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MULTIPLE REACTION MONITORING MASS SPECTROMETRY FOR THE DISCOVERY AND QUANTIFICATION OF O-GLCNAC MODIFIED PROTEINS

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ABSTRACT

O-linked-N-acetylglucosamine (O-GlcNAc) is a post-translational modification regulating proteins involved in a variety of cellular processes and diseases. Unfortunately, O-GlcNAc remains challenging to detect and quantify by shotgun mass spectrometry (MS) where it is time-consuming and tedious. Here, we investigate the potential of Multiple Reaction Monitoring Mass Spectrometry (MRM-MS), a targeted MS method, to detect and quantify native O-GlcNAc modified peptides without extensive labeling and enrichment. We report the ability of MRM-MS to detect a standard O-GlcNAcylated peptide and show that the method is robust to quantify the amount of O-GlcNAcylated peptide with a method detection limit of 3fmol. In addition, when diluted by 100-fold in a trypsin-digested whole cell lysate, the O-GlcNAcylated peptide remains detectable. Next, we apply this strategy to study glycogen synthase kinase-3 beta (GSK-3β), a kinase able to compete with O-GlcNAc transferase and modify identical site on proteins. We demonstrate that GSK-3β is itself modified by O-GlcNAc in human embryonic stem cells (hESC). Indeed, by only using gel electrophoresis to grossly enrich GSK-3β from whole cell lysate, we discover by MRM-MS a novel O-GlcNAcylated GSK-3β peptide, bearing 3 potential O-GlcNAcylation sites. We confirm our finding by quantifying the increase of O-GlcNAcylation following hESC treatment with an O-GlcNAc hydrolase inhibitor. This novel O-GlcNAcylation could potentially be involved in an auto-inhibition mechanism. To the best of our knowledge, this is the first report utilizing MRM-MS to detect native O-GlcNAc modified peptides. This could potentially facilitate rapid discovery and quantification of new O-GlcNAcylated peptides/proteins.

KEYWORDS

O-GlcNAc, Multiple Reaction Monitoring, Mass Spectrometry, glycogen synthase kinase-3 beta

INTRODUCTION

O-linked N-acetylglucosamine (O-GlcNAc) is a monosaccharide post-translational modification reversibly added on serine and threonine of nucleocytoplasmic proteins.[1] O-GlcNAc has often been reported as interplaying with phosphorylation.[2] Indeed, O-GlcNAc and phosphorylation can either compete to modify identical sites or cooperate to modify adjacent sites on the same protein.[3] Furthermore, most of the reported O-GlcNAcylated proteins are also known to be phosphorylated,[4] suggesting that O-GlcNAcylated proteins might be as abundant as phosphoproteins. To date, thousands nucleocytoplasmic proteins have been reported as O-GlcNAc modified in viruses and most of eukaryotic multi-organisms (fungi, plants, animals).[5, 6] O-GlcNAc regulates protein stability, sub-cellular localization and binding.[7] In addition, O-GlcNAc modified proteins are involved in a variety of cellular and physiological processes (signalling,[8] apoptosis,[9] stress response,[10] cell cycle,[11] gene transcription,[7] development[12]) and diseases (cancer,[13] diabetes,[14] cardiovascular disease,[15] Alzheimer's disease[16]). Overall, O-GlcNAc is considered to be one of the most abundant and important post-translational modifications and its study will have a significant impact on numerous biological fields.[5]

However, research on O-GlcNAc has been hampered due to issues associated with the modification: lability and low stoichiometry.[17, 18] Those issues are exemplified in shotgun Mass Spectrometry (MS) where signals from ionized O-GlcNAcylated peptides are often suppressed in favor of non-modified peptides.[19] To circumvent this problem, many techniques have been developed to enrich O-GlcNAcylated peptides before shotgun MS. These enrichment techniques rely on immunoprecipitation using specific antibodies[20] or affinodetection using specific lectins such as the wheat germ agglutinin (WGA).[21] More refined techniques involve chemically labeling O-GlcNAc followed by specific enrichment [22, 23, 24] These labeling and enrichment techniques coupled to shotgun MS have been

instrumental for the large-scale discovery of numerous O-GlcNAcylated proteins. In contrast to shotgun MS, a targeted MS method would be beneficial when working on a pre-selected protein of interest. Firstly, a targeted MS might require lesser labeling and enrichment steps than shotgun MS, which would make the sample preparation less time-consuming and tedious (e.g. 2 full days are required to complete O-GlcNAc labeling through chemical substitution).[23] Secondly, a targeted MS approach might be able to directly quantify the amount of native O-GlcNAcylated peptide, whereas quantification has so far only by reported on labeled peptides.[25, 26, 27]

Multiple Reaction Monitoring (MRM) is a targeted MS method used to detect and quantify expected peptides of predetermined fragmentation masses.[28] MRM is usually associated with a liquid chromatography (LC)-coupled to a triple-quadruple (QQQ) mass spectrometer. The first mass analyzer (Q1) filters masses corresponding to the expected precursor ions, interested ions are then selected specifically and fragmented in the collision chamber (Q2), and fragment ions matching the expected fragment masses are searched for in Q3.[29] MRM-MS reports the intensity traces of precursor-fragment ions couples, also called transition couples, during LC elution.[29] Transition traces originated from the same precursor simultaneously co-elute, therefore confirming the existence of the expected peptide. In addition, the integration of peptide transition traces gives an area value proportional to the amount of the peptide thus enabling MRM-MS to be used for quantification purposes without need for peptide labeling.[30] Beside quantification, the other advantage of MRM is its ability to detect low amount of peptides in a complex mixture.[31] In this regard, MRM-MS has been reported as suited for the study of post-translational modifications such as oxidation,[32] acetylation,[33] ubiquitinylation,[34] and phosphorylation.[35, 36] The of MRM-MS to detect O-GlcNAcylated peptide was previously reported.[37] There, Yuzwa S.A. *et al.* successfully detected an O-GlcNAcylated peptide from human Tau protein recombinantly expressed in *E. Coli*. However MRM-MS has yet to be tested on native O-GlcNAcylated proteins. In addition, there has not been an extensive study reporting MRM-MS characteristics applied to O-

GlcNAc detection, such as: fragmentation pattern, method detection limit, quantification potential and purity required to detect native O-GlcNAcylated peptides.

