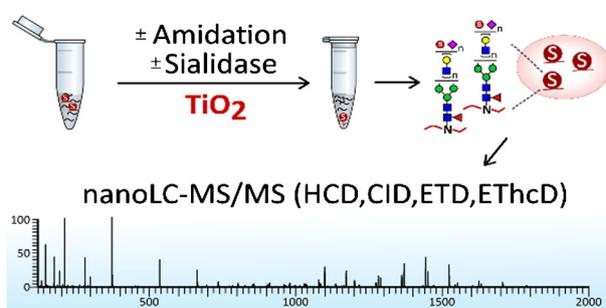


Distinctive and Complementary MS² Fragmentation Characteristics for Identification of Sulfated Sialylated *N*-Glycopeptides by nanoLC-MS/MS Workflow

Chu-Wei Kuo, Shih-Yun Guu, Kay-Hooi Khoo

Institute of Biological Chemistry, Academia Sinica, 128, Academia Road, Sec. 2, Nankang, Taipei, 11529, Taiwan



Abstract. High sensitivity identification of sulfated glycans carried on specific sites of glycoproteins is an important requisite for investigation of molecular recognition events involved in diverse biological processes. However, aiming for resolving site-specific glycosylation of sulfated glycopeptides by direct LC-MS² sequencing is technically most challenging. Other than the usual limiting factors such as lower abundance and ionization efficiency compared to analysis of non-

glycosylated peptides, confident identification of sulfated glycopeptides among the more abundant non-sulfated glycopeptides requires additional considerations in the selective enrichment and detection strategies. Metal oxide has been applied to enrich phosphopeptides and sialylated glycopeptides, but its use to capture sulfated glycopeptides has not been investigated. Likewise, various complementary MS² fragmentation modes have yet to be tested against sialylated and non-sialylated sulfoglycopeptides due to limited appropriate sample availability. In this study, we have investigated the feasibility of sequencing tryptic sulfated *N*-glycopeptide and its MS² fragmentation characteristics by first optimizing the enrichment methods to allow efficient LC-MS detection and MS² analysis by a combination of CID, HCD, ETD, and EThcD on hybrid and tribrid Orbitrap instruments. Characteristic sulfated glyco-oxonium ions and direct loss of sulfite from precursors were detected as evidences of sulfate modification. It is anticipated that the technical advances demonstrated in this study would allow a feasible extension of our sulfoglycomics analysis to sulfoglycoproteomics.

Keywords: Sulfated sialylated glycopeptide, Sulfoglycopeptides, Sulfoglycomics, Titanium dioxide, EThcD, HCD, Orbitrap Fusion Tribrid, Mass spectrometry

Received: 4 December 2017/Revised: 7 February 2018/Accepted: 8 February 2018/Published Online: 11 April 2018

Introduction

High sensitivity mass spectrometry (MS)-based identification of sulfate modification on a diverse range of sialylated mammalian *N*-glycans remains technically challenging despite its well-appreciated biological importance (see [1]). Time and again, glycan array screening revealed that many

endogenous glycan-binding proteins including members of the important classes of selectins and Siglecs bind the sulfated counterparts of their cognate ligands at significantly higher affinity. The prime examples are the 6-sulfo sialyl Le^x expressed on the high endothelial venule of peripheral lymph node that mediates lymphocyte homing [2, 3], and the 6-sulfo- α -2,6-sialyl LacNAc on human B cells that constitutes a higher affinity ligand of human CD22, an inhibitory co-receptor of B cells [4, 5]. In most cases, whether a terminal glyco-epitope or glycotope would be presented at optimal density, valency and accessibility to function as a proper recognition code is critically dependent on the underlying glycan structures and specific site localization on particular glycoprotein carriers.

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s13361-018-1919-9>) contains supplementary material, which is available to authorized users.

Correspondence to: Kay-Hooi Khoo; e-mail: kkhoo@gate.sinica.edu.tw

Moreover, additional modifications such as sulfate would attenuate the binding affinity by conferring negative charge at or near the contact sites, to the extent that it may ultimately fine tune and determine its pathophysiological consequences.

To address the occurrence and functional consequences of sulfate modifications, we have recently established the enabling sample preparation and MS techniques for identifying sulfated glycans, thus making significant headways in sulfoglycomics [6, 7]. However, for reasons elaborated above, a grand challenge in all glycomic studies is whether the identified, biologically relevant glycotopes can be further localized to which protein carriers at which sites in a follow-up glycoproteomic venture [8, 9]. To date, while many heavily *O*-glycosylated mucins are known to carry sulfated *O*-glycans, only a handful of glycoproteins have been biochemically characterized to carry sulfated *N*-glycans and far too few were directly demonstrated at glycopeptide level by MS. It is not known if there is generally a low sulfation level for most *N*-glycosylated sites, or the distribution of sulfated *N*-glycans has a distinctive protein site-specific preference. To answer such critical questions, a reliable and highly sensitive method that can confidently detect the typically low abundant sulfated *N*-glycopeptides among the complex proteomic digests is needed.

The key limiting factors in all MS-based glycoproteomics are satisfactory glycopeptide enrichment, diagnostic ions to filter, select and identify a glycopeptide, sufficient peptide backbone cleavage ions to allow unambiguous sequencing and site localization, and computational tools to facilitate data analysis. These issues have each been intensively studied and discussed recently [10–13]. Although many different variations of enrichment methods and formats have been developed, the principles largely converged on selective capture based on lectin affinity or higher hydrophilicity and/or larger size imparted by the extra glycan moiety carried on the peptide backbone. In the context of sulfated glycopeptides, no suitable lectin or universally applicable antibody is currently available. On the other hand, their extra negative charge makes it attractive for enrichment methods based on ionic interactions or metal chelation such as the titanium dioxide (TiO₂) method originally developed for phosphopeptides but has since been adapted to sialoglycopeptides [14, 15]. It is, however, untested and unknown if sulfated glycopeptides can actually be selectively captured by TiO₂ and whether these can be discriminated from equally negatively charged sialylated glycopeptides. We have previously shown that sulfated glycopeptides from recombinant erythropoietin (rEPO) produced in BHK 21 cells could be efficiently captured by amine-functionalized magnetic nanoparticles, along with other glycopeptides without any apparent specificity or enrichment preference [16]. Toyoda et al. demonstrated a strategy by utilizing ion exchange chromatography to enrich sulfated *N*-glycopeptides from bovine luteinizing hormone after limited protease digest and modification with acetohydrazide that neutralized all carboxylic groups [17]. Unfortunately, its applicability to capturing intact sulfated sialylated glycopeptides has not been demonstrated.

With or without prior enrichment, detection of glycopeptides is facilitated by the abundant diagnostic glyco-oxonium ions resulting from facile glycosidic cleavage at GlcNAc [18, 19]. In particular, HexNAc⁺ at *m/z* 204.0867 and Hex-HexNAc⁺ at *m/z* 366.1396 are almost universally produced by conventional beam type collision induced dissociation (CID) as implemented on a Q/TOF instrument or, more recently, by higher energy collisional dissociation (HCD) on various Orbitrap-based instruments. Positive detection of these oxonium ions at high mass accuracy are now commonly used in many data analysis tools as a criterion to filter out true glycopeptide MS² spectra and/or positively contributing to identification scores [11]. Their detection by HCD MS² in the initial LC-MS/MS data-dependent acquisition event on a hybrid or tribrid Orbitrap instrument is also commonly defined as criteria to trigger additional product-dependent acquisition of other MS² data such as ion trap CID or electron transfer dissociation (ETD) [20, 21]. In general, glycopeptides carrying different terminal sialylated and/or fucosylated glycotopes, with and without other non-saccharide substituents, are expected to yield the corresponding diagnostic oxonium ions [19, 22], but the prospect of detecting diagnostic sulfated glyco-oxonium ions indicative of sulfated *N*-glycopeptides for selective data acquisition or analysis has not been investigated.

Although HCD and other beam type or ion trap CID MS² alone may be sufficient for glycopeptide identification, current state-of-the-art glycoproteomics increasingly relies on complementary use of all three MS² modes, namely HCD, trap CID, and ETD, available on the hybrid LTQ-Orbitrap or tribrid Orbitrap Fusion instruments [13, 23, 24]. It is now well appreciated that ion trap CID provides more fragment ions corresponding to sequential losses of glycosyl residues from the precursor, with very few peptide sequence informative *b* and *y* ions. HCD fragmentation is generally similar to trap CID but typically yields more of the peptide backbone cleavages and the oxonium ions without the low mass cutoff problem associated with trap CID. ETD, on the other hand, would retain the intact glycan but produces a more complete series of *c* and *z* peptide cleavage ions. This is particularly useful for site localization when sequencing *O*-glycopeptides [23], but this fragmentation mode is generally less efficient and better suited for glycopeptides of higher charge density with overall low *m/z* values. Supplemental activation is thus often introduced to promote more fragmentation. Recently, this takes on a new activation mode on the Orbitrap Fusion, which itself offers a much improved ETD performance. In the so-called EThcD, additional HCD at lower energy than that of standalone HCD was applied after initial ETD activation [25, 26]. The fragmentation characteristics of glycopeptides under these different MS² modes have been well studied and documented [12, 13, 23, 27] but not extended to sulfated *N*-glycopeptides.

As a prerequisite for any meaningful MS-based sulfoglycoproteomics, we have set out in this work to investigate the LC-MS² characteristics of sulfoglycopeptides on both the hybrid and tribrid Orbitrap instruments, focusing on the sulfated *N*-glycopeptides derived from tryptic digests of a

largely non-sialylated rEPO from BHK21 cells and the highly sialylated bovine thyroglobulin (bTG). We demonstrated that TiO₂ could be used to enrich the sulfated *N*-glycopeptides, but better specificity was attained for sialylated glycopeptides. Sulfated glyco-oxonium ions, particularly that of sulfo LacNAc (Hex-HexNAc), could indeed be detected under HCD and EThcD and used as criterion for selective filtering of sulfoglycopeptide MS² spectra. Direct loss of a sulfite moiety from the precursors under trap CID and EThcD would constitute another important cue for verifying the presence of sulfate substitution. Formation of these diagnostic ions would be adversely affected by sialylation but could be partly alleviated. Other than these features, the overall MS² fragmentation pattern for *N*-glycopeptides as currently known for the different modes of MS² is not significantly altered by carrying an extra sulfate, and thus, their reliable sequencing and identification are subjected to the same practical considerations and shortcomings.

