Published in final edited form as:

Bioanalysis. 2014 October ; 6(19): 2571–2580. doi:10.4155/bio.14.239.

Proteomic approaches for site-specific O-GlcNAcylation analysis

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Abstract

O-GlcNAcylation is a dynamic protein post-translational modification of serine or threonine residues by an *O*-linked monosaccharide N-acetylglucosamine (*O*-GlcNAc). *O*-GlcNAcylation was discovered three decades ago and its significance has been implicated in several disease states, such as metabolic diseases, cancer and neurological diseases. Yet it remains technically challenging to characterize comprehensively and quantitatively because of its low abundance, low stoichiometry and extremely labile nature under conventional collision-induced dissociation tandem MS conditions. Herein, we review the recent advances addressing these challenges in developing proteomic approaches for site-specific *O*-GlcNAcylation analysis, including specific enrichment of *O*-GlcNAc peptides/proteins, unambiguous site-determination of *O*-GlcNAc modification and quantitative analysis of *O*-GlcNAcylation.

O-GlcNAcylation, a distinctive form of *O*-glycosylation [1,2], occurs on serine or threonine residues by adding a monosaccharide, N-acetylglucosamine (GlcNAc) via the side chain hydroxyl group (Figure 1). Protein *O*-GlcNAc is dynamically controlled by *O*-GlcNAc transferase, *O*-GlcNAcase and UDP-GlcNAc. *O*-GlcNAc transferase adds *O*-GlcNAc to the Ser/Thr residues in proteins using UDP-GlcNAc as the donor, while *O*-GlcNAcase removes *O*-GlcNAc from proteins [3]. UDP-GlcNAc is the product of the hexosamine biosynthetic pathway and its concentration affects *O*-GlcNAcylation [4]. Therefore, *O*-GlcNAcylation is often deemed a nutrient sensor and is now recognized as an important mechanism for modulating cell responses to the constantly changing nutrient environment [5,6].

Initially *O*-GlcNAc was detected using UDP-[³H]-galactose radioactive methods [7]. With the development of MS-based proteomic approaches [8], over 1000 proteins are known to be

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Financial & competing interests disclosure

Portions of this work were supported by NIH grants U24-CA-160019 from the National Cancer Institute Clinical Proteomic Tumor Analysis Consortium (CPTAC) and NIGMS Biomedical Technology Research Resource P41GM103493, and an Inter-agency Agreement from the US Department of Defense through the Henry M. Jackson Foundation under MIPR2DO89M2058. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

O-GlcNAcylated from nearly all functional classes [9], including transcription factors [10], histones [11], cytoskeletal proteins [12], proteasome proteins [13], metabolic enzymes [14], kinases [15] and other signaling proteins. Aberrant *O*-GlcNAcylation has been observed in cancers [16–18] and a number of other human diseases, including diabetes [19] and Alzheimer's disease [20]. The significance of *O*-GlcNAc was also highlighted by a recent study suggesting that the regulation of PKF1 by *O*-GlcNAc is key for rewiring of cancer cell's metabolism [21,22]. The function of *O*-GlcNAc in metabolism was also recently reviewed [23]. *O*-GlcNAcylation has also been reported in the regulation of other biological processes, such as cell signaling [24] and stress response [25]. The interplay of *O*-GlcNAcylation with phosphorylation [9] or ubiquitination [26] also potentially has significant biological impacts for cellular regulation.

However, proteomic analysis of *O*-GlcNAc still presents significant challenges. First, *O*-GlcNAc is of low stoichiometry and generally low abundance. Although some proteins may be abundantly modified [20,27], the frequency of *O*-GlcNAc among the whole proteome has been estimated to be far below that of phosphorylation [28,29]. Second, the extremely labile nature of *O*-GlcNAc makes it difficult to determine modification sites by conventional tandem MS (MS/MS), which uses collision-induced dissociation (CID). Third, there are many forms of *O*-glycosylation, including *O*-GalNAc [30,31], *O*-Mannose [32], *O*-Fucose [33] and so on. The other *O*-glycosylation species share much similarity with *O*-GlcNAc in that they can all undergo β -elimination and Michael addition (BEMAD), be easily lost in CID fragmentation and may show similar affinity for **lectins**. Moreover, the *O*-GalNAc modification [34] (Tn antigen) has the exact same mass as *O*-GlcNAc modification because of the same chemical composition, causing ambiguity in MS detection. Combining with subcellular fractionation, most of the time *O*-GlcNAc and other *O*-linked sugars may be differentiated, because the latter are mostly present in the Golgi/endoplasmic reticulum and extracellular proteins [35].

