concomitantly the *N*- and *O*-glycosylation sites. This information proved a requirement for the annotation of the complex PTM profiles observed in the native intact protein mass spectra. In addition to the characterization of *N*- and *O*-glycosylation, it proved possible to characterize the substantial *C*-mannosylation (carbon-linked mannoses) as featured on terminal complement proteins and properdin (Fig. 3). Pronker et al. reported the use of ETciD for the localization of *C*-mannosylation (carbon-linked mannoses) to specific amino acid residues of myelin-associated glycoprotein [50], whereas Maynard et al. demonstrated the presence of *S*-linked GlcNAcylation (GlcNAc residues attached to the sulfur of a cysteine) on 14 sites across 11 murine membrane proteins by EThcD [51].

As final point, hybrid fragmentation schemes have also recently been combined with various metabolic and isobaric stable isotope labeling strategies for analyte quantification. Parker et al. applied an HCD-pd-EThcD *N*-glycoproteomics workflow to the analysis of adipocyte membrane glycoproteins using a stable-isotope labeling with amino acids in cell culture (SILAC) approach [52]. Woo et al. established azido sugar metabolic labeling for subsequent ETD/EThcD analysis [53], a concept what was followed up by alkynyl sugars for the study of the sialylated glycoproteome [54]. The application of isobaric tags specifically for EThcD (namely, *N*,*N*-dimethyl leucine) was recently demonstrated by Chen et al. in an application on the characterization and quantification of *N*-glycopeptides from a breast cancer cell line [55].

3. Future perspectives

In recent years the field of glycoproteomics is expanding its scope from *N*-glycan analysis to glycopeptide analysis and even intact glycoprotein analysis [14]. In analyzing glycopeptides, it has become apparent that non-standard CID-based fragmentation schemes are much needed, and reports are accumulating that show the use of hybrid fragmentation methodologies. In our definition these broadly include all methods that fragment a selection of precursor ions with multiple types of fragmentation, either by altogether different dissociation mechanisms or by switching energy levels. The stepping in collision energy, the use of orthogonal fragmentation methods and the combination of fragmentation mechanisms have all shown substantial contributions to the high-density and confident site-specific assignment of glycoconjugates – extending the coverage of the glycoproteome as a consequence. These non-standard fragmentation techniques have also allowed the simultaneous analysis of phosphorylated and sulfonated glycopeptides, and glycoconjugates of not only *N*- and, *O*-linkage, but also of the *S*- and *C*-linked variants that are reported less frequently [56–58]. While hybrid fragmentation may not be optimal for all experimental setups (longer analysis time per analyte could lead to a loss of sensitivity, site information may be lost if CID/HCD removes *O*-glycans from the peptide altogether), groups also report definite benefits for the quality and number of glycosites/glycoforms detected (e.g., by EThcD compared to HCD alone; Fig. 2) [33] and richer MS^2 spectra for site-specific localization when glycosylation markers remain associated (e.g., by EThcD over CID and HCD; Fig. 3) [46].

Having said this, it has to be noted that current-day mass spectrometric glycoproteomics techniques are not yet capable of unraveling all biologically relevant aspects of glycosylation, and it is typically not yet possible to obtain full peptide sequence coverage for a given protein. For glycosylation it remains particularly difficult to resolve by mass spectrometry alone the stereoisomerism between monosaccharides (for example GlcNAc *vs.* GalNAc, mannose *vs.* galactose), the linkages between monosaccharides (α-linked *vs.* β-linked galactosylation, α2,3- *vs.* α2,6-linked sialylation), as well as the complex branching patterns that structures may adopt (hybrid-type *vs.* α-galactosylated, a third antenna on the α1,3-branch *vs.* the α1,6-branch). For released glycan analysis, chromatographic and electrophoretic separation technologies have started to separate these molecules, HILIC, CE and especially PGC providing good

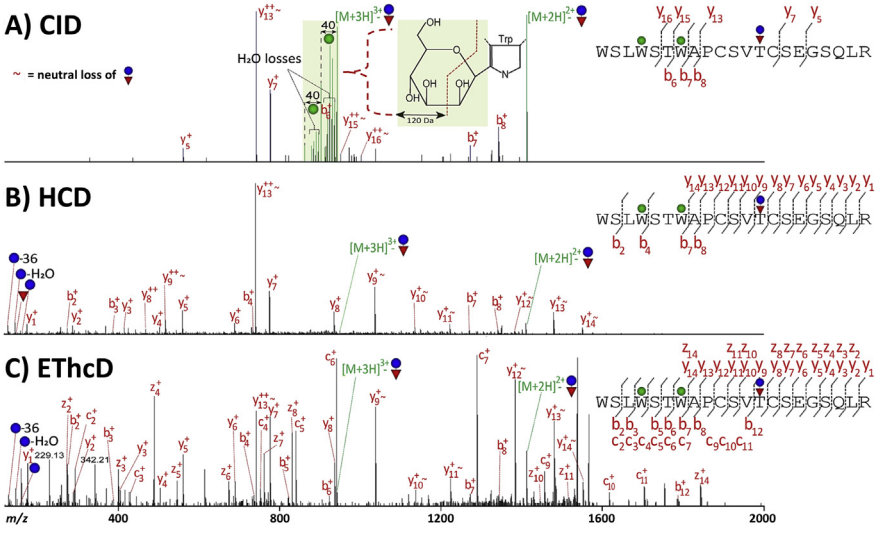

Fig. 3. Exemplary MS^2 spectra of a tryptic glycopeptide derived from properdin, containing two *C*-mannoses and a Fuc-Hex disaccharide. A) CID fragmentation shows the 120 Da loss (indicated with mass differences of 40 for triply charged ions), which is typical for aromatic *C*-glycosides. The fragmentation process is accompanied with multiple H_2O losses. B) HCD fragmentation generates C-terminal y-ions providing higher sequence coverage. Diagnostic ions in the lower mass region indicate the presence of glycans in the peptide and may be used for the sequential triggering of ETD. C) EThcD spectrum, containing next to y- and b-ions also c- and z-ions. These latter ions retain the labile *O*-glycan and may therefore be instrumental in determining its accurate position on the peptide. All MS^2 spectra were analyzed by Byonic software (Protein Metrics Inc.). The EThcD spectrum was reprinted and modified with permission from Ref. [46], under the Creative Commons 4.0 license (https://creativecommons.org/licenses/by/4.0/).