Experimental

Materials

Thyroglobulin from bovine thyroid (bTG) was obtained from Sigma-Aldrich (St. Louis, MO). Recombinant erythropoietin (rEPO) from BHK21 cells was purchased from Advanced Gene Technology Corp. (Taiwan). ZIC-HILIC® (Zwitterionic Hydrophilic Interaction Liquid Chromatography) ProteaTip Sample Prep Kit was from Protea Biosciences (Morgantown, WV), titanium dioxide TiO₂ (Titansphere, 5 μm) from GL Sciences Inc. (Japan), peptide *N*-glycosidase F (PNGase F) and *Arthrobacter ureafaciens* neuraminidase from Roche Diagnostics (Mannheim, Germany), and sequencing grade, modified trypsin from Promega (Madison, WI).

In-Solution Proteolytic Digestions

Proteins were reduced with 10 mM dithiothreitol at 37 °C for 1 h, alkylated with a final concentration of 50 mM iodoacetamide in 25 mM ammonium bicarbonate buffer for 1 h in the dark at room temperature. After that, the concentration of dithiothreitol was adjusted to 50 mM and proteins were treated overnight with sequencing grade trypsin at an enzyme-to-substrate ratio of 1:50 at 37 °C. The digested products were then diluted with formic acid to a final concentration with 0.1% for further experiments.

Release and Permethylolation of *N*-Glycans

One hundred forty microgram bTG protein was dissolved in 6 M urea with 25 mM ammonium bicarbonate buffer and reduced by 10 mM dithiothreitol at 37 °C for 1 h, followed by alkylation with 50 mM iodoacetamide at room temperature for 1 h in the dark. The sample was further diluted with 25 mM ammonium bicarbonate buffer to a final concentration of 1 M urea and digested overnight with trypsin at 37 °C. After heat denaturation, the peptides were treated overnight with

PNGaseF at 37 °C to release *N*-glycans. Released *N*-glycans were isolated from peptides by passing through a C₁₈ Sep-Pak cartridge (Waters) in 5% acetic acid and dried down by SpeedVac. Slurry of ground NaOH pellets in dimethyl sulfoxide was added to dried glycan samples in glass tube, followed by addition of methyl iodide. The reaction was carried out at room temperature for 30 min with gentle vortex and then terminated by dropwise addition of water. The permethylated glycans were extracted into chloroform, washed repeatedly with water, and then dried down under a stream of nitrogen.

MALDI-MS Profiling of Permethylated *N*-Glycans

The permethylated glycan samples were mixed 1:1 with either 2,5-dihydroxybenzoic acid (DHB) matrix solution (10 mg/mL in 50% acetonitrile) for positive ion mode detection or 3,4-diaminobenzophenone (DABP) matrix (10 mg/mL in 50% acetonitrile) for negative ion mode on a MALDI target plate. Data acquisition was performed manually on the MALDI TOF/TOF 5800 system (Sciex) and operated in the reflectron mode. The laser intensity was set at 4500, and 5000 shots were used to generate one spectrum.

Glycopeptide Enrichment by TiO₂

Procedures used to enrich sulfated glycopeptides were modified according to the methods used to enrich sialoglycopeptides [28]. Briefly, peptide mixtures were diluted with TiO₂ loading buffer (1 M glycolic acid, 80% acetonitrile, 1% trifluoroacetic acid (TFA)) in 1:10 ratio, added to TiO₂ beads, and incubated for 30 min in a vortex mixer. The supernatant was then removed after centrifugation, and the beads were washed with loading buffer for 1 min by incubation in the mixer. After settling down the beads by centrifugation, the supernatant was removed and the beads washed sequentially with washing buffer I (80% acetonitrile, 1% TFA) and washing buffer II (2% acetonitrile, 0.1% TFA) before drying down in a SpeedVac. The bound glycopeptides were eluted by incubating with elution buffer (1.5% ammonia in water) for 20 min in the mixer, then collected into the supernatant after centrifugation and dried down.

Sialic Acid Modification with Acetohydrazide/EDC

The glycopeptides were derivatized according to procedures described in previous work [17] but with some changes. Peptide mixtures were dried down and dissolved in 100 μL of 1 M acetohydrazide (adjusted to pH 3.5–4 with 0.5 N HCl) for 10 s with vortex. One hundred microliters of 0.5 M *N*-ethyl-*N'*-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) in 1:1 water/methanol solution was then added to the sample solution. The mixtures were vortexed for 10 s, and the solution evaporated using SpeedVac. The pellet was resuspended in 500 μL of 1:1 water/methanol solution, vortexed, and evaporated again. Finally, the pellet was redissolved in 0.1% formic acid for cleanup.

Analysis of Glycopeptides by LC-MS²

Samples for LC-MS² analyses were cleaned up using ZipTip C₁₈ (Millipore), dried down, and redissolved in 0.1% formic acid. For analysis of sulfated glycopeptides with various fragmentation modes, data were performed on an LTQ-Orbitrap Elite or an Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Scientific), fitted with a PicoView nanospray interface (New Objective) and a nanoACQUITY UPLC system (Waters) (for LTQ-Orbitrap Elite) or Easy-nLC 1200 (for Orbitrap Fusion Lumos Tribrid) (Thermo Scientific). For nanoACQUITY UPLC system, peptide mixtures were loaded onto a 75 $\mu\text{m} \times 250$ mm nanoACQUITY UPLC BEH130 column packed with C18 resin (Waters) and were separated at a flow rate of 300 nL/min using a linear gradient of 5 to 55% solvent B (95% acetonitrile with 0.1% formic acid) in 35 min. Solvent A was 0.1% formic acid in water. For Easy-nLC 1200, peptide mixtures were loaded onto an Acclaim PepMap RSLC 25 $\text{cm} \times 75$ μm i.d. column (Dionex) and separated at a flow rate of 300 nL/min using a gradient of 5 to 45% solvent B (100% acetonitrile with 0.1% formic acid) in 60 min. Solvent A was 0.1% formic acid in water. The mass spectrometer was operated in the data-dependent mode. Briefly, survey full-scan MS spectra were acquired in the Orbitrap with the resolution set to 120,000. The most intense ions (Elite) or highest charge state ions (Lumos) were sequentially isolated for MS² analysis using the following settings: CID MS² fragmentation and detection in the ion trap on Elite with AGC target at 5×10^4 , isolation window 3, collision energy (%) 35; HCD MS² with detection in the Orbitrap on Elite with AGC target at 5×10^4 , isolation window 3, collision energy (%) 30, resolution 30,000; HCD MS² with detection in the Orbitrap on Fusion Lumos with AGC target at 5×10^4 , isolation window 2, collision energy (%) 28. An HCD-pd-ETD workflow was employed to additionally trigger ETD events upon detecting the HCD product ions at m/z 204.0867, 138.0545, and 366.1396. Supplemental activation was enabled on the LTQ-Orbitrap Elite, and the ETD MS² ions were detected in ion trap. Similar HCD-pd-ETD was enacted on the Orbitrap Fusion Lumos using the same HCD product ions to trigger ETD. For ETD, the calibrated charge-dependent parameter was used and supplemental activation was allowed with a 15% SA collision energy. The ETD ions were detected in the Orbitrap at a resolution of 60,000.

Results and Discussion

While direct LC-MS/MS analysis of the proteolytic digests of an isolated glycoprotein is highly desirable, some form of glycopeptide enrichment is inevitable for analysis at the glycoproteomic level. Even for single glycoprotein, efficient enrichment of sulfated glycopeptides would be required since the degree of sulfation is typically low. We thus set out to evaluate if the more commonly used glycopeptide enrichment method would allow satisfactory recovery of sulfated glycopeptides. rEPO produced in BHK21 cells is unique in that it is

highly sulfated with very little sialylation [6] and hence provide an ideal sample to evaluate if the negatively charged sulfate carried on the glycans can be relied on for enrichment in the same manner that sialylation (and phosphorylation) allows their selective capture by TiO₂. Once the characteristic features including the MS² patterns were established, a more complex sulfated and sialylated glycoprotein, bovine thyroglobulin (bTG), was employed to further evaluate how these may differ when sialylation co-exists with sulfation to give terminal sialylated sulfo LacNAc glycotopes, from the perspectives of glycopeptide enrichment, chromatography on reverse phase (RP) C18 capillary column, and the differential fragmentation patterns under different MS² modes.

Enrichment of Sulfated Glycopeptides

We first attempted to enrich sulfated glycopeptides with commercialized ZIC-HILIC tip according to recommended procedures. Applying to tryptic peptides derived from sulfated rEPO glycoprotein, we detected only sulfated *O*-glycopeptides but no sulfated *N*-glycopeptides (data not shown) after ZIC-HILIC enrichment, suggesting that this procedure may not be suitable for general sulfated glycopeptide enrichment. We then adopted conditions used to enrich sialylated glycopeptides by TiO₂ [15] to the same sample. With 5% TFA in glycopeptide sample loading step, only the sulfated *O*-glycopeptides could be efficiently retained by TiO₂ (Fig. S1D) and not the sulfated *N*-glycopeptides (Fig. S1A). We found that decreasing the acid concentration in loading step from 5 to 1% TFA or even 0.1% significantly increased the signals detected for not only sulfated *O*-glycopeptides (Fig. S1E and F) but also sulfated *N*-glycopeptides from rEPO (Fig. S1B and C). However, the use of 0.1% TFA in sample loading step significantly increased the non-specific binding of peptides. Therefore, 1% TFA was chosen as the optimized sample loading condition for TiO₂ enrichment in all subsequent work.