To date proteomic studies of *O*-GlcNAc modifications have been largely focused on method development. The most successful *O*-GlcNAc profiling study to date detected 1750 sites at the murine synapse [29]. Herein we review the current proteomic approaches for analysis of *O*-GlcNAc and their limitations and discuss some techniques that may further advance this field.

Enrichment of *O*-GlcNAc

Efficient, specific **enrichment of *O*-GlcNAc** peptides/proteins is desired for successful proteomic study of *O*-GlcNAc. Currently available enrichment methods for *O*-GlcNAc peptides or proteins can be divided into two categories: chemoenzymatic methods using a chemical handle for enrichment; and, affinity-enrichment methods using lectins or antibodies.

Chemoenzymatic methods

In the chemoenzymatic methods, *O*-GlcNAc is typically linked to a chemical handle, such as an azide [14], acetone [36] or alkyne [37], through enzyme-catalyzed reactions, then the chemical handle is linked with biotin, phosphate or resin for enrichment (Figure 2).

In the first step, the chemical handle is transferred to GlcNAc *in vitro* or *in vivo*. The *in vitro*-labeling strategy was developed by Khidekel *et al.* and Clark *et al.*, in which a ketogalactose [36] or azidogalactose [14] was transferred onto the *O*-GlcNAc moiety using an engineered GalT1 enzyme with a Y289L mutation. The *in vivo*-labeling strategy was developed by Vocadlo *et al.* and Zaro *et al.*, in which a GlcNAc modified with azide (GlcNAz) [38] or alkyne (GlcNAIk) [37] is accommodated by the enzymes of the GlcNAc salvage pathway, and then incorporated into proteins by *O*-GlcNAc transferase in living cells. However, *in vivo* labeling is restricted to cultured cells, while *in vitro* labeling is applicable to all types of samples, such as tissues. The choice of azide or alkyne to be the chemical handle is based on the fact that they are small, inert under physiological conditions and their reactions, similar to **click chemistry**, are specific and robust.

In the second step, the chemical handle is linked to a biotin or phosphate moiety or directly to a resin via click chemistry. Application of click chemistry in *O*-GlcNAc analysis was exemplified by the research of Wang *et al.* [39] and Alfaro *et al.* [40]. The term click chemistry usually refers to Cu-catalyzed azide–alkyne cycloaddition [41], which was introduced in 2002 [42]. Click chemistry usually offers great specificity and high reaction efficiency and the side reactions can be minimized by fine-tuning of the catalyst system [43].

Avidin–biotin chromatography, which is based on the strong interaction between streptavidin and biotin, is often used in the enrichment step. Target peptides are eluted from the column after the nonspecific peptides are washed away. However, the elution of biotinylated *O*-GlcNAc peptides from avidin chromatography is often difficult owing to the strong biotin–avidin interaction. To tackle this challenge, cleavable linkers, such as disulfide linker [44], acid cleavable linker [45], azobenzene linker [46] and photocleavable linker [39], were introduced to release the enriched molecules. Among these cleavable linkers, the photocleavable linker is attractive in principle because it needs no extra reagent for elution. First adopted by Wang *et al.*, photocleavable linkers allow efficient release of enriched peptides from the avidin resin upon UV radiation [39]. This chemical/enzymatic photochemical cleavage enrichment method offers a relatively good specificity and sensitivity by taking advantage of the high specificity of enzymatic tagging, click chemistry and biotin–avidin chromatography, and the highly efficient photochemical cleavage. Another advantage of this method is that *O*-GlcNAc-modified peptides are tagged with a basic moiety, aminomethyltriazolacetyl galactosamine (AMT-GalNAz) as a result of photocleavage. This basic tag may promote **electron transfer dissociation (ETD)** and also increase peptide fragment efficiency of higher energy collision induced dissociation (HCD) by producing two additional diagnostic oxonium ions (300.13 and 503.21 *m/z*) for the intact sugars compared with that from untagged native *O*-GlcNAc peptides in HCD and CID. These advantages provide increased confidence and specificity (i.e., *O*-GalNAcylated peptides cannot be enriched by this method) of *O*-GlcNAcylated peptide identification and site localization. With this method, Wang *et al.* identified 141 *O*-GlcNAcylation sites in 64 proteins from <15 µg of spindle and midbody proteins [39,47]. Recently we have adopted this approach for enriching *O*-GlcNAc peptides using approximately 100 µg of tryptic peptides from complex mouse cerebrocortical brain tissue [40]. In a single LC–MS/MS

analysis, 249 *O*-GlcNAcylated proteins were identified with 358 unambiguous *O*-GlcNAc modification sites determined.