separation of isomeric structures [5,7]. Exoglycosidase sequencing and lectin capturing can help to resolve some of these aspects for MS (depending on the availability and the specificity of these molecules), but further advances are required for obtain full information on the glycan structure.

Interestingly, hybrid fragmentation experiments (in this case energy-stepping CID) have shown that oxonium ions generated in the lower *m/z* end of mass spectra can be indicative of, for example, the difference between GlcNAc and GalNAc residues on relatively simple structures, as well as linkage variants of sialylation [18,19]. It can be imagined that other structural aspects of glycosylation may be determined by in-depth fragmentation as well, especially in MS^n experiments in which individual branches and antennae can be isolated and characterized. Of note, glycan-based fragment ions may also be used to score the likelihood of a fragmentation pattern to be derived from a glycopeptide precursor, an important requirement for large-scale glycoproteomics experiments and automated data analysis [26,59–61].

Another exciting application of modern-day MS technology is the additional separation of ions inside the mass spectrometer, made possible by ion mobility (IM)-MS [62,63]. For small sugar molecules (e.g. fragments) IM has shown to separate structural differences such as α- and β-anomerism and β1,3- *vs.* β1,4-linked galactosylation [62,63]. In all cases, development in the field would require well-characterized glycan and glycopeptide standards, including tri- and tetraantennary glycan species with multiple types of sialylation, acetylation, sulfonation, phosphorylation, glucuronylation, and other motifs. Recent efforts in glycoengineering may prove enabling in this regard [27,64,65].

Chemical derivatization also has the potential to enhance the MS analysis of glycoconjugates, but this still requires substantial method development. For analysis by MALDI-TOF-MS and ion trap-MS with CID, chemical modification of carboxylic acid residues has shown highly beneficial for the stabilization and ionization propensity of sialylated glycopeptides (and may in some cases distinguish sialic acid linkage isomers) [66,67], and with hybrid fragmentation one example has been reported in which sialylated and sulfated glycopeptides were modified by acethydrazide amidation [42]. Another derivatization strategy, permethylation, has been widely employed in released glycan analysis to enhance ionization and fragmentation [68], and recently papers have been appearing in which permethylation proved achievable for glycopeptide mixtures as well [69]. For hybrid MS, these approaches may provide additional oxonium or cross-ring ions to perform selection for product-dependent MS^n, or different fragmentation pathways with orthogonal information on glycan structure. One critical aspect here is the flexibility of databases and proteomics search actions, as mass shifts will take place on the glycan and the peptide.

In addition to expanding the structural information on the glycan, it is imperative to increase the sequence coverage of the originating proteins, one direction being the optimization of sample preparation [43,44]. It has been shown that a careful selection of proteases is of great benefit for bottom-up and middle-down proteomics, and developments are ongoing for the discovery, development and exploitation of new binding sites and specificities. For *N*-glycoproteomics, a recently reported example includes flavastacin, which cleaves C-terminally of an *N*-glycosylated asparagine [70], while for *O*-glycoproteomics new developments include an enzyme that cleaves N-terminally of *O*-glycosylated serine/threonine residues (OgpA) [71].

Similar developments are ongoing amongst the methodologies for fragmentation, with the aim of increasing sequence coverage and fragment types. An exciting prospect for glycoproteomics is the development of AI-ETD, in which ions are pre-activated and partially dissociated by infrared radiation after which further fragmentation is achieved by ETD [72], which is gaining traction for the analysis of complex sample types [10]. Another promising direction is the development and application of UVPD [73–75]. With this technique, molecules are photodissociated with semi-random sites of reactivity, and dissociation can occur both on the peptide and the glycan portion of the conjugate [76]. While application of the method has so far been reported for glycans, peptides and glycopeptides [73–75], a combination of the technique with other modes of dissociation (e.g., HCD) still needs to be further investigated. Both AI-ETD and UVPD are expected to be especially beneficial for middle-down proteomics, as they exhibit efficient fragmentation [77]. Their applicability to native and top-down glycoproteomics still needs to be ascertained.

To summarize, hybrid fragmentation mechanisms have shown major benefits within the field of glycoproteomics, as have the surrounding developments in sample preparation and data interpretation. We anticipate that the future of glycoproteomics will be in a combined analysis of glycans, glycopeptides and intact glycoproteins especially, by virtue of their different levels of throughput and information content [14]. The further development of in-depth fragmentation techniques will be critical for all of these directions, and advancements are expected in glycoform structural characterization and the sequence coverage of peptides, and we envision the dissociation of ever larger glycoconjugates.

Dedication

This paper is dedicated by AJRH to the late Hanfa Zou, Dalian Institute of Chemical Physics, a friend and exceptionally creative scientist in analytical chemistry and a great mentor and collaborator for many throughout the world.

Declarations of interest

None.

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