LC-MS² Detection of Sulfated Glycopeptides

For rEPO produced in BHK21 cells, the monosulfated glycopeptides could be readily detected as multiply protonated species (Fig. 1a), typically in more than one charge states and accompanied by minor forms of ammonium/sodium adducts, after a simple step of TiO₂ enrichment. The non-sulfated glycopeptides were, however, not excluded by the enrichment, whereas disulfated ones could be additionally detected. It is noted that the additional presence of a sulfate moiety would significantly increase the retention time of the tryptic glycopeptides on a RP C18-nanoLC capillary column, as evidenced from an overlapping extracted ion chromatogram plot of the non-sulfated, monosulfated, and disulfated *N*-glycopeptides carrying the same peptide backbone and non-sialylated, LacNAc₄-containing complex type *N*-glycan (Fig. 1b). In contrast, extra LacNAc units would shift the elution to slightly earlier time as it increases the overall hydrophilicity by the glycan size.

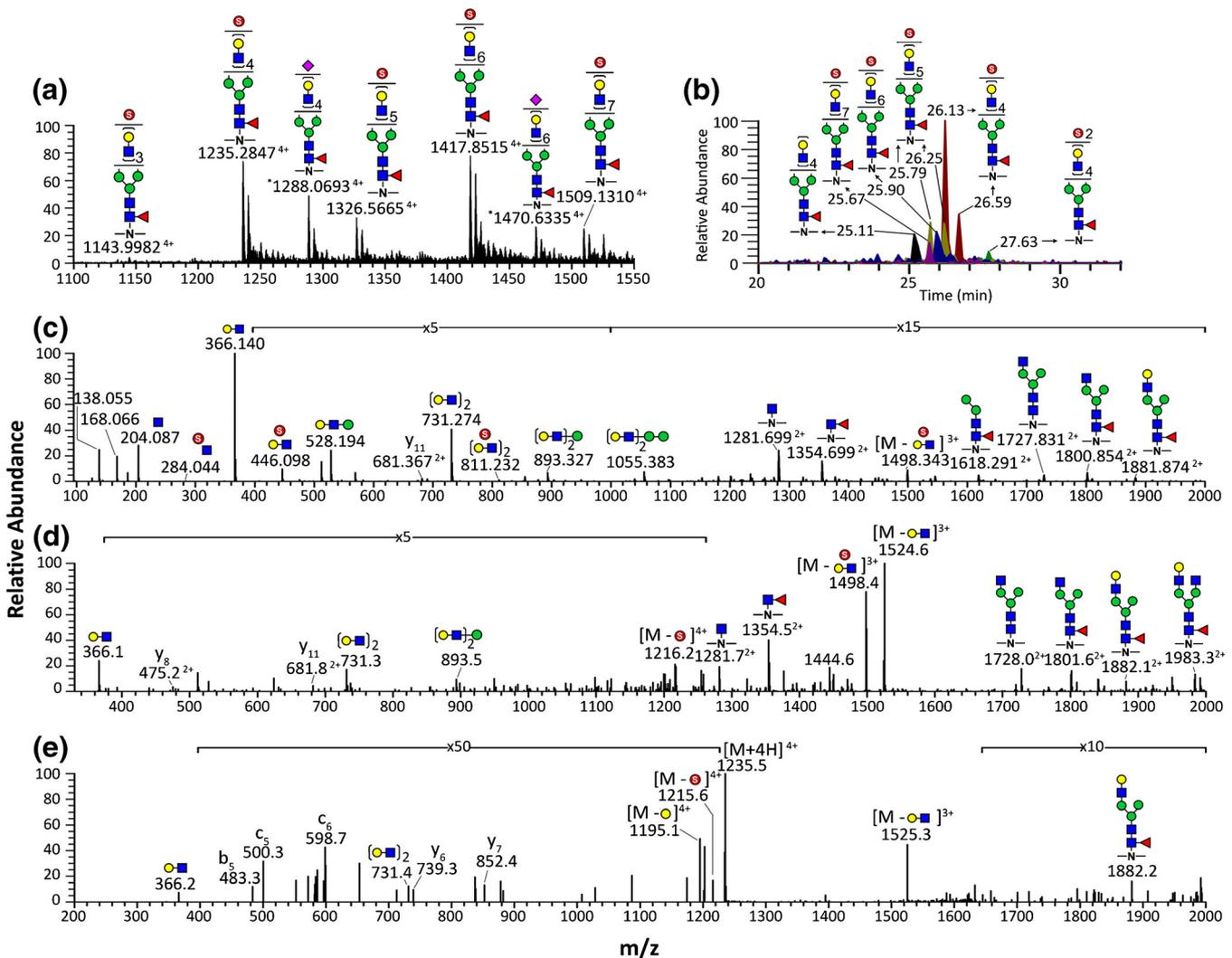


Figure 1. LC-MS and MS² of TiO₂-enriched *N*-glycopeptides from EPO expressed in BHK21 cells. **(a)** A snapshot MS profile averaged from 12 survey MS scans (~1 min) showing the major sulfated glycoforms of the tryptic *N*-glycopeptide GQALLV-NSSQPWEPLQLHVDK. Peaks labeled with an asterisk “*” are non-sulfated but sialylated. The monosulfated glycoforms, which vary in carrying four to seven LacNAc units eluted closely on RP C18-nanoLC capillary column within a short span of 1.5 min, as shown by the overlapping extracted ion chromatograms in **(b)**. On the other hand, the non-sulfated and disulfated counterparts of the major LacNAc₄-containing sulfo-*N*-glycopeptide were eluted at 1 min earlier and 1.5 min later, respectively. As demonstrated by the MS² spectra for the [M + 4H]⁴⁺ precursor of the LacNAc₄-containing sulfo-*N*-glycopeptide at *m/z* 1235.2847, the diagnostic sulfated glyco-oxonium ions (*m/z* 284.044, 446.098, and 811.232) were readily produced only by HCD MS² **(c)**, whereas loss of a sulfite moiety (*m/z* 1216) was commonly detected in CID MS² **(d)** and ETD MS² **(e)** spectra. The cartoon annotation used here and all other figures are based on the SNFG (symbol nomenclature for glycans) system [29], the details of which can be found at NCBI [<http://www.ncbi.nlm.nih.gov/books/NBK310273/>]

As expected, HCD MS² afforded a series of fragment ions corresponding to intact peptide backbone retaining one or more of the glycosyl residues including the so-called Y1 ion at *m/z* 1281.699²⁺ (Fig. 1c) for the quadruply charged precursor at *m/z* 1235.2847⁴⁺ selected for exemplary MS² analyses. More importantly, very low abundant oxonium ions for sulfated HexNAc (*m/z* 284.044), LacNAc (*m/z* 446.098), and di-LacNAc (*m/z* 811.232) could be detected by Orbitrap at high resolution and mass accuracy to be used as cues to filter out HCD MS² spectra of sulfated glycopeptides for a rapid survey of the presence of sulfated glycopeptides (Fig. 1b), in analogy

to the use of the common oxonium ions at *m/z* 204 and 366 to filter out glycopeptide MS² spectra [19, 24]. Measured here at a mass accuracy less than 3 ppm, their detection also allows discriminating sulfated from isobaric phosphorylated ions. Such sulfated glyco-oxonium ions appears not to be sufficiently produced by trap CID MS² (Fig. 1c) or ETD MS² (Fig. 1d) on a hybrid LTQ-Orbitrap Elite instrument. Instead, a characteristic loss of a sulfite moiety (80 u) and sulfated LacNAc (445 u) from the quadruply charged precursor was detected for CID MS², and similar loss of sulfite was also observed for ETD MS², whereas HCD only afforded loss of sulfated LacNAc. For

disulfated glycopeptides, two consecutive losses of sulfites, or a sulfite and a sulfated LacNAc, could be observed by trap CID MS² (Fig. S2). The same pattern was observed for the rEPO *O*-glycopeptides (Fig. S3), pointing towards a common pattern of sulfite and/or sulfated LacNAc loss from the precursor as a diagnostic feature for identification of sulfated glycopeptides by MS². This is equally true for the disulfated glycopeptides from bovine glycoprotein hormone carrying two sulfated lactiNAc termini, where loss of sulfite and sulfated lactiNAc by CID MS² was readily detected (Fig. S4).

Other than these sulfate-specific ions, the overall MS² pattern of the sulfoglycopeptides generally follows that understood for non-sulfated glycopeptides [12, 13, 27], namely HCD and CID yielded mostly Y series of ions due to extensive losses of glycosyl residues, with very few peptide backbone cleavage b or y ions. On the contrary, favorable ETD produced mainly c and z peptide cleavage ions, with occasional loss of glycosyl residues attributable to the applied supplemental activation. It should, however, be noted that probably due to higher performance and efficiency of the EThcD in the newer generation Orbitrap Fusion series, the sulfated glyco-oxonium ion at *m/z* 446.098 could be detected (see later examples and Fig. 4) and similarly mass measured at high mass accuracy, and thus could equally be used to filter out EThcD MS² spectra of sulfated glycopeptides from a large LC-MS/MS dataset.

Sialylated Sulfated N-Glycans of Bovine Thyroglobulin

Most sulfated glycotopes functionally implicated in biological processes are sialylated, such as sulfo sialyl LacNAc or sulfo sialyl LeX [30]. Having established the enrichment and MS² fragmentation characteristics of non-sialylated, sulfated glycopeptides from rEPO, we next turned to bTG, a major protein of thyroid secretory epithelium cells with a molecular weight of 330 kDa, as a source of sialylated and sulfated glycopeptides. Thirteen out of 14 putative *N*-glycosylation sites of bTG have previously been confirmed to be *N*-glycosylated, of which 9 sites were inferred to carry hybrid or complex type *N*-glycans [31]. Although sulfate modification on the *N*-glycans of bTG is well supported by identification of Gal-3-SO₄ and GlcNAc-6-SO₄ [32], the overall sulfated *N*-glycan profile and whether there is any site-specific distribution of the sulfated glycoforms remain undefined.