In addition to biotin–avidin chromatography, alternative approaches have been reported for enriching azide (or other chemical handle)-containing *O*-GlcNAc peptides. In a study by Parker *et al.*, *O*-GlcNAc peptides were first given an azide handle and then linked with phosphor-alkyne reagent and enriched by TiO₂ chromatography, allowing a total of 42 *O*-GlcNAc peptides to be identified [48]. In another study, Merel *et al.* used a cyclooctyne resin to enrich azide-containing peptides [49]. The 1,3-dipolar cycloaddition reaction between azide and cyclooctynes does not need a copper catalyst and was given the name ‘copper-free click chemistry’ [50]. In another method, azido glycopeptides were captured by immobilized alkyne on the surface of magnetic silica particles and then released into solution by cleavage of a disulfide bond [51]. More recently, metabolic labeling of cells by azide-modified GlcNAc followed by capturing of *O*-GlcNAcylated proteins by an alkyne resin was used for identifying 1500 *O*-GlcNAcylated proteins; 185 *O*-GlcNAc sites were determined by peptides released through β -elimination [52]. Nishikaze *et al.* also introduced an approach using reversible hydrazide chemistry for enrichment of *O*-GlcNAc [53]. In this method, *O*-GlcNAc was transferred with GalNAz and then linked with 3-ethynyl-benzaldehyde via click chemistry. Reversible hydrazide chemistry was then exploited to enrich this aromatic aldehyde-modified peptide. This method was demonstrated in a proof-of-principle manner using a tryptic digest of a *O*-GlcNAc protein, bovine α -crystallin.

Affinity enrichment using lectins or antibodies

Besides chemoenzymatic methods, affinity enrichment is another popular alternative for analyzing protein *O*-GlcNAcylation. The specific affinity to different carbohydrates makes lectins ideal for enriching *O*-GlcNAc. The natural lectin wheat germ agglutinin (WGA), which binds to *N*-acetyl-D-glucosamine and sialic acid, has been commonly used for enrichment of *O*-GlcNAc [54]. In WGA-based weak affinity chromatography, *O*-GlcNAc-modified glycopeptides migrate slower than unmodified peptides and can, thus, be enriched [54]. Using this approach, Vosseller *et al.* identified 145 *O*-GlcNAc peptides from postsynaptic density preparations [54]. More recently, the same group further advanced their workflow by improving the lectin affinity chromatography with WGA immobilized on silica beads and by fractionation of the enriched peptides with high pH reversed-phase LC, which allowed the identification of 1750 sites of *O*-GlcNAcylation from proteins extracted from 30 mg of murine synaptosomes [29]. This study illustrates the benefit of **2D-LC** for achieving broad coverage [55]. Besides WGA, a synthetic lectin with enhanced selectivity for *O*-GlcNAc has been recently reported [56], which may provide further improvements for *O*-GlcNAc profiling.

O-GlcNAc antibodies are also useful for enrichment of *O*-GlcNAc proteins. It is desirable to have a high-affinity and pan-specific antibody for *O*-GlcNAc, but to date such an antibody has not become available. Recently, Teo *et al.* obtained a large panel of *O*-GlcNAc-specific IgG monoclonal antibodies [57]. After immunoprecipitation with these antibodies, 200 *O*-GlcNAc proteins were identified from a large-scale shotgun proteomics study. These antibodies appear to have different but overlapping local peptide determinants in addition to

their *O*-GlcNAc dependence. The potential application of these antibodies in analyzing *O*-GlcNAc towards comprehensive coverage of *O*-GlcNAc modifications has yet to be demonstrated.

Identification & site determination of *O*-GlcNAc by MS

Conventional LC–MS/MS using CID has been commonly applied to identify *O*-GlcNAc; however, *O*-GlcNAc site localization remains difficult under CID conditions owing to the high susceptibility of neutral loss of the *O*-GlcNAc moiety. The BEMAD approach [58] or ETD method have been reported to largely overcome this issue.

