# Specific Enrichment and Identification of Azide Modified Glycoproteins using the Staudinger Ligation

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## Abstract

Among more than 100 types of post-translational modifications, glycosylation is the most common with several diseases being associated with the aberrant forms. It is for these reasons that the development of methods for the study of glycosylation is becoming increasingly important. Sialic acid expression on cell surface glycoproteins and the modification of intracellular proteins by *O*-linked β-N-acetylglucosamine (*O*-GlcNAc) are significant disease state post-translational modifications. Cell surface sialic acid is known to mediate a variety of cellular interactions during cell development, differentiation and tumor progression. It has been shown that enhanced sialyltransferase (ST) activity, which results in hypersialylation, is implicated in up-regulation of metastatic potential. The *O*-GlcNAc modification affects cell transcription, translation, and signaling. Several studies have shown the possible role *O*-GlcNAc plays in neurodegenerative diseases and diabetes. In this study the enrichment capabilities of the Staudinger ligation were used to examine the incorporation and presentation of sialic acid and *O*-GlcNAc. The Staudinger ligation is a reaction between an azide functionality and a phosphine derivative to yield an amide bond. Incorporating azide-modified mannose, a sialic acid precursor, and azide-modified *N*-acetylglucosamine, cell culture can produce modified glycoproteins. FLAG-phosphine, used for its immunochemical versatility, acts as a probe for the capture of the azide-modified glycoproteins. By exploiting these techniques, sialic acid and *O*-GlcNAc modified glycoproteins were identified by combining immunoprecipitation and LC-MS/MS. To our knowledge, this is the first reported instance of the utilization of a modified Staudinger ligation to enrich for modified glycoconjugates, making available a powerful new tool for glycoprotein analysis.

## Overview

The relationship between cellular glycosylation and human disease has been studied for many years **(1)**. In spite of this, the molecular elucidation of glycan function and structure has been slow due to the relative paucity of appropriate analytical techniques. Research efforts, however, have shown that sialic acid expression on cell surface glycoproteins and the modification of intracellular proteins by *O*-linked β-N-acetylglucosamine (*O*-GlcNAc) are significant disease state post-translational modifications. Sialic acid expression in cancer cells can affect invasiveness, metastasic activities essential for growth, and development and survival of an organism **(2)**. The most frequently described change in glycosylation pattern associated with cancer is the presence of highly branched, heavily sialylated structures **(3)**. In addition, recent research has demonstrated the critical role *O*-GlcNAc modifications may play in diabetes and Alzheimer's disease **(4)**. Thus a tool to monitor the differential expression of sialic acid on cell surfaces and one that can also monitor internal *O*-GlcNacylation will be of immense value to researchers investigating the relationship between glycan expression and disease states.

"Metabolic oligosaccharide engineering", a method used to modulate cell-surface carbohydrates by the biosynthetic incorporation of unnatural monosaccharides, has emerged as a useful tool to monitor cell surface glycans. This technology employs the concept of bioorthogonality by using a set of functional azide groups and a complementary set of phosphine compounds. In summary, an azide modified sugar may enter a cell and progress through to ultimate protein glycosylation which then might be probed by a funtionalized phosphine (via bonding by Staudinger ligation) **(5)**. Specifically, unnatural N-α-azidoacetylmannosamine ($Ac_4$ManNAz) can be metabolized to N-α-azido sialic acid (NeuNAz) and then incorporated into cell-surface glycoproteins. N-a-azidoacetylglucosamine ($Ac_4$GlcNAz) **(Figure 1)** may be incorporated into internally presented glycoproteins. In this trial, the modified glycoprotein is probed with a FLAG-phosphine derivative, thereby allowing full implementation of the FLAG-Antibody system of tools. By exploiting these techniques, sialic acid and *O*-GlcNAc modified glycoproteins were identified by combining immunoprecipitation and LC-MS/MS. To our knowledge, this is the first reported instance of the utilization of a modified Staudinger ligation to enrich for modified glycoconjugates, making available a powerful new tool for glycoprotein analysis.

## Azidosugars incorporated into glycoproteins

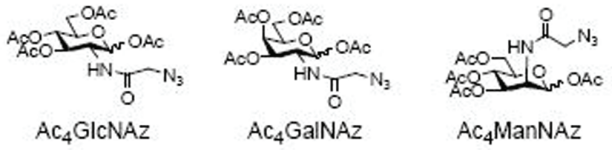


**Figure 1**

## Materials

All materials were obtained from or prepared at Sigma-Aldrich, unless noted.

$Ac_4$ManNAz and $Ac_4$GlcNAz **(Cat. No. A7605 and A7355)**

FLAG-Phosphine **(Cat. No. GPHOS1)**

Cell Dissociation Solution **(Cat. No. C5914)**

RIPA Buffer **(Cat. No. R0278)**

Monoclonal ANTI-FLAG® BioM2 antibody produced in mouse **(Cat. No. F9291)**

Streptavidin-Agarose **(Cat. No. S1638)**

Tris-Buffered Saline with 3% nonfat milk **(Cat. No. T8793)**

Tris Buffered Saline, with Tween® 20, pH 8.0 **(Cat. No. T9039)**

Monoclonal ANTI-FLAG® M2-Peroxidase (HRP) antibody produced in mouse **(Cat. No. A8592)**

Chemiluminescent Peroxidase Substrate-3 **(Cat. No. CPS3)**

## Applications