To facilitate glycopeptide mapping and identification, the total *N*-glycans were first released from bTG, permethylated and analyzed by MALDI-MS in positive and negative ion mode for non-sulfated and sulfated glycans, respectively. We have previously shown that permethylation will retain the sulfate moiety and hence the negative charges imparted [6]. On the other hand, sialic acids will be methyl esterified and thus charge neutralized. As such, the sulfated permethylated glycans could be separated from the non-sulfated permethylated glycans by virtue of simple ion exchange-based solid-phase extraction cartridge [33, 34]. However, for larger *N*-glycans such as those derived from bTG, and when

separation into two populations is not needed, simple cleanup after permethylation by chloroform/water partitioning was found to retain all non-sulfated and sulfated permethylated *N*-glycans in the organic phase and could then be selectively detected by MALDI-MS. In positive ion mode, all peaks detected could be assigned as monosodiated molecular ions (Fig. 2a). Other than the high mannose structures, the major complex type *N*-glycans as inferred from their masses and deduced glycosyl compositions correspond to core fucosylated, biantennary, triantennary, and tetraantennary structures bearing an extra Gal or NeuAc at the various LacNAc termini, with no obvious sulfate modification. However, when switching to negative ion mode, a series of signals could be detected and assigned as [M-H]⁻ of their monosulfated counterparts (Fig. 2b). Among the most abundant ones are the monosulfated, monosialylated, and disialylated biantennary and triantennary core fucosylated *N*-glycans, with and without extra Gal residue. In addition, some minor sulfated species could only be detected as [M-H]⁻ in negative ion mode, including those at *m/z* 2023, 2198, 2490, and 3750 (see tentative assignment on Fig. 2b).

LC-MS² Characteristics of Sialylated Sulfoglycopeptides

Overall, bTG is not only a good source of sialylated sulfoglycopeptides but, due to its large size, also provides a high complexity of non-sulfated sialoglycopeptide and peptide mixtures to test the efficiency of sulfated glycopeptide enrichment. It appears that after TiO₂ enrichment, both non-sulfated and sulfated sialylated glycopeptides were detected by MS (Fig. 3a), whereas the sialylated sulfoglycopeptides were hardly detected without enrichment. Based on the criteria of detecting the diagnostic oxonium ion at *m/z* 366.140 above an ion intensity of 100 as evidence of glycopeptides, only about 40% of all HCD MS² in non-enriched sample could be attributed to glycopeptides, which increased to around 74% after enrichment. Consistent with that observed for the non-sialylated *N*-glycopeptides from rEPO, additional sulfate moiety on otherwise identical sialylated glycopeptides increased their retention time on a RP-C18 nanoLC column (Fig. 3b inset).

Comparing the trap CID MS² of a non-sulfated (Fig. 3b) to a sulfated (Fig. 3c) sialylated *N*-glycopeptide revealed that pairs of fragment ion signals with the mass difference corresponding to a sulfite group were only detected in the CID MS² spectrum of the sialylated sulfoglycopeptide. These could be attributed to either a direct loss of sulfated glycosyl moiety or additional loss of a sulfite moiety accompanying independent loss of glycosyl residues. Unlike the case with non-sialylated sulfoglycopeptides (Fig. 1d), a direct loss of sulfite from the precursor was not always observed (Figs. 3c and S5C). Its production was somewhat dependent on the exact structure of the sialylated multiantennary *N*-glycan itself rather than the peptide backbone (Fig. S6). In contrast, a direct loss of a sulfite moiety from the precursor could be detected under EThcD for all cases of bTG sialylated sulfoglycopeptides examined (Figs. 3e, 4, and S5D), with and without also inducing losses of other sulfated glycosyl

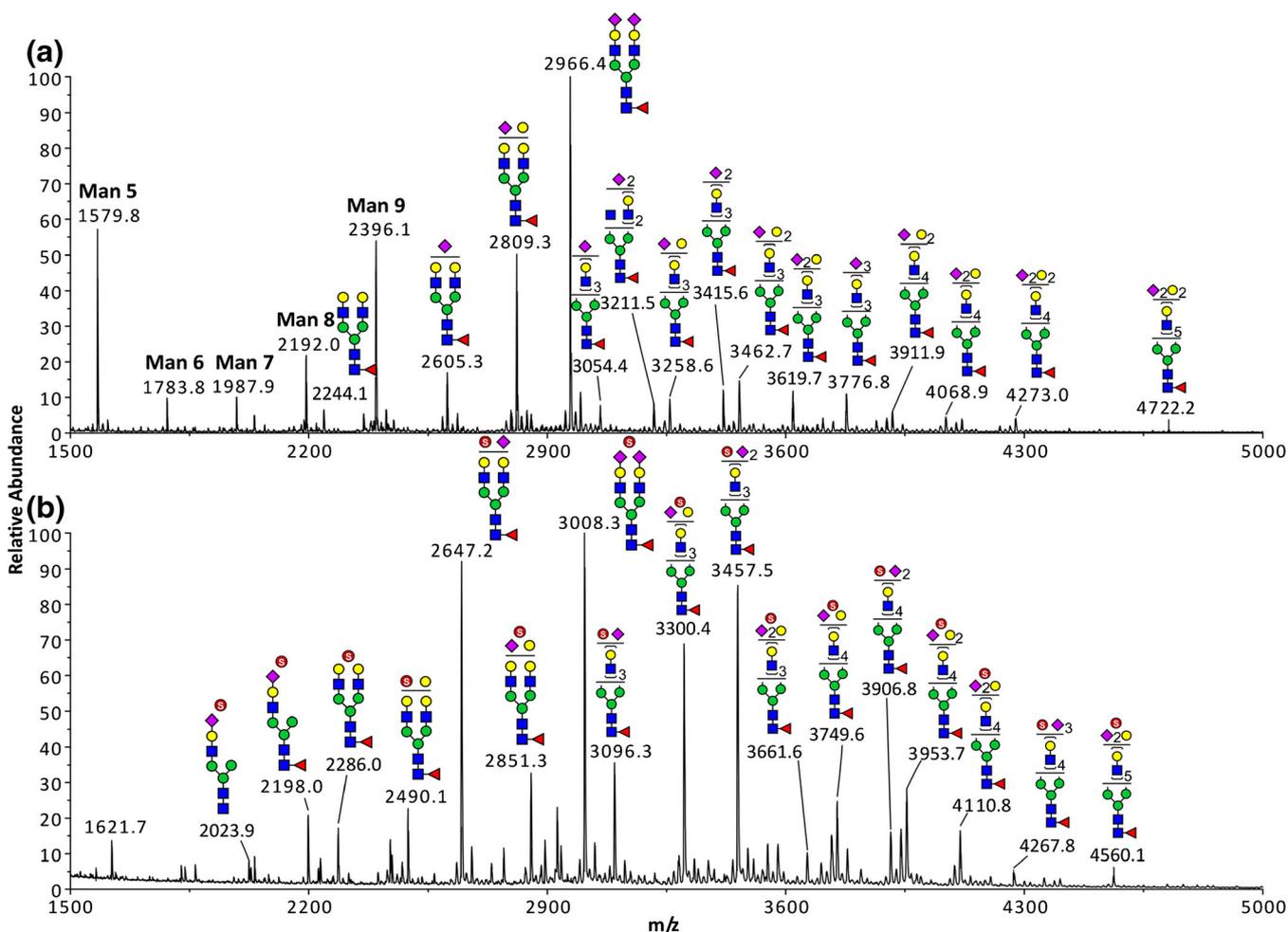


Figure 2. MALDI MS profile of permethylated *N*-glycans from bTG in positive (a) and negative ion mode (b). Tentative assignments of the major peaks were based on glycosyl composition deduced from the molecular masses, and prior knowledge on the presence of sialylated or α -galactosylated LacNAc termini [31, 32]. No attempt was made to verify the structure nor to define the linkage and stereochemistry. A comprehensive and detailed structural study including the minor peaks detected is beyond the scope of this work

moieties. On the other hand, loss of both sulfite and sialic acid that was common by trap CID (Fig. S6) was not observed under EThcD (Fig. 4) for the same set of sialylated sulfoglycopeptides.

Sulfated oxonium ion corresponding to sialylated sulfo LacNAc was never detected while that of sulfo LacNAc could be observed in the HCD MS² spectra (Fig. 3d) but typically at very low intensity. Not only would EThcD on an Orbitrap Fusion commonly afford loss of sulfite from the precursor itself but also direct loss of a sulfo LacNAc along with production of an oxonium ion of sulfo LacNAc when a non-sialylated sulfo LacNAc antenna is present among the multiantennary sialylated complex type *N*-glycans (Fig. S5D). This is true for all three sites of bTG found in this work to carry such sulfo sialylated *N*-glycans (Fig. 4), indicating that production of the diagnostic sulfo LacNAc oxonium ion by EThcD on Orbitrap Fusion is also not peptide backbone but rather glycan dependent. Overall, it appears that direct loss of sulfite under EThcD is the most consistent common feature indicative of sulfoglycopeptides irrespective of sialylation status and the exact glycan structures. However, in cases when good EThcD

MS² data could not be obtained, surveying for loss of sulfite, sulfite + sialic acid, or sulfo LacNAc from sialylated sulfoglycopeptides by CID MS² will be a viable option. On the other hand, searching for a non-favorable sulfo LacNAc oxonium ion in the CID MS² spectra is not advisable, not least due to potential one third low mass cutoff, while doing that for HCD and EThcD MS² spectra is useful and worthy but not foolproof.