BEMAD strategy

The BEMAD strategy was first reported by Wells *et al.* for the precise determination of sites of *O*-GlcNAcylation [58]. In a solution of pH 12–12.5, the *O*-GlcNAc peptide undergoes a β -elimination reaction, producing a double bond, and dithiothreitol or biotin pentylamine [58], cystaemine [59] or biotin–cystaemine [60] can be added onto the newly formed double bond through Michael addition (Figure 3). The resulting peptide is stable in CID and can provide enough fragment ions for mapping the site of *O*-GlcNAc modification. Recently, Nika *et al.* optimized the BEMAD procedure of *O*-GlcNAc peptides on reversed-phase supports [59]. In this approach, peptides were first immobilized on reversed phase (C18) supports, and undergo β -elimination and Michael addition using Barium hydroxide as base and 2-aminoethanethiol as nucleophile. Most recently, Hahne *et al.* showed that β -elimination alone can allow the identification of *O*-GlcNAc [52]. Despite these efforts, the concern of side reactions and efficiency of the reaction seems to prevent this approach from being applied for large-scale profiling of *O*-GlcNAcylation.

Direct detection methods using different dissociation modes

CID is the most commonly used MS/MS technique. In CID, a feature for *O*-GlcNAc peptides with GlcNAc loss and GlcNAc oxonium ion (204.09 *m/z*) was observed due to the labile nature of the *O*-glycosidic bond in the gas phase [61]. This feature was exploited by Hahne *et al.* to identify hundreds of new *O*-GlcNAc proteins from published proteome data [28]. Although this feature helps to identify *O*-GlcNAc peptides (i.e., presence of the *O*-GlcNAc modification), *O*-GlcNAc site localization remains difficult in CID. For determination of the exact site(s) of *O*-GlcNAc modification, ETD is often necessary.

Unlike CID, ETD [62] is not based on collisions with neutral gas molecules. In ETD, electrons are transferred from radical anions to peptides or proteins, causing the latter fragment to form *c* and *z* ions in a nonergodic process, thus preserving labile post-translational modifications (PTMs) in the daughter ions, enabling unambiguous and confident determination of the modification sites for *O*-GlcNAc [63]. The efficiency of ETD is often decisive for successful site determination and ETD generally works better for relatively longer peptides carrying multiple charges. This can be problematic for peptides generated by trypsin digestion owing to their smaller average size (approximately 10 amino acid residues) and charge (typically +2) [64], leading to a lower success rate of identification by ETD [65]. Many previous ETD studies using trypsin (instead of Lys-C to generate longer

peptides) seem to have benefited from the miscleavages in guanidine-assisted trypsin digest (partial digestion) [29,40]. Tryptic peptides that have one or two missed cleavage sites are more suitable for ETD and often give extensive *c* and *z* ions series. The concept of partial digestion for ETD was also demonstrated by Deng *et al.* in their phosphoproteome profiling study of mitochondria, in which 185 phosphorylation sites were only identified through partial digestion and ETD while 45 sites were identified only by complete digestion and CID [66]. The use of multiple proteases, such as trypsin and GluC [67], may enhance sequence coverage of site determination by ETD. The use of LysN in conjunction with the ETD analysis of *O*-GlcNAc is potentially promising, since LysN cleaves proteins at the amino side of lysine, producing predominant *c*-type fragment ions in ETD [68]. Another strategy to improve ETD performance is to modify tryptic peptides to facilitate the generation of higher charge peptide ions in electrospray ionization. Both of the carboxyl groups [69,70] and the amine groups [64,71] can be modified with basic tags, such as tertiary or quaternary amine groups. As mentioned earlier, *O*-GlcNAc peptides were tagged by a basic tag in the chemical/enzymatic photochemical cleavage enrichment method, which may contribute to enhanced ETD performance [39]. Moreover, further improvement can be obtained by the use of supplementary activation [65] in ETD of these peptides [40].

In addition to the use of ETD, CID and HCD may be complementary for obtaining better coverage of *O*-GlcNAc peptides. Analysis of the same precursor ions by both CID and ETD has been advocated as a superior method for identification of labile phosphopeptides [72] and *O*-GlcNAc peptides [40]. HCD has also been combined with ETD to obtain accurate site assignment for *O*-GlcNAc [73]. For example, a more efficient use of ETD was reported by triggering the ETD only when the *O*-GlcNAc reporter ion was detected by HCD [74].

Quantification of *O*-GlcNAc

In addition to identification, quantitative analysis for the dynamic *O*-GlcNAc modifications in cellular systems is essential for understanding signaling pathways regulated by *O*-GlcNAcylation and for identifying functional *O*-GlcNAc sites and studying potential crosstalk with other PTMs, such as phosphorylation [75].