Here, we investigated the use of MRM-MS to detect native O-GlcNAcylated peptides. We first demonstrated that MRM-MS is able to robustly detect and quantify the amount of a standard O-GlcNAcylated peptide. We also showed that O-GlcNAcylated peptide detection by MRM-MS is not affected by the presence of a complex peptide mixture. We then chose to apply this methodology to characterize GSK-3β, a kinase related to neurodegenerative diseases[38] and cancers.[39, 40] We here demonstrated that GSK-3β is bearing O-GlcNAc modifications in human embryonic stem cells (hESC) *via* MRM-MS; we were able to discover a novel O-GlcNAcylated GSK-3β peptide (bearing 3 potential O-GlcNAcylation sites) without extensive enrichments. To the best of our knowledge, this is the first report demonstrating that MRM-MS can be used to analyze native O-GlcNAcylated peptides from a complex mixture. Accordingly, we believe that MRM-MS could be integrated with existing O-GlcNAc-related analytical methodologies for discovery and quantification purposes.

EXPERIMENTAL SECTION

Reagents and Standards

PUGNAc (Sigma-Aldrich) was reconstituted in water. Standard O-GlcNAcylated CREB peptide (TAPT(S-O-GlcNAc)TIAPG, M=1117.5 g.mol^{-1}) (Invitrogen) was reconstituted in 40 mM ammonium bicarbonate (pH 8). Before LC-MRM-MS analysis, the standard O-GlcNAcylated CREB peptide was further diluted in LC-MS buffer to the final composition: 1 % formic acid, 2 % methanol. Antibodies were purchased from the following suppliers: anti-O-GlcNAc antibody (CTD110.6, Convance); anti-GSK-3β antibody (27C13, Cell signaling).

Mixtures preparation

Mixtures of O-GlcNAcylated peptides from human embryonic stem cell (hESC) lysate were prepared by trypsin-digestion of 5 µg of whole hESC lysate as described below. The tryptic digest from whole hESC lysate was mixed with the standard O-GlcNAcylated CREB peptide at the following ratios 5:95; 2.5:97.5; 1:99 (O-GlcNAcylated peptide: hESC lysate) in LC-MS buffer (1 % formic acid, 2 % methanol).

Identification of O-GlcNAcylated GSK-3β peptides

Identification of O-GlcNAcylated GSK-3β peptides was performed as follows. Briefly, hESC cultures were supplemented with or without 100 µM of PUGNAc for 24 h before being harvested.[23, 41] After cell lysis and protein quantification, 20 µg of whole hESC lysate was loaded into a well of NuPAGE® 4-12 % SDS-PAGE gel (Life Technologies) and separated by electrophoresis. SDS-PAGE gel were then incubated with Coomassie blue solution (1 % coomassie brilliant blue A250, 10 % acetic acid, 30 % methanol in water) for 30 min. The stained SDS-PAGE gel were then subjected to several washes (10 % acetic acid, 30 % methanol in water) to reach the desired dye intensity. A gel band of 2 mm wide around 45 kDa was cut for subsequent proteolysis. For this purpose, gel pieces were incubated at 4ºC in washing solution (50 % acetonitrile, 25 mM ammonium bicarbonate) for 24 h. Excised bands were then incubated at 37ºC under shaking for 20 min in fresh washing solution followed by incubation at room temperature for 10 min in acetonitrile, and then dried down with Savant SpeedVac AES2010 Centrifugal Evaporator (Thermo) followed by incubation at 56ºC for 1 h in 20 mM DTT (protein reduction step). After removal of the DTT solution, excised bands were incubated at room temperature in the dark for 45 min in 55 mM Iodoacetamide (protein alkylation step) and then washed at room temperature with a serie of 10 min incubations: acetonitrile, 100 mM ammonium bicarbonate, acetonitrile; and finally

dried with Savant SpeedVac. Excised bands were then incubated at 37ºC with shaking for 16 h in a solution of 0.02 $\mu g.\mu L^{-1}$ trypsin (Promega), 25 mM ammonium bicarbonate (digestion). Alternatively to trypsin, incubation in 0.02 $\mu g.\mu L^{-1}$ of Glu-C (Promega) in 100 mM Tris HCl, pH 7.8 was used for digestion. Following digestion, peptides were extracted with a serie of 10 min incubations in the following extraction solutions: acetonitrile with sonication, 100 mM ammonium bicarbonate and acetonitrile. The extraction solutions were combined and dried with Savant SpeedVac. The peptides were finally reconstituted in 10 µL of LC-MS buffer (1 % formic acid, 2 % methanol) to be used for MS analysis. Finally, the total peptide mixture extracted from the single band cut was reconstituted in 10 µL of LC-MS buffer (1 % formic acid, 2 % methanol). Finally, 2 µL of this solution was injected on LC-MRM-MS for analysis.