Finally, it is of interest to note that the relative abundance of the major sulfated glycoforms detected for each of the three sites of bTG was significantly different (Fig. S7). Site at Asn₂₂₅₁ preferred disialylated biantennary *N*-glycan. Asn₁₇₇₆ carried mostly monosialylated biantennary *N*-glycan, whereas Asn₄₉₅ preferred monosialylated and disialylated triantennary *N*-glycans. Taken together, the major sulfated and sialylated *N*-glycans contributed by all three sites are consistent with the picture gained from analysis of the released permethylated sulfated *N*-glycans (Fig. 2b). Although not meant to be a comprehensive analysis, our data suggest that there are site-specific distributions of the sulfated, sialylated *N*-glycans on

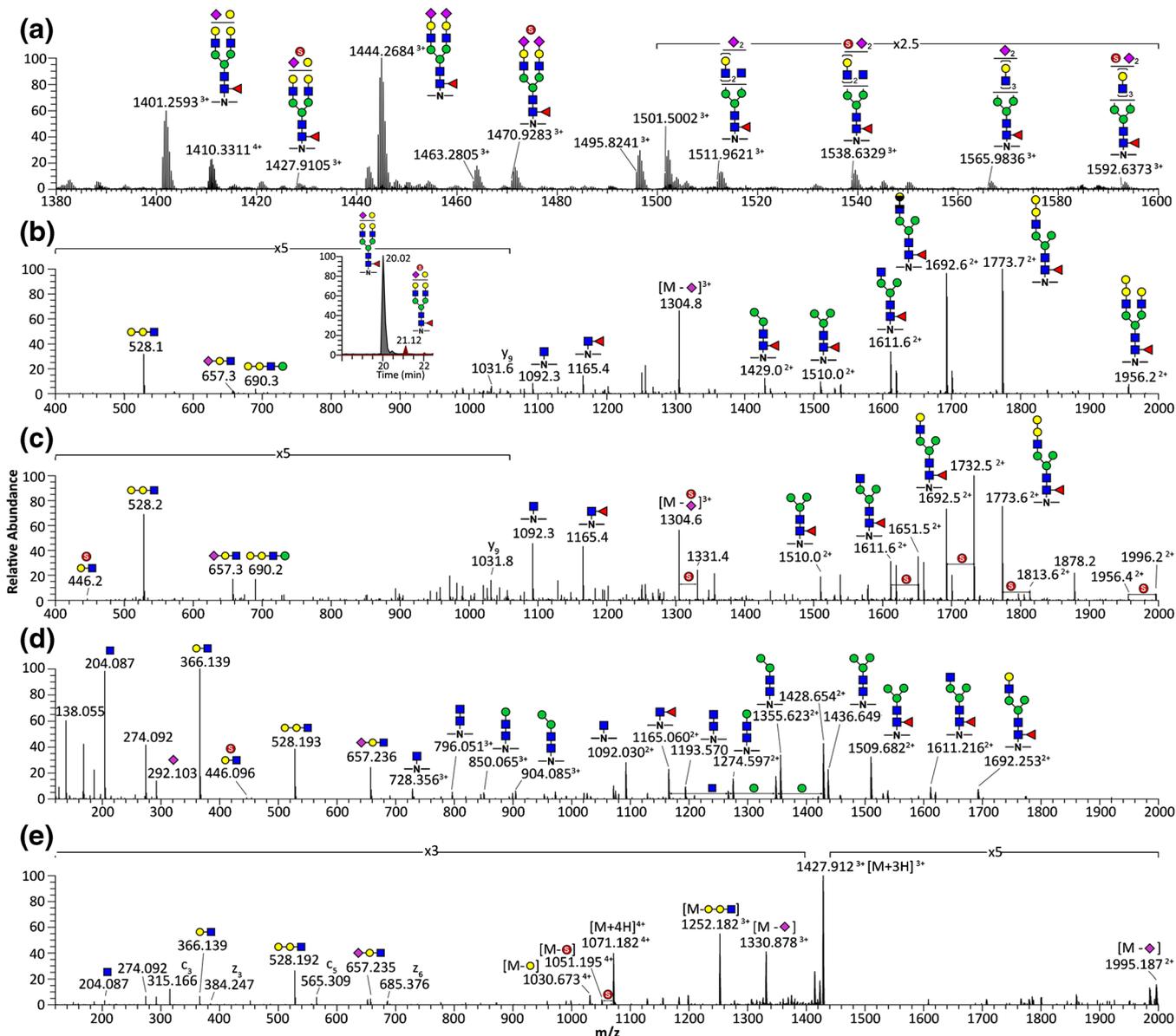


Figure 3. LC MS and MS² of TiO₂-enriched *N*-glycopeptides from bTG. (a) A snapshot MS profile averaged from 21 survey MS scans (~2 min) showing the major glycoforms of the tryptic *N*-glycopeptide APEHLNWTGSWEATKPR. Both sialylated and sulfo sialylated glycopeptides were equally enriched by TiO₂, with the latter eluted at about a minute later on a RP C18-nanoLC capillary column (b). CID MS² of the non-sulfated *N*-glycopeptide at *m/z* 1401.2593³⁺ (b) and its sulfated counterpart at *m/z* 1427.9105³⁺ (c) demonstrated that the mass difference of 80 u between pairs of signals could only be detected in MS² of sulfated glycopeptide due to loss of sulfite. The HCD MS² of the same sialylated sulfoglycopeptide at a higher charge state (*m/z* 1071.185⁴⁺) afforded a very minor oxonium ion of sulfated LacNAc at *m/z* 446.096 (d), whereas the corresponding ETHcD MS² acquired on Orbitrap Fusion showed a characteristic loss of sulfite (e) but produced no sulfated LacNAc oxonium ion

three out of the nine *N*-glycosylation sites known to carry complex type *N*-glycans. Non-sulfated sialoglycopeptides were indeed detected for these other sites but not the sulfated ones at any comparable level. The identification of the diagnostic MS² features of sulfated glycopeptides allowed us to search through a large dataset of sulfated and non-sulfated glycopeptide MS² spectra, only a small portion of which can lead to confident ID when using search engine such as Byonic (data not shown).

Further Considerations in Removing Interference from Sialic Acids

For a more universal application without resorting to a hybrid or tribrid Orbitrap instrument capable of trap CID and ETHcD, it would be highly desirable to be able to rely on a diagnostic sulfo LacNAc oxonium ion by HCD MS² alone akin to Q/TOF CID fragmentation to identify a sulfoglycopeptide, irrespective of the sialylation status. Prior removal of sialic acids may seem like a viable option given the success with the sulfated

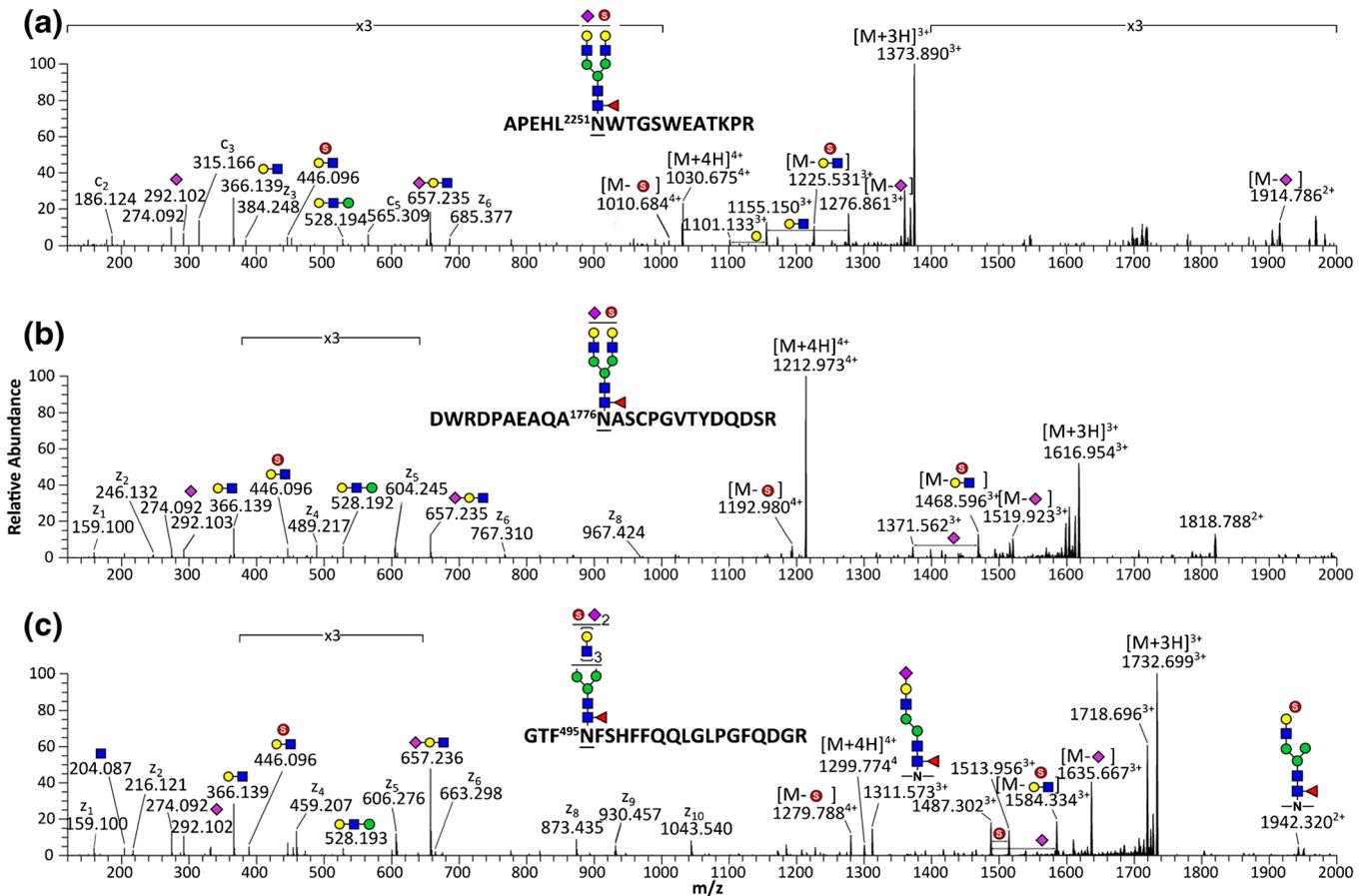


Figure 4. EThcD MS² spectra of select sialylated sulfoglycopeptides from bTG with different tryptic peptide backbone. EThcD MS² were acquired on the quadruply charged precursors at (a) m/z 1030.671⁴⁺, (b) m/z 1212.968⁴⁺, and (c) m/z 1299.773⁴⁺, the peptide sequences of which are as annotated along with the carried glycans. The high mass accuracy detection of sulfated LacNAc oxonium ion at m/z 446.096 coupled with characteristic loss of sulfite moiety from the precursor by EThcD, both measured at < 3 ppm on Orbitrap Fusion, provided direct evidence of sulfate modification