Global relative quantification

In principle, *O*-GlcNAc-modified peptides can be quantified by both label-free and stable isotope-labeling-based methods. The label-free method, relying on signal intensity or spectrum count, is conceptually simple and straightforward compared with isotope-labeling methods; however, the reproducibility of overall workflow and platform often presents greater challenges for the label-free methods to achieve reliable quantification.

A number of isotopic-labeling strategies are potentially applicable for relative quantification of *O*-GlcNAc. Stable isotope-labeling with amino acids in cell culture has been broadly applied in quantitative proteomic analysis and was first adopted by Wang *et al.* for quantitative analysis of *O*-GlcNAc [76,77]. Isotope labeling can also be introduced in BEMAD using deuterated dithiothreitol [78], or in chemoenzymatic enrichment through dimethylation of the *O*-GlcNAc peptides [79]. Isobaric tag for relative and absolute quantitation [80,81] and tandem mass tag [82] labeling are both promising methods for

quantitative analysis of *O*-GlcNAc, with the added advantage of sample multiplexing, hence improved throughput. HCD MS/MS is usually required for isobaric tag for relative and absolute quantitation- and tandem mass tag-based quantification, although Rosa *et al.* reported the utility of combining tandem mass tag labeling and ETD for relative quantification of *O*-GlcNAc modification [82]. More recently, a promising method called neutron encoding (NeuCode) was reported, which exploits the subtle mass differences (5–40 mDa) between stable isotopes [83]. These differences, caused by nuclear binding energy variation, are not distinguishable with normal resolution but distinguishable with ultra-high resolution (>200,000) in MS. NeuCode labels can be introduced by either metabolic labeling or chemical tagging to facilitate highly multiplexed quantification [84].

Stoichiometry & targeted quantification

As a dynamic PTM, stoichiometry or site occupancy of *O*-GlcNAc is also of great interest. *O*-GlcNAc stoichiometry measurements have mostly relied on a gel-based method developed by Rexach *et al.* [85]. In this method, a PEG tag (2 or 5 kDa) was linked to the *O*-GlcNAc motif through an enzymatically tagged ketogalactose with the reaction between the aminoxy and ketone groups, as a result *O*-GlcNAcylated protein will show a certain mass shift on gel-electrophoresis corresponding to the extent of *O*-GlcNAcylation. By immunoblotting, one can quantify the *in vivo* *O*-GlcNAc stoichiometry, determining the protein to be non-, mono-, di- or multiply glycosylated. Using this method, *O*-GlcNAcylation was shown to be induced at Serine 529 of phosphofructokinase 1 in response to hypoxia [21]. In addition, phosphorylation could also be rapidly visualized by immunoblotting with antibodies. Thus, direct readout of phosphorylation and *O*-GlcNAcylation on the same protein becomes possible. With this approach, interplay between these two PTMs on CREB was studied in details by Rexach *et al.* [27]. However, this mass tagging and electrophoresis-blotting method does not allow site-specific quantification and relies on the quality of the primary antibodies.

Recently, the potential of multiple reaction monitoring-MS to detect and quantify native *O*-GlcNAc peptides were reported [86]. This study showed that multiple reaction monitoring is robust to quantify *O*-GlcNAc peptides with a detection limit of 3 fmol. In principle, targeted MS using both *O*-GlcNAc-modified and unmodified synthetic peptides as internal standards should allow accurate targeted quantification of *O*-GlcNAc and its site occupancy for interested sites.

Future perspective

Although significant advances have been made to improve the proteomic analysis of *O*-GlcNAcylation, technical challenges remain for in-depth, quantitative study of *O*-GlcNAcylation and its regulation in biology and pathologies.

Further advances in the enrichment approaches for *O*-GlcNAc proteins and peptides will be essential for effective *O*-GlcNAc analysis. The current two main enrichment strategies, the chemoenzymatic and the affinity enrichment, both have their own advantages and disadvantages. The chemoenzymatic methods, exemplified by chemical/enzymatic photochemical cleavage, offer potential for achieving enrichment with high specificity;

however, the current protocols are still quite labor-intensive. The overall sample recovery is a point of concern owing to the multiple steps of the reaction, including azide labeling, click chemistry and biotin–avidin chromatography. There are also potential side reactions such as Cu⁺-mediated oxidation of proteins [53] and copper-mediated non-specific binding of proteins to alkyne–biotin reagent or alkyne resin [52] during click chemistry. Despite these limitations, we anticipate the chemoenzymatic approaches will continue to be improved and optimized to enable more efficient global quantitative profiling of *O*-GlcNAc modifications. For example, those unwanted side reactions might be largely averted through the adoption of copper-free click chemistry [50] or using a reversed-click chemistry configuration, that is, labeling *O*-GlcNAc with alkyne first, and using azide–biotin reagent in click chemistry [37,87].