MRM programming

Transition couples (precursor-fragment ions couples) values were calculated for each peptide of interest using Pinpoint Software (Thermo Scientific). Transition couple values can be found in the supplementary tables: Standard O-GlcNAcylated CREB peptide (Supporting information Table 1); tryspin-digested ACTIN peptides (Supporting information Table 2); tryspin digested GSK-3β peptides (Supporting information Table 3) and Glu-C-digested (in phosphate buffer) GSK-3β peptides (Supporting information Table 4).

LC-MRM-MS analysis

Peptide samples were injected to nano-LC-MRM-MS. Peptide separation was performed with nanoACQUITY UltraPerformance LC® (Waters) using solvent A (water, 0.1 % formic acid); solvent B (Acetonitrile, 0.1 % formic acid); C18 trap column (Waters Nanoacquity

UPLC 180µm x 20mm 5µM symmetry C18); and an analytical column (Waters Nanoacquity UPLC 75µm x 200mm 1.7µm BEH130 C18) kept at 35°C.

Samples containing the standard O-GlcNAcylated CREB peptide were loaded onto the trap column with 99.5 % of solvent A at 6 µl/min for 3 min and eluted on the analytical column using a linear gradient from 2 % to 40 % solvent B for 15 min at a flow rate of 0.3 µl/min. Samples used for GSK-3β identification and quantification were loaded onto the trap column with 99 % of solvent A at 6 µl/min for 6 min and eluted on the analytical column using a linear gradient from 2 % to 30 % solvent B for 45 min followed by another linear gradient from 30 % to 40 % of solvent B for 10 min at a flow rate of 0.3 µl/min.

Eluted peptides were then sent on a TSQ vantage triple stage quadrupole mass spectrometer (Thermo Scientific). Fixed parameters were set as follows: 220°C interface heater temperature; 1700 V electrospray needle; 0.4 Da peak width for Q1; 1.3 mTorr Argon pressure in collision cell; 20 ms dwell time per transition for a maximum of 120 transitions. Peptide-specific tuned parameters determined by Pinpoint software (Thermos Scientific) were: S-lens value; collision energy up to a maximum of 50 V; declustering potential. Acquired data were analyzed on Pinpoint Software (Thermos Scientific) for detection and integration of LC-MRM-MS traces.

Additional protocols

Additional information (hESC culture, Protein harvest, Immunoprecipitation, SDS-PAGE gel and Western blots) can be found in the Supporting Information section.

RESULTS AND DISCUSSION

MRM-MS to detect a standard O-GlcNAcylated CREB peptide

We used a commercially available standard O-GlcNAcylated CREB peptide to investigate MRM-MS detection using the transition couples values listed in Supporting information Table 1. As O-GlcNAc is often reported to be lost during fragmentation in Q2[19], we first compared signal intensities between transitions (precursor-fragment ions) of ions retaining and of ions losing O-GlcNAc. As expected, the transition of ions losing O-GlcNAc all presented a higher intensity than the transition of ions retaining O-GlcNAc (noted with an asterisk on the figure), with an intensity difference ranging from 42 to 1205 folds (Figure 1.A). In addition, transitions of peptide backbone with O-GlcNAc loss and of O-GlcNAc oxonium ion were among the most intense transitions, supporting the observation of a loss of O-GlcNAc during the fragmentation in Q2. Even though we are able to detect O-GlcNAc peptide based on ions retaining O-GlcNAc (Supporting Information Figure 1), we chose to focus on transitions of fragment ions losing O-GlcNAc because it gave higher intensity signals (Figure 1.B).

Next, we determined for the standard O-GlcNAcylated CREB peptide, the method detection limit defined as "the minimum concentration of a substance that can be measured and reported with 99% confidence".[42] Even though as little as 0.5 pg (0.5 fmol) of peptide gave rise to a good signal to noise ratio (7,000 folds in average), the transition traces were not clearly resolved (Figure 2.A, B). Whilst 1 or 2 clear transitions are often reported for peptide detection, we used a more stringent threshold of 4 clear transitions to define the method detection limit. On this basis, 2.5 pg (3 fmol) was chosen as our method detection limit.

Furthermore, by integrating all LC-MRM-MS traces, a calibration curve (Relative area/peptide amount) was drawn with a coefficient of determination of 0.996 (Figure 2.C). The calibration curve therefore suggests that MRM-MS is a robust method to detect and quantify the amount of O-GlcNAcylated peptide by traces integration.

We then tested if the detection of O-GlcNAcylated peptide would be affected by the presence of a complex mixture. The control O-GlcNAcylated peptide was mixed with trypsin-digested whole cell lysate obtained from human embryonic stem cells (hESC). Two series

containing 2.5 pg and 12.5 pg of O-GlcNAc peptide were respectively investigated with a ratio O-GlcNAc peptide: hESC lysate from 5:95 to 1:99. The presence of hESC lysate was confirmed by the detection of transition couples values (listed in Supporting information Table 2) corresponding to two ACTIN peptides obtained after a trypsin digestion (see LC-MRM-MS traces in Supporting Information Figure 2). With a ratio as low as 1:99 (O-GlcNAc peptide: hESC lysate), MRM-MS was still able to detect 12.5 pg (Figure 3.A) and 2.5 pg (Figure 3.B) of the control O-GlcNAcylated peptide. In addition, the LC-MRM-MS traces of the O-GlcNAcylated peptide were clear of non-specific peak during the whole LC run (Figure 3.C, D). Even though the noise slightly increased with a higher ratio of 1:10,000; the O-GlcNAcylated peptide remained clearly detectable by MRM-MS (Supporting Information Figure 3).