glycopeptides from rEPO reported above. In principle, when devoid of sialic acid, the sulfated glycopeptides may also be specifically enriched due to their carrying negative charge. On the other hand, loss of a sulfite or production of a sulfo LacNAc oxonium ion is not sufficiently specific to identify the presence of sialylated sulfoglycotopes. It is worth investigating if neutralizing the negative charge imparted by sialic acid instead of removing it may promote formation of a diagnostic sulfo sialylated oxonium ion. For these purposes, the tryptic digest of bTG was either treated with neuraminidase to remove the sialic acids or subjected to acetohydrazide/EDC modification of the sialic acids to neutralize their negative charge. By the latter approach, it was anticipated that reducing the interaction of sialic acids and the carboxylic groups of Asp and Glu with TiO₂ may accentuate the negative charge contributed by the sulfate and thus favoring the selective capture of sulfoglycopeptides.

Surprisingly, in the absence of sialylated glycopeptides after neuraminidase treatment, many non-glycopeptides were captured by TiO₂ alongside the glycopeptides (Fig. 5a, inset). Among the 1721 HCD MS² spectra acquired, only 59% was

contributed by glycopeptides based on the criterion of detecting the oxonium ion at m/z 366.140 within 10 ppm (Fig. 5a, upper). The proportion of non-specific capture by TiO₂ of non-glycopeptides (41%) was thus actually higher than that afforded when most of the bTG glycopeptides were sialylated (26%). However, on the positive side, formation of sulfo LacNAc oxonium ion at m/z 446.098 was promoted among the non-sialylated sulfoglycopeptides. Although the ion intensities of m/z 446.098 were generally low comparing to those of m/z 366.140, the selectivity was high due to high mass accuracy afforded by Orbitrap detection. In fact, 23 out of 25 spectra (2% of 1023 HCD MS² spectra containing m/z 366.140, Fig. 4a, lower) extracted by virtue of m/z 446.098 could indeed be manually verified as contributed by sulfoglycopeptides of bTG. In contrast, only very few HCD MS² spectra contained the diagnostic oxonium ion at m/z 446.098 when the sialic acid was not first removed to promote its formation. The impact on CID (Fig. 5b) and ETD MS² (Fig. 5c) was somewhat expected and corroborated the observation that in the absence of sialic acid, direct loss of sulfite from the precursor was prominent and could thus be reliably used as important cues to identify MS² spectra of sulfoglycopeptides.

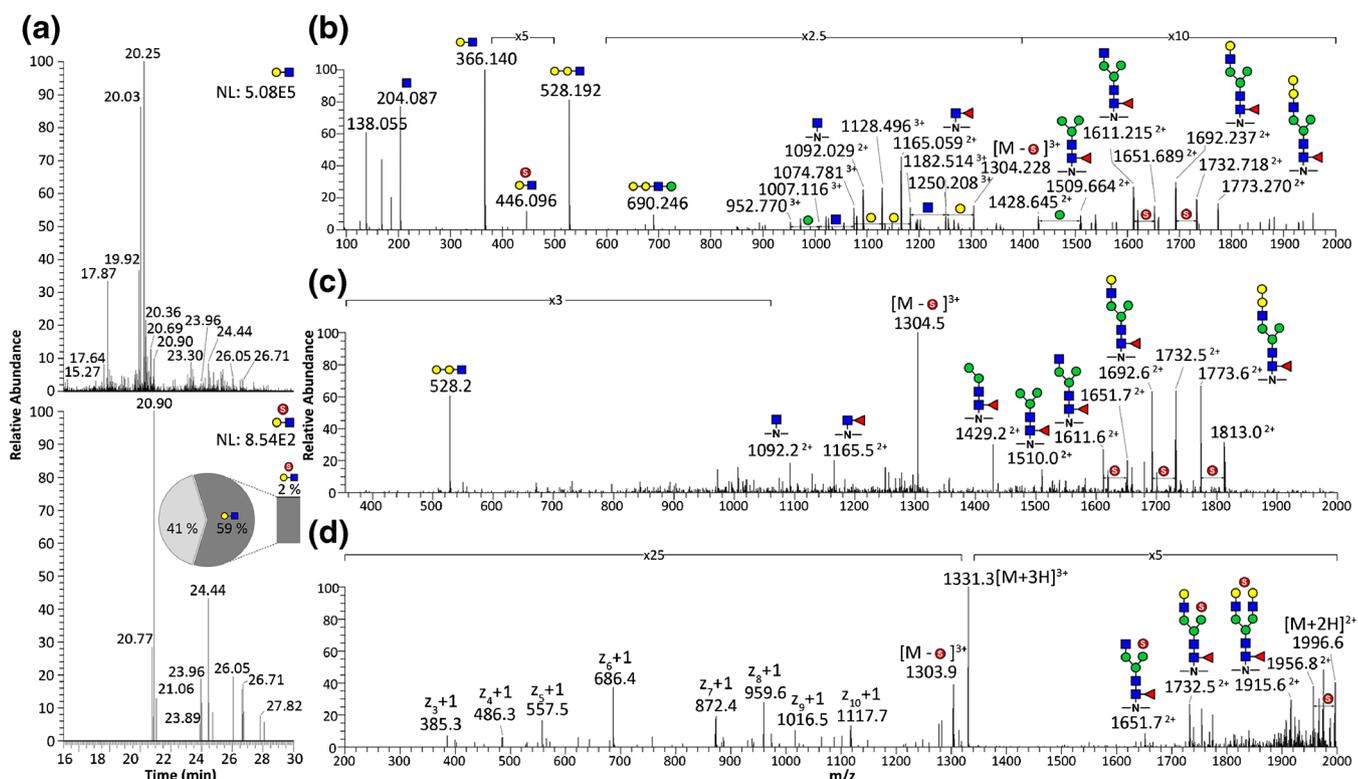


Figure 5. LC-MS² analyses of neuraminidase treated, TiO₂-enriched *N*-sulfoglycopeptides from bTG. **(a)** Extracted ion chromatograms for the HCD MS² ion at *m/z* 366.140 (upper) versus 446.096 (lower) at 10 ppm accuracy, showing the relative number of triggered HCD MS² events containing the selected ions and their respective intensities. Fifty-nine percent of all HCD MS² spectra acquired contained the LacNAc oxonium ion at *m/z* 366.140 with intensity > 100, to be considered as contributed by glycopeptides, of which only 2% also contained the sulfated LacNAc oxonium ion at *m/z* 446.096 to be considered as sulfoglycopeptides (inset). As exemplified by MS² analyses of the sulfoglycopeptide from bTG (APEHLNWTGSWEATKPR with HexNAc₄Hex₆Fuc₁Sulfate₁) at *m/z* 1330.879³⁺, prior removal of sialic acids by neuraminidase treatment significantly promoted the detection of the sulfated LacNAc oxonium ion at *m/z* 446.096 by HCD **(b)**, and direct loss of a sulfite moiety from the precursor by CID **(c)** and ETD with supplemental activation **(d)**, on a hybrid LTQ-Orbitrap instrument. Additional loss of sulfite from other primary fragment ions were also obvious for HCD and CID MS² while more c and z ions were detected in ETD compared with those afforded by sialylated sulfoglycopeptides

When the sialic acid was neutralized and modified by acetoimidazole/EDC, enrichment efficiency of glycopeptides by TiO₂ resembled that of non-derivatized sample, with around 73% of the HCD MS² spectra enumerated as glycopeptides based on the same criterion of oxonium ion at *m/z* 366.140. It is likely that while it did not contribute to capturing only the sulfated sialoglycopeptides, it reduced non-specific interactions with the carboxylic side chains of non-glycosylated peptides. Direct loss of sulfite moiety from the precursors by CID and ETD MS² was similarly promoted (Fig. 6c, d) although loss of the modified sialic acid was still prominent by CID MS². Overall, it can be concluded that stabilizing the sialic acid by neutralizing it, or the removal of it, would facilitate direct loss of sulfite moiety from the precursors by CID and ETD MS² and that formation of peptide c and z ions would be further promoted in the latter. This echoes the initial observation from the analysis of the sulfoglycopeptides from rEPO, which were largely without sialic acids. However, formation of sulfo sialylated oxonium ions by HCD MS² were never observed even when the sialic acid was modified (Fig. 6b). In the same spectrum, oxonium ions corresponding to derivatized sialic

acid at *m/z* 348.140 (instead of *m/z* 292.103) and sialyl LacNAc at *m/z* 713.279 (instead of *m/z* 657.236) were detected, which indicated that sialic acid modification itself did not prevent formation of oxonium ion containing it. It thus appears that the strategy of sialic acid modification will not confer any significant advantage in so far as facilitating enrichment, promoting diagnostic sulfo sialylated oxonium ion, or improving peptide backbone fragmentation pattern. With the advent of EThcD on Orbitrap Fusion, sulfo LacNAc oxonium ion could now be readily detected and used as an important criterion alongside loss of sulfite to identify ±sialylated sulfoglycopeptides without the additional steps of removing or modifying the sialic acids (Fig. 4).