Affinity-based enrichment is conceptually much simpler and hence has the potential to be more robust and reproducible. Since no complex chemical reactions were involved, there is no concern for side reactions. However, the currently available affinity approach often does not offer high specificity and the ability to work with small-sized samples has yet to be demonstrated. With the advances of novel affinity reagents, such as anti-*O*-GlcNAc aptamer [88], the global profiling using affinity enrichment may become much more effective.

Besides enrichment, the next challenge of *O*-GlcNAc analysis is the site determination, since the modifications are often found in regions rich in serine or threonine residues, which suggests multiple possible sites for modification. The advances in MS instrumentation that allow multiple fragmentation modes (e.g., HCD and ETD) and accurate mass measurements should greatly facilitate site determination.

Overall, we anticipate that there will be significant advances in the overall workflow of *O*-GlcNAc analysis (including enrichment, site determination and quantification) to enable robust, site-specific and quantitative analysis of *O*-GlcNAc modifications in cells and tissues within the next few years. With these advances, the significance of *O*-GlcNAc in biology and pathologies and the potential crosstalk with other PTMs, such as phosphorylation, will be increasingly recognized.

Acknowledgments

The experimental work described herein was performed in the Environmental Molecular Sciences Laboratory, a national scientific user facility sponsored by the US Department of Energy (DOE) Office of Science Biological and Environmental Research and located at Pacific Northwest National Laboratory, which is operated by Battelle Memorial Institute for the DOE under Contract DE-AC05-76RL0 1830.

Key terms

<i>O</i>-GlcNAcylation	A distinctive form of <i>O</i> -linked glycosylation occurring on serine or threonine residues by adding a monosaccharide, N-acetylglucosamine (GlcNAc), via the side chain hydroxyl group
Lectin	Proteins with high affinity to specific carbohydrates

Enrichment of <i>O</i>-GlcNAc	The process of enriching or isolating <i>O</i> -GlcNAc-modified proteins or peptides from complex biological samples by affinity or chemical approaches
Click chemistry	Cu-catalyzed azide–alkyne cycloaddition; it is a robust, efficient and specific ligation method that is being widely applied in chemistry and biology researches
Electron transfer dissociation (ETD)	A tandem MS technique, in which an electron is transferred to the cation of peptide or protein from anion, then the peptide or protein ion dissociates to <i>c</i> and <i>z</i> ions, while preserving most of the labile post-translational modifications, such as <i>O</i> -GlcNAcylation and phosphorylation
2D-LC	A separation approach where samples sequentially pass through two LC (usually orthogonal to each other) separation stages to maximize their separation

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Executive summary

Background

- *O*-GlcNAc is an important protein post-translational modification that is technically challenging to characterize by LC–MS/MS because of its low abundance and labile nature.

Enrichment of *O*-GlcNAc

- Enrichment of *O*-GlcNAc modified proteins or peptides is a crucial step for successful analysis by LC–MS/MS. The two main current strategies are chemoenzymatic methods and lectin-affinity enrichment.

Identification & site determination of *O*-GlcNAc by MS

- For identification of *O*-GlcNAc peptides, the *O*-GlcNAc moiety provides a signature reporter ion in collision-induced dissociation. β -elimination and Michael addition or electron transfer dissociation methods are necessary to determine the site of *O*-GlcNAc modification.

Quantification of *O*-GlcNAc

- Quantification of *O*-GlcNAc, while essential, is still not well demonstrated. Both changes in relative abundance and stoichiometry are important aspects of *O*-GlcNAcylation.

Future perspective

- The significance *O*-GlcNAc should be increasingly recognized with the continued advances of analytical approaches for *O*-GlcNAc profiling.

Figure 1.
Dynamic *O*-GlcNAcylation of Ser/Thr residues in proteins.

Figure 2.
The chemoenzymatic strategy for enrichment of *O*-GlcNAcylated peptides.

Figure 3. The BEMAD reactions for characterizing *O*-GlcNAc peptides by MS/MS
BAP: Biotin pentylamine; DTT: Dithiothreitol.