MRM-MS to discover a novel O-GlcNAcylated GSK-3β peptide

After completing the proof-of-concept experiments on O-GlcNAcylated peptide standard, we focused our attention on employing MRM-MS technology to detect previously unreported native O-GlcNAcylated peptides. Glycogen synthase kinase-3 beta (GSK-3β) protein was chosen as candidate for two reasons. First, GSK-3β kinase activity is related to diseases (neurodegenerative diseases,[38] cancer[39, 40]), and competes with O-GlcNAc transferase to modify identical sites on some proteins.[43, 44] Second, GSK-3β has also been reported to be modified[45] and regulated[46] by O-GlcNAc modification. To date, the O-GlcNAcylation site of GSK-3β is still unknown.

Therefore, GSK-3β O-GlcNAcylation in hESC was investigated. By using an anti-O-GlcNAc antibody to purify hESC O-GlcNAcylated proteins, GSK-3β was immunoprecipitated (Figure 4 IB: Anti-GSK-3β, lower panel). In addition, by treating hESC with PUGNAc (an O-GlcNAc hydrolase inhibitor)[23, 41] to increase global level of O-GlcNAcylation (Figure 4 IB: Anti-O-

GlcNAc, upper panel), the amount of precipitated GSK-3β was increased (Figure 4 IB: Anti-GSK-3β, lower panel); which is consistent with GSK-3β being O-GlcNAc modified.

GSK-3β O-GlcNAc modification sites were predicted based on human GSK-3β protein sequence (Uniprot accession number: P49841) by using a web-based bioinformatics resource: the database of O-GlcNAcylated proteins and sites (dbOGAP).[47] Six predicted O-GlcNAcylation sites were found on GSK-3β by using this prediction tool (Supporting Information Table 5).

The predicted GSK-3β O-GlcNAcylation sites were then investigated by MRM-MS. For that, GSK-3β enrichment was kept to a minimum: only 20 µg of whole hESC lysate was separated through gel electrophoresis; and based on the molecular weight of GSK-3β, a gel slice ~45 kDa was excised and processed for proteolysis using either trypsin or Glu-C digestion. The presence and digestion of GSK-3β was confirmed by the detection of transition couples values (listed in Supporting Information Table 3, 4) corresponding to the expected non-modified GSK-3β peptides following trypsin digestion (Supporting Information Figure 4.A) and Glu-C digestion (Supporting Information Figure 4.B). Similarly, by taking the transition couples values for the predicted modified peptides (in Supporting Information Table 3, 4), we identified by MRM-MS the overlapping peptides: VT^{38}TVVATPGQGPDRPQEVSYTDTK+O-GlcNAc from trypsin digestion (Figure 5.A, B) and GSKVT^{38}TVVATPGQGPD+O-GlcNAc from Glu-C digestion (Figure 5.C, D) that corresponds to GSK-3β T^{38}-O-GlcNAc peptides. The detection of those two overlapping peptides not only validates the identification of GSK-3β O-GlcNAcylated peptides but also helps in narrowing down the potential O-GlcNAcylation sites to 3 possibilities which are T^{38}, T^{39}, T^{43}.

Next, we used the identified peptide (GSKVTTVVATPGQGPD + O-GlcNAc) to demonstrate the use of MRM for quantifying changes in O-GlcNAcylation, between hESC non-treated and treated with PUGNAc [23, 41] (Figure 6.A). A non-modified GSK-3β peptide (YTPTARLTPLE) was used as a MS loading control (Figure 6.B). By integrating the (GSKVTTVVATPGQGPD

+ O-GlcNAc) peptide trace and normalizing the area value with (YTPTARLTPLE), the amount of (GSKVTTVVATPGQGPD + O-GlcNAc) peptide was shown to increase by 2.5 fold following PUGNAc treatment (Figure 6.C). Concordantly, an increase of 2.6 fold was observed for the overlapping trypsin digested O-GlcNAcylated peptide (VTTVVATPGQGPDRPQEVSYTDTK + O-GlcNAc) following PUGNAc treatment (Supporting Information Figure 5).

In contrast to (GSKVTTVVATPGQGPD + O-GlcNAc), the amount of its corresponding non-modified peptide (GSKVTTVVATPGQGPD) was not significantly affected by PUGNAc treatment (Supporting Information Figure 6). This is not surprising since the amount of O-GlcNAcylated peptide might be negligible in comparison of its corresponding non-modified peptide; indeed the stoichiometry of O-GlcNAc on specific proteins is usually low (<10%).[5, 48]

CONCLUSIONS

O-GlcNAc modificiation is relevant for a variety of biological and physiological processes. However, significant technical difficulties remain in the detection of modification sites. In this study, we investigated the potential use of MRM-MS to detect and quantify O-GlcNAc modification.