Conclusions

In this work, we have shown that ±sialylated sulfoglycopeptides could be captured by TiO₂ in an enrichment step with a slightly modified sample loading conditions. However, the enrichment efficiency or specificity was not improved upon removal of the

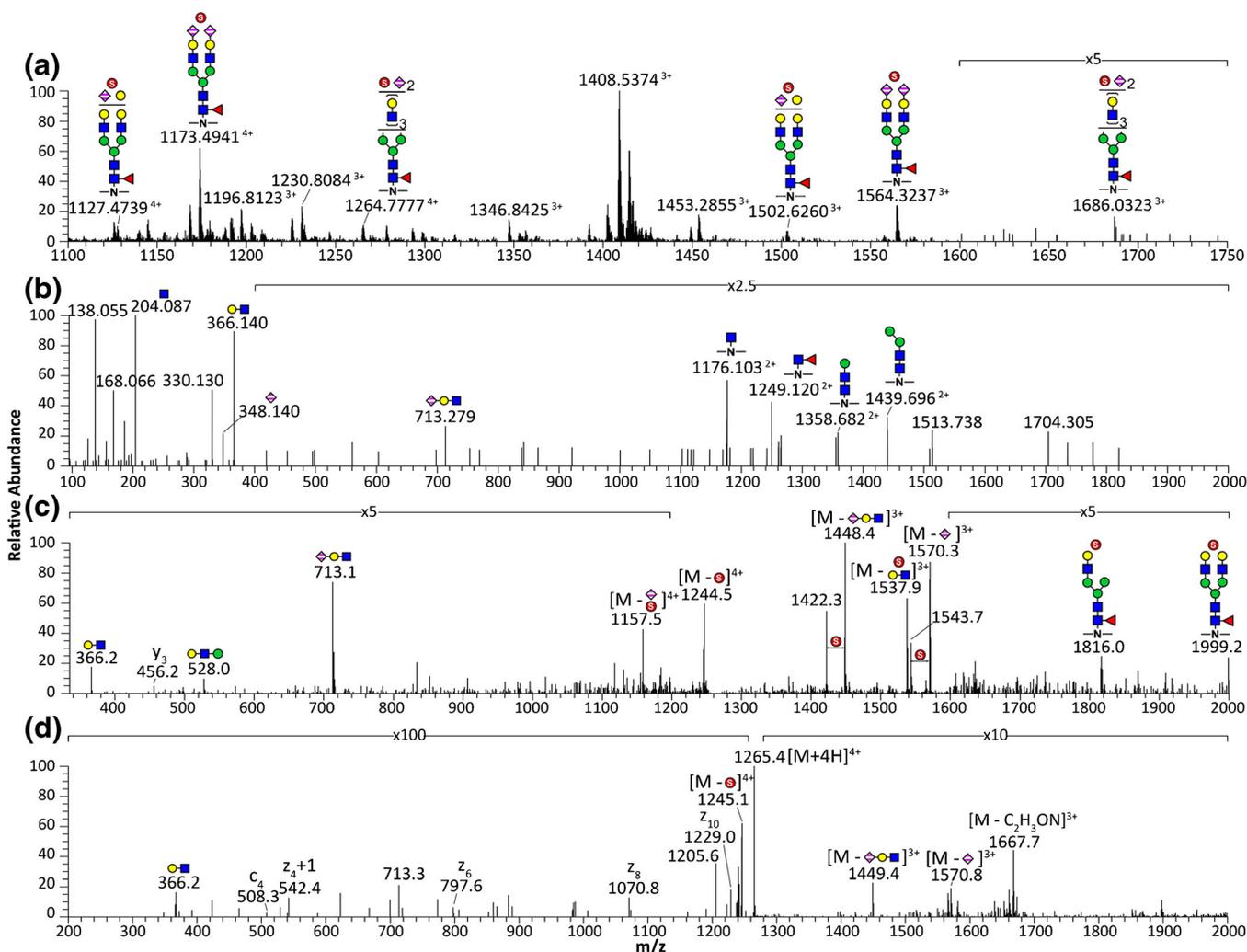


Figure 6. LC-MS² analyses of chemically derivatized, TiO₂-enriched *N*-sulfoglycopeptides from bTG. **(a)** A snapshot MS profile averaged from four survey MS scans (~13 s) showing the major glycoforms of the sialic acid-modified tryptic *N*-glycopeptide APEHLNWTGSWEATKPR. The peak at *m/z* 1408.5374 could not be assigned as one of the expected sulfoglycopeptides. **(b)** HCD MS² of the sialylated sulfoglycopeptide at *m/z* 1264.7777⁴⁺ afforded the oxonium ions corresponding to derivatized sialic acid (*m/z* 348.140) and/or sialyl LacNAc (*m/z* 713.279) but no sulfated LacNAc nor sulfo sialylated oxonium ion. Direct loss of a sulfite moiety from the precursor was equally observed for CID MS² **(c)** and ETD MS² with supplemental activation **(d)** on an LTQ-Orbitrap Elite instrument

negative charges contributed by sialic acids. In the absence of a more efficient method that can specifically enrich out the usually rather low abundant sulfoglycopeptides from non-sulfated glycopeptides irrespective of the sialylation status, diagnostic MS² ions that can positively confirm their presence will be very important. Unfortunately, reliable formation of sulfo LacNAc oxonium ion by HCD MS² was only observed with non-sialylated sulfoglycopeptides. When the sulfoglycopeptides were sialylated, both HCD and EThcD on an Orbitrap Fusion Tribrid could produce the sulfo LacNAc oxonium ion but somewhat dependent on the antennary structures of the sialylated sulfoglycans carried. These could be mass measured at high resolution and mass accuracy by Orbitrap (<3 ppm in this work) and used as an important criterion to filter out bona fide sulfoglycopeptide MS² spectra, as well as to discriminate against the isobaric phosphorylation. Direct loss of a sulfite moiety from

the precursor by EThcD could be relied on as an independent or corroborative evidence, whereas a dual considerations for loss of sulfite and sulfite + sialic acid can be adopted for trap CID MS² data, all of which can be mass measured at high mass accuracy on an Orbitrap. The latter would assist cases when productive EThcD was not acquired, or in instrument that does not offer the EThcD option.

Any identification of glycopeptide by a combination of HCD, CID, and EThcD is still not a trivial issue [13, 24] even when the MS² data can now be searched against database using a few software programs developed by academic groups or the commercially available Byonic [11, 35]. These usually allow for additional considerations of sulfate, or can be configured accordingly either as a modification, adduct, or by expanding the glycan library used. It nevertheless increases the search scope and adds to the associated uncertainties. We showed here

that, in general, the presence of sulfate did not significantly alter the kind of fragment ions and information one can derive from different MS² modes and thus subject to the same successes and pitfalls in MS²-based glycopeptide identifications. The ability to distinguish the presence of a sulfate on glycopeptide from other glycosyl combinations or peptide modifications would therefore be critical to reduce the likely false assignments. In this context, the use of basic tripeptide for ion pairing on sulfated glycopeptides that was reported to not only allow discriminating against isobaric phosphorylation but would also enhance trap CID fragmentation to yield more informative glycosidic cleavage ions [36, 37] is worth exploring. In particular, it remains to be investigated how the useful features afforded by ion pairing may similarly extend to more complex sialylated sulfoglycopeptides with longer peptide backbones typically encountered in glycoproteomics. We have established the basics, but the field is still wide open for incorporating any experimental approach that may improve on the complementary use of HCD and EThcD now available on the high mass accuracy Orbitrap instruments, to enable a more efficient and definitive identification of sialylated sulfoglycopeptides.

Funding Information

This work was financially supported by an Academia Sinica Investigator Award grant to KKH and partly by the Research and Development Program of New Drugs and Vaccines for Critical Diseases of Academia Sinica and Ministry of Science and Technology (MOST 104-0210-01-09-02, MOST 105-0210-01-13-01, MOST 106-0210-01-15-02), Taiwan.