We first showed that MRM-MS can be used as a qualitative method for the detection of O-GlcNAcylated protein. Unfortunately, most of O-GlcNAc moiety is lost during fragmentation in Q2, which is in accordance with previous reports done with shotgun MS.[19, 49] As fragment ions retaining O-GlcNAc might not be distinguishable from background noises, MRM method might not be ideal to directly pinpoint the exact O-GlcNAc modification site on a peptide. Nonetheless, as the method was able to specifically select precursor ions corresponding to O-GlcNAcylated peptides and detect their corresponding transitions, MRM-MS is able to detect O-GlcNAcylated peptides. We also reported a method detection limit of 3 fmol for the

standard O-GlcNAcylated CREB peptide. This method detection limit was not affected by the presence of a complex peptide mixture with a percentage as low as 1 % for the O-GlcNAcylated peptide. In comparison, O-GlcNAc stoichiometry has been reported to vary from 100 % to as low as 2 %.[18] Our data therefore supports that MRM-MS could be used to identify O-GlcNAcylated peptide from biologically samples where O-GlcNAc peptides only represent a small percentage of the total mixture. Furthermore, using this method, we discovered a novel O-GlcNAcylated GSK-3β peptide following a simple gel electrophoresis separation without the need for additional labeling or enrichment. As a result, MRM-MS doesn't require as extensive labelings or enrichments as the ones often reported for MS shotgun process.[20, 21, 22, 23] In this regard, MRM-MS could be integrated with existing O-GlcNAc analytical techniques as an early discovery tool to quickly narrow down potential O-GlcNAcylation sites on a specific protein.

Beside qualitative detection, we demonstrated that MRM-MS method can be applied to robustly quantify the amount of O-GlcNAcylated peptides without the need for labeling. As O-GlcNAc research often involves comparing protein O-GlcNAcylation level between cell compartments,[50] mutant cells,[51] drug-treated cells,[52] and stem cell differentiation;[12] a quantification instrument such as MRM-MS will be beneficial for future O-GlcNAc research. Furthermore, as MRM-MS is also able to identify and quantify phosphorylated peptides;[35, 36] MRM can become a powerful tool to investigate the interplay between phosphorylation and O-GlcNAcylation.

We also reported GSK-3β O-GlcNAcylation in hESC. This modification might regulate GSK-3β kinase function in hESC as reported previously in human kidney HEK cells.[46] In addition, we narrowed down the potential O-GlcNAc modification site to three threonine residues: T^{38}, T^{39} and T^{43}. Interestingly, a phosphorylation site on an adjacent serine (S^{25}) was previously reported;[53] suggesting that there might be interplay between these two modifications. Furthermore, MRM-MS identified O-GlcNAcylated sites could be used to predict O-GlcNAc

function. For example, the domain of the GSK-3β O-GlcNAcylated site identified in our study was previously reported to be involved in a GSK-3β kinase auto-inhibition mechanism.[54] Hence, we hypothesize that in hESC GSK-3β O-GlcNAcylation may be involved in a similar mechanism.

For future investigation, our reported MRM method could be improved to enable the discovery of O-GlcNAc modification sites. Indeed, reports have shown the advantage of a β-elimination/Michael addition of DTT (BEMAD) reaction to substitute O-GlcNAc by dithiothreitol which contrary to O-GlcNAc, is stable through fragmentation.[23, 24] By using BEMAD on O-GlcNAcylated peptides, MRM-MS method could then pinpoint the exact dithiothreitol substitution sites. Alternatively to BEMAD, using a mass spectrometer equipped with electron transfer dissociation (ETD) fragmentation might enable MRM method to detect native O-GlcNAcylation sites. Indeed, in contrast to the collision induced dissociation (CID) fragmentation used in this study, ETD fragmentation relies on charge transfer from anions[55] and have been reported to preserve O-GlcNAc modification through fragmentation.[43, 56] In addition, ETD fragmentation is known to produce c- and z-ions which might then be used as additional transition couples to verify peptide detection by MRM-MS.[57, 58]

To conclude, we demonstrated that MRM-MS is a robust analytical method for qualitative and quantitative assessments of O-GlcNAcylated peptides issued from native proteins. We are confident that this new tool will be invaluable for the field of O-GlcNAc research.

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ABREVIATIONS

The abbreviations used are as follows:

GSK-3β	Glycogen synthase kinase-3 beta
hESC	Human embryonic stem cells
LC	Liquid chromatography
O-GlcNAc	O-linked N-acetylglucosamine
MRM	Multiple Reaction Monitoring
MS	Mass Spectrometry

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FIGURES AND FIGURES LEGENDS

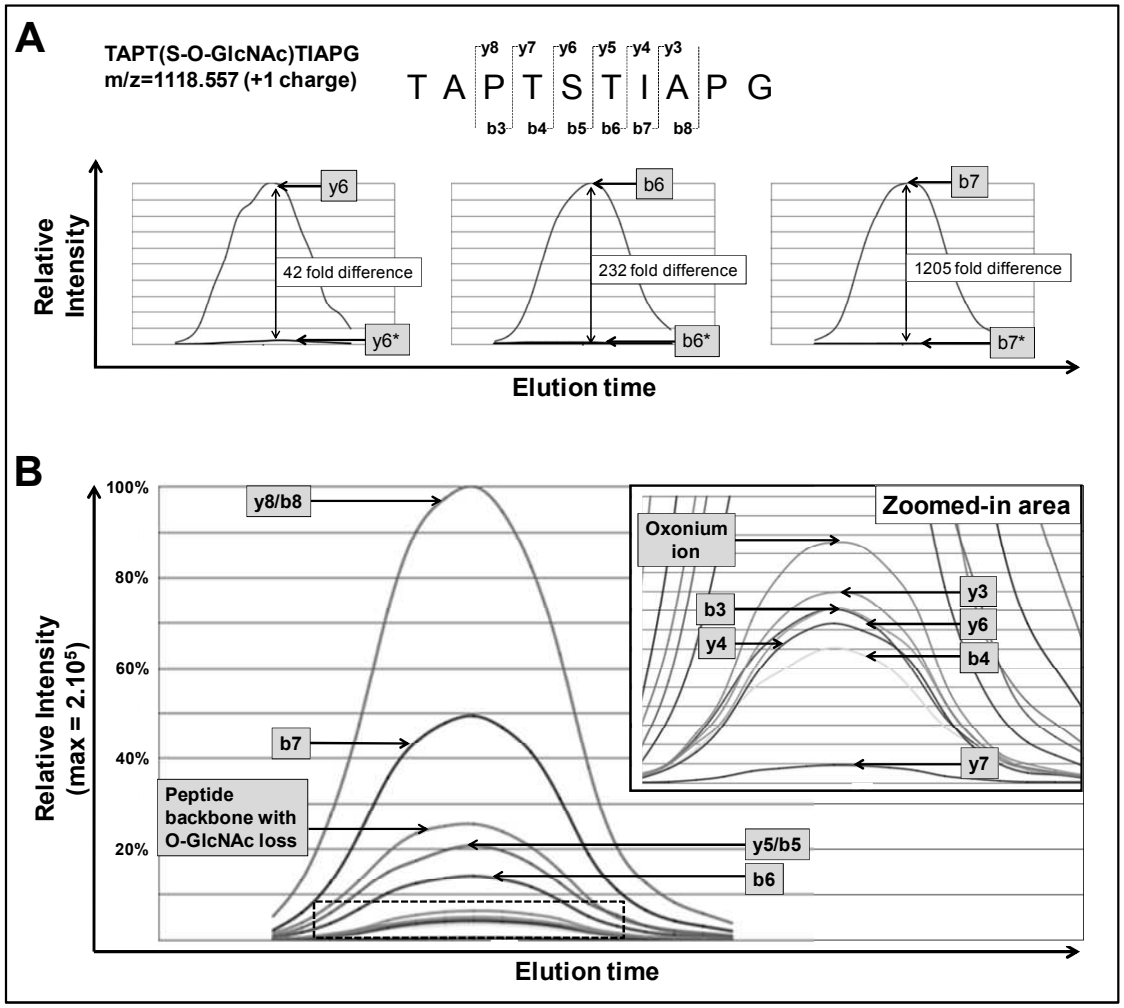


Figure 1. A. Comparison of representative LC-MRM-MS traces between transitions of ions retaining (*) and transitions of ions losing O-GlcNAc. **B.** Representative LC-MRM-MS traces from an injection of 250 pg of

standard O-GlcNAcylated CREB peptide with transitions of +1 charged precursor coupled to fragment ions losing O-GlcNAc modification. The encircled area with doted lines represents the zoomed-in area at the top right corner.

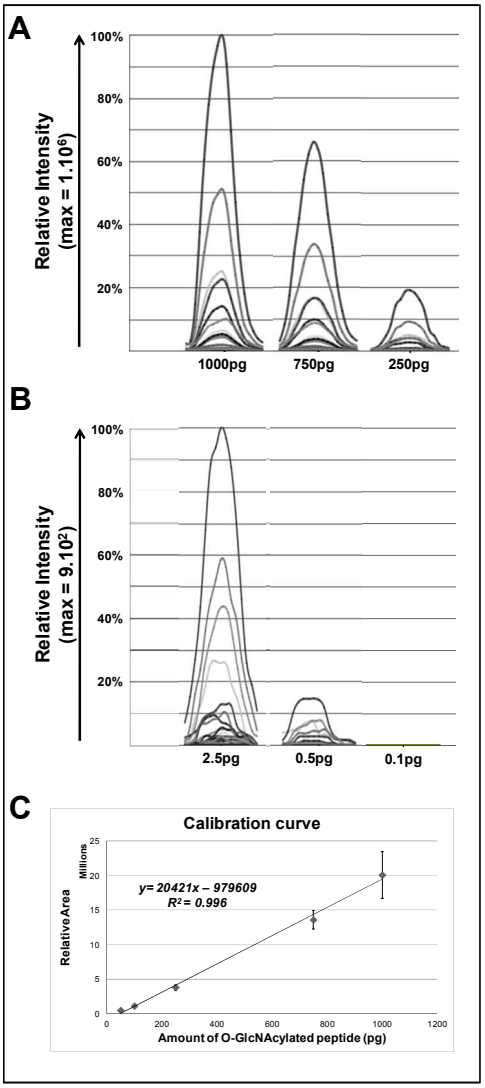


Figure 2. Comparison of representative LC-MRM-MS traces from **A.** 1000 pg to 250 pg; and **B.** 2.5 pg to 0.1 pg of standard O-GlcNAcylated CREB peptide. **C.** Calibration curve of standard O-GlcNAcylated CREB peptide with 1000; 750; 250; 100; 50 pg inputs. Each sample was run in triplicate. The area values were obtained by integrating LC-MRM-MS traces.

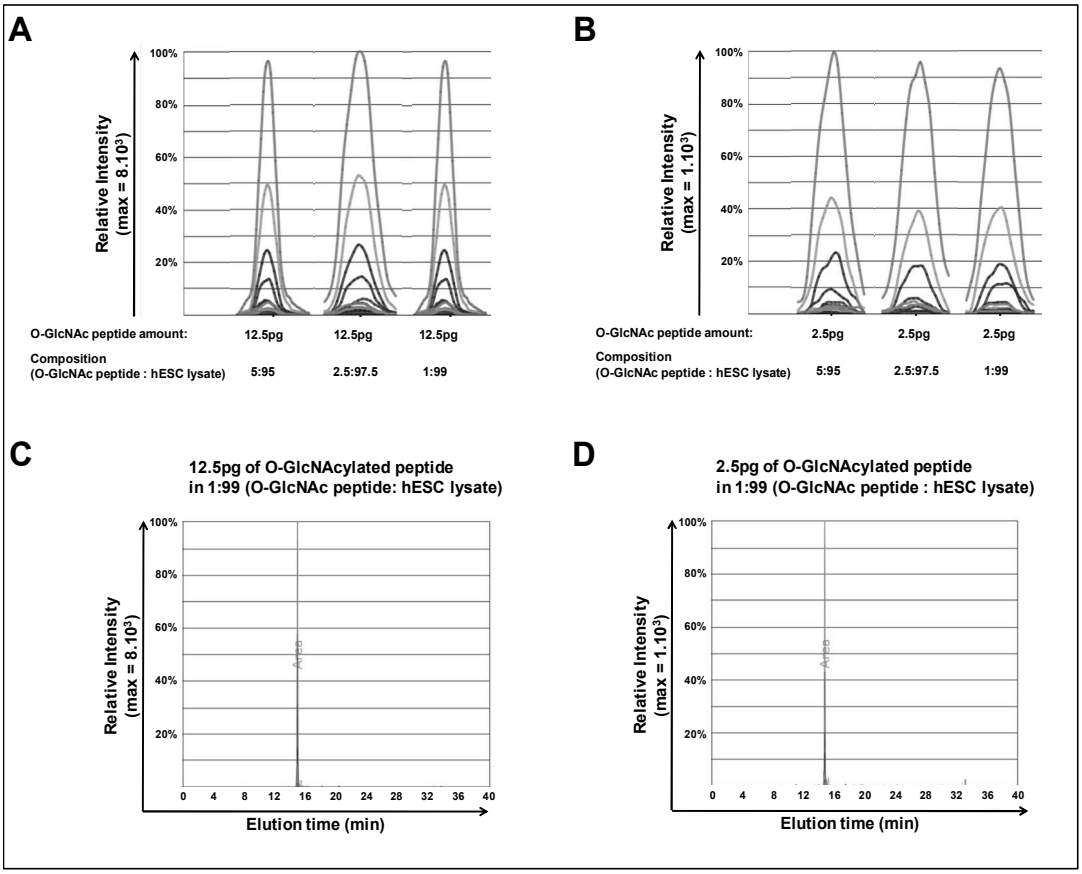


Figure 3. LC-MRM-MS traces from O-GlcNAcylated peptide: hESC lysate mixture in the ratios: 5:95; 2.5:97.5 and 1:99 for respectively **A.** 12.5 pg and **B.** 2.5 pg of standard O-GlcNAcylated CREB peptide. LC-MRM-MS traces for the whole 40 min LC run of mixture 1:99 (O-GlcNAcylated peptide: hESC lysate) containing respectively **C.** 12.5 pg; and **D.** 2.5 pg of standard O-GlcNAcylated CREB peptide. The trace corresponding to the O-GlcNAcylated peptide elutes at 14.9 min.

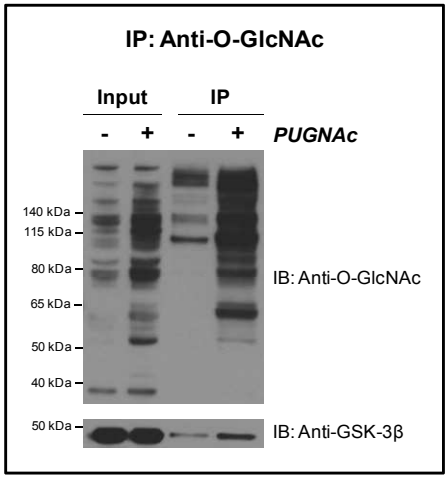


Figure 4. Western blots of immunoprecipitated (IP) O-GlcNAcylated proteins from hESC treated without (-) or with (+) 100 µM of PUGNAc for 24 h.

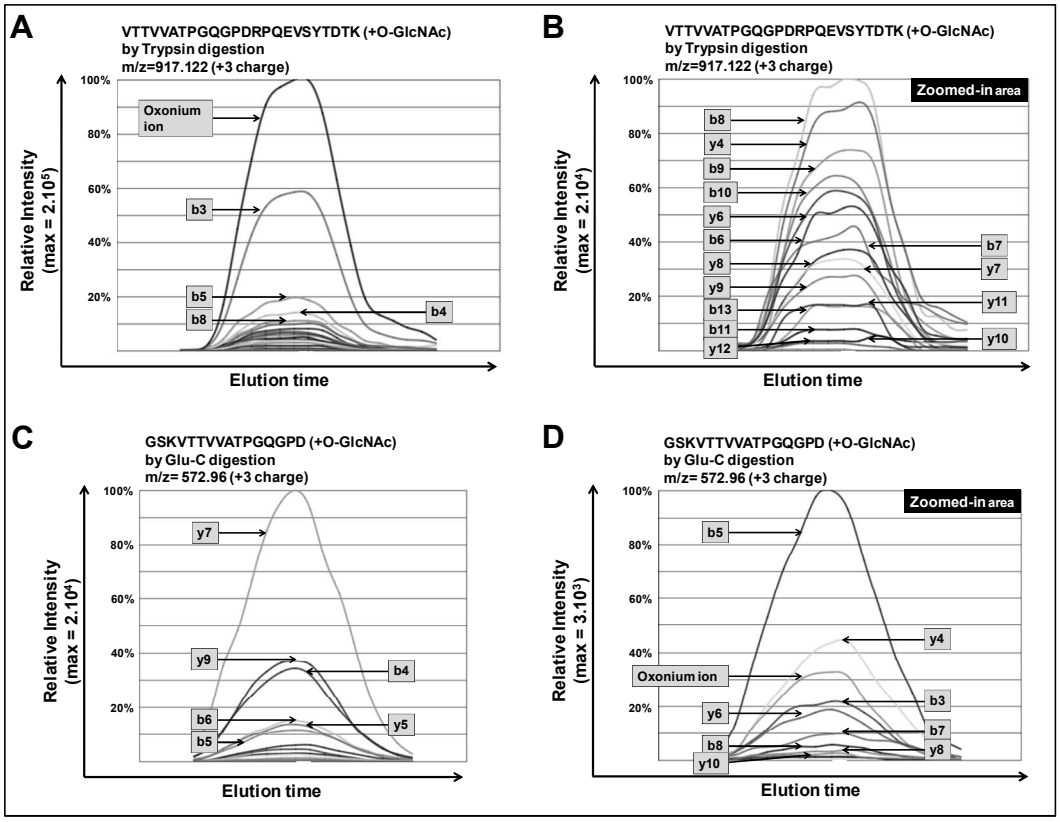


Fig5. LC-MRM-MS traces of O-GlcNAcylated GSK-3β peptides by **A. and B.** trypsin digestion (VTTVVATPGQGPDRPQEVSYTDTK+O-GlcNAc); and **C. and D.** Glu-C digestion (GSKVTTVVATPGQGPD+O-GlcNAc).

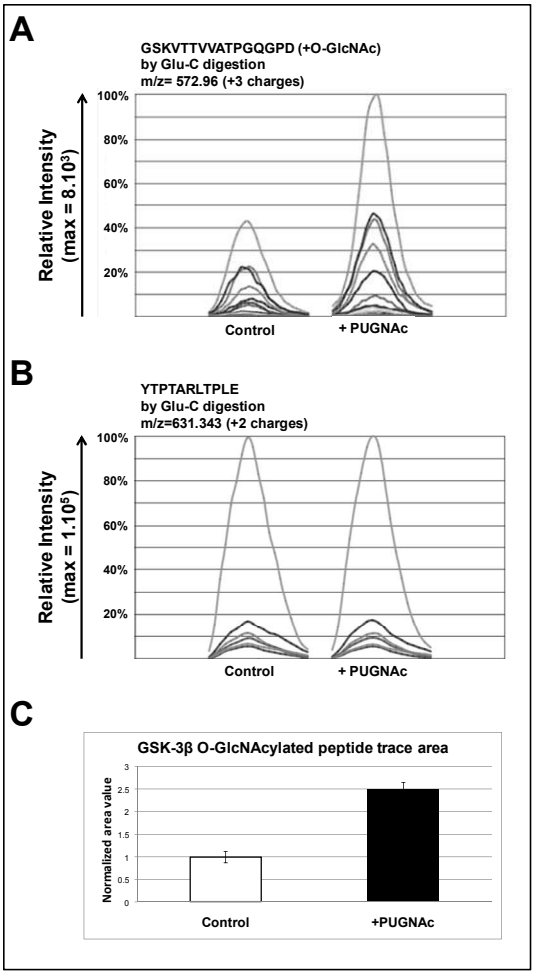
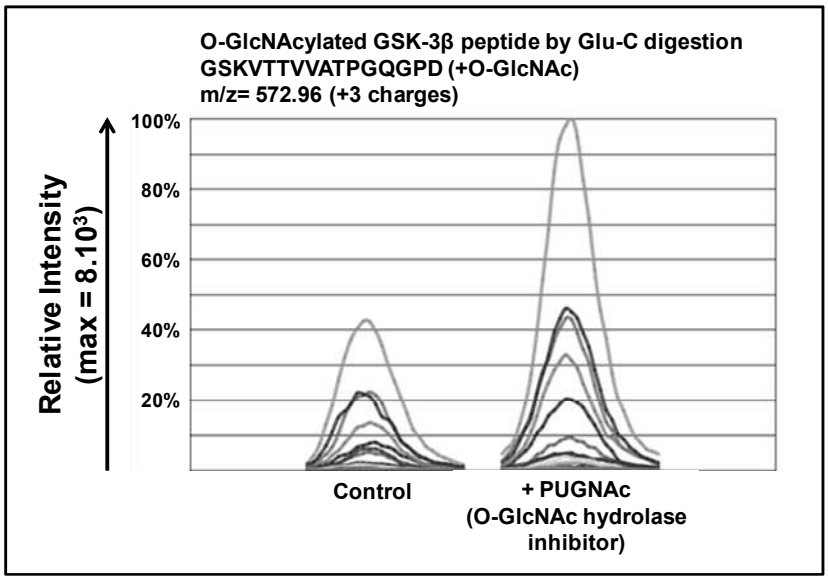


Fig6. Comparison between hESC non-treated (Control) and hESC treated with 100 µM of PUGNAc for 24h (+ PUGNAc). Representative LC-MRM-MS traces of **A.** Glu-C digested O-GlcNAcylated GSK-3β peptides (GSKVTTVVATPGQGPD+O-GlcNAc); and of **B.** Glu-C digested GSK-3β peptide (YTPTARLTPLE) used as MS loading control. **C.** Comparison of the area values from the integration of O-GlcNAcylated GSK-3β peptides traces normalized by (YTPTARLTPLE) peptide.

FOR TOC only:



Abstract Graphic.