References

- Chen, J.Y., Huang, H.H., Yu, S.Y., Wu, S.J., Kannagi, R., Khoo, K.H.: Concerted mass spectrometry-based glycomic approach for precision mapping of sulfo sialylated N-glycans on human peripheral blood mononuclear cells and lymphocytes. *Glycobiology*. **28**, 9–20 (2018)
- Rosen, S.D.: Ligands for L-selectin: homing, inflammation, and beyond. *Annu. Rev. Immunol.* **22**, 129–156 (2004)
- Mitoma, J., Bao, X., Petryanik, B., Schaerli, P., Gauguet, J.M., Yu, S.Y., Kawashima, H., Saito, H., Ohtsubo, K., Marth, J.D., Khoo, K.H., von Andrian, U.H., Lowe, J.B., Fukuda, M.: Critical functions of N-glycans in L-selectin-mediated lymphocyte homing and recruitment. *Nat. Immunol.* **8**, 409–418 (2007)
- Kimura, N., Ohmori, K., Miyazaki, K., Izawa, M., Matsuzaki, Y., Yasuda, Y., Takematsu, H., Kozutsumi, Y., Moriyama, A., Kannagi, R.: Human B-lymphocytes express alpha2-6-sialylated 6-sulfo-N-acetylglucosamine serving as a preferred ligand for CD22/Siglec-2. *J. Biol. Chem.* **282**, 32200–32207 (2007)
- Macauley, M.S., Kawasaki, N., Peng, W., Wang, S.H., He, Y., Arlian, B.M., McBride, R., Kannagi, R., Khoo, K.H., Paulson, J.C.: Unmasking of CD22 co-receptor on germinal center B-cells occurs by alternative mechanisms in mouse and man. *J. Biol. Chem.* **290**, 30066–30077 (2015)
- Yu, S.Y., Wu, S.W., Hsiao, H.H., Khoo, K.H.: Enabling techniques and strategic workflow for sulfoglycomics based on mass spectrometry mapping and sequencing of permethylated sulfated glycans. *Glycobiology*. **19**, 1136–1149 (2009)
- Cheng, C.W., Chou, C.C., Hsieh, H.W., Tu, Z., Lin, C.H., Nycholat, C., Fukuda, M., Khoo, K.H.: Efficient mapping of sulfated glycotopes by negative ion mode nanoLC-MS/MS-based sulfoglycomics analysis of permethylated glycans. *Anal. Chem.* **87**, 6380–6388 (2015)
- Khoo, K.H.: From mass spectrometry-based glycosylation analysis to glycomics and glycoproteomics. *Adv. Neurobiol.* **9**, 129–164 (2014)
- Rudd, P., Karlsson, N.G., Khoo, K.H., Packer, N.H.: Glycomics and glycoproteomics. In: Varki A, Cummings RD, Esko JD, Stanley P, Hart GW, Aebi M, Darvill AG, Kinoshita T, Packer NH, Prestegard JH, Schnaar RL, Seeberger PH (eds.). Cold Spring Harbor (NY), (2015–2017)
- Thaysen-Andersen, M., Packer, N.H.: Advances in LC-MS/MS-based glycoproteomics: getting closer to system-wide site-specific mapping of the N- and O-glycoproteome. *Biochim. Biophys. Acta.* **1844**, 1437–1452 (2014)
- Hu, H., Khatri, K., Zaia, J.: Algorithms and design strategies towards automated glycoproteomics analysis. *Mass. Spectrom. Rev.* (2016)
- Nilsson, J.: Liquid chromatography-tandem mass spectrometry-based fragmentation analysis of glycopeptides. *Glycoconj. J.* **33**, 261–272 (2016)
- Thaysen-Andersen, M., Packer, N.H., Schulz, B.L.: Maturing glycoproteomics technologies provide unique structural insights into the N-glycoproteome and its regulation in health and disease. *Mol. Cell. Proteome. MCP.* **15**, 1773–1790 (2016)
- Engholm-Keller, K., Larsen, M.R.: Titanium dioxide as chemo-affinity chromatographic sorbent of biomolecular compounds—applications in acidic modification-specific proteomics. *J. Proteome.* **75**, 317–328 (2011)
- Palmisano, G., Lendal, S.E., Engholm-Keller, K., Leth-Larsen, R., Parker, B.L., Larsen, M.R.: Selective enrichment of sialic acid-containing glycopeptides using titanium dioxide chromatography with analysis by HILIC and mass spectrometry. *Nat. Protoc.* **5**, 1974–1982 (2010)
- Kuo, C.W., Wu, I.L., Hsiao, H.H., Khoo, K.H.: Rapid glycopeptide enrichment and N-glycosylation site mapping strategies based on amine-functionalized magnetic nanoparticles. *Anal. Bioanal. Chem.* **402**, 2765–2776 (2012)
- Toyoda, M., Narimatsu, H., Kameyama, A.: Enrichment method of sulfated glycopeptides by a sulfate emerging and ion exchange chromatography. *Anal. Chem.* **81**, 6140–6147 (2009)
- Huddleston, M.J., Bean, M.F., Carr, S.A.: Collisional fragmentation of glycopeptides by electrospray ionization LC/MS and LC/MS/MS: methods for selective detection of glycopeptides in protein digests. *Anal. Chem.* **65**, 877–884 (1993)
- Halim, A., Westerlind, U., Pett, C., Schorlemer, M., Ruetschi, U., Brinkmalm, G., Sihlbom, C., Lengqvist, J., Larson, G., Nilsson, J.: Assignment of saccharide identities through analysis of oxonium ion fragmentation profiles in LC-MS/MS of glycopeptides. *J. Proteome Res.* **13**, 6024–6032 (2014)
- Saba, J., Dutta, S., Hemenway, E., Viner, R.: Increasing the productivity of glycopeptides analysis by using higher-energy collision dissociation-accurate mass-product-dependent electron transfer dissociation. *Int. J. Proteome.* **2012**, 560391 (2012)
- Singh, C., Zampronio, C.G., Creese, A.J., Cooper, H.J.: Higher energy collision dissociation (HCD) product ion-triggered electron transfer dissociation (ETD) mass spectrometry for the analysis of N-linked glycoproteins. *J. Proteome Res.* **11**, 4517–4525 (2012)
- Yagi, H., Kuo, C.W., Obayashi, T., Ninagawa, S., Khoo, K.H., Kato, K.: Direct mapping of additional modifications on phosphorylated O-glycans of alpha-dystroglycan by mass spectrometry analysis in conjunction with knocking out of causative genes for dystroglycanopathy. *Mol. Cell. Proteome. MCP.* **15**, 3424–3434 (2016)
- Darula, Z., Medzihradsky, K.F.: Analysis of mammalian O-glycopeptides—we have made a good start, but there is a long way to go. *Mol. Cell. Proteome. MCP.* (2017)
- Wu, S.W., Pu, T.H., Viner, R., Khoo, K.H.: Novel LC-MS² product dependent parallel data acquisition function and data analysis workflow for sequencing and identification of intact glycopeptides. *Anal. Chem.* **86**, 5478–5486 (2014)
- Frese, C.K., Altelaar, A.F., van den Toorn, H., Nolting, D., Griep-Raming, J., Heck, A.J., Mohammed, S.: Toward full peptide sequence coverage by dual fragmentation combining electron-transfer and higher-energy collision dissociation tandem mass spectrometry. *Anal. Chem.* **84**, 9668–9673 (2012)
- Yu, Q., Wang, B., Chen, Z., Urabe, G., Glover, M.S., Shi, X., Guo, L.W., Kent, K.C., Li, L.: Electron-transfer/higher-energy collision dissociation (EThcD)-enabled intact glycopeptide/glycoproteome characterization. *J. Am. Soc. Mass. Spectrom.* (2017)

27. Wührer, M., Catalina, M.I., Deelder, A.M., Hokke, C.H.: Glycoproteomics based on tandem mass spectrometry of glycopeptides. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **849**, 115–128 (2007)
28. Palmisano, G., Lendal, S.E., Larsen, M.R.: Titanium dioxide enrichment of sialic acid-containing glycopeptides. *Methods Mol. Biol.* **753**, 309–322 (2011)
29. Varki, A., Cummings, R.D., Aebi, M., Packer, N.H., Seeberger, P.H., Esko, J.D., Stanley, P., Hart, G., Darvill, A., Kinoshita, T., Prestegard, J.J., Schnaar, R.L., Freeze, H.H., Marth, J.D., Bertozzi, C.R., Etzler, M.E., Frank, M., Vliegthart, J.F., Lutteke, T., Perez, S., Bolton, E., Rudd, P., Paulson, J., Kanehisa, M., Toukach, P., Aoki-Kinoshita, K.F., Dell, A., Narimatsu, H., York, W., Taniguchi, N., Kornfeld, S.: Symbol nomenclature for graphical representations of glycans. *Glycobiology*. **25**, 1323–1324 (2015)
30. Kannagi, R., Ohmori, K., Chen, G.Y., Miyazaki, K., Izawa, M., Sakuma, K.: Sialylated and sulfated carbohydrate ligands for selectins and siglecs: involvement in traffic and homing of human memory T and B lymphocytes. *Adv. Exp. Med. Biol.* **705**, 549–569 (2011)
31. Rawitch, A.B., Pollock, H.G., Yang, S.X.: Thyroglobulin glycosylation: location and nature of the N-linked oligosaccharide units in bovine thyroglobulin. *Arch. Biochem. Biophys.* **300**, 271–279 (1993)
32. Spiro, R.G., Bhojroo, V.D.: Occurrence of sulfate in the asparagine-linked complex carbohydrate units of thyroglobulin. Identification and localization of galactose 3-sulfate and N-acetylglucosamine 6-sulfate residues in the human and calf proteins. *J. Biol. Chem.* **263**, 14351–14358 (1988)
33. Cheng, P.F., Snovida, S., Ho, M.Y., Cheng, C.W., Wu, A.M., Khoo, K.H.: Increasing the depth of mass spectrometry-based glycomic coverage by additional dimensions of sulfoglycomics and target analysis of permethylated glycans. *Anal. Bioanal. Chem.* **405**, 6683–6695 (2013)
34. Khoo, K.H., Yu, S.Y.: Mass spectrometric analysis of sulfated N- and O-glycans. *Methods Enzymol.* **478**, 3–26 (2010)
35. Lisacek, F., Mariethoz, J., Alocci, D., Rudd, P.M., Abrahams, J.L., Campbell, M.P., Packer, N.H., Stahle, J., Widmalm, G., Mullen, E., Adamczyk, B., Rojas-Macias, M.A., Jin, C., Karlsson, N.G.: Databases and associated tools for glycomics and glycoproteomics. *Methods Mol. Biol.* **1503**, 235–264 (2017)
36. Zhang, Y., Jiang, H., Go, E.P., Desaire, H.: Distinguishing phosphorylation and sulfation in carbohydrates and glycoproteins using ion-pairing and mass spectrometry. *J. Am. Soc. Mass Spectrom.* **17**, 1282–1288 (2006)
37. Irungu, J., Dalpathado, D.S., Go, E.P., Jiang, H., Ha, H.V., Bousfield, G.R., Desaire, H.: Method for characterizing sulfated glycoproteins in a glycosylation site-specific fashion, using ion pairing and tandem mass spectrometry. *Anal. Chem.* **78**, 1181–1190 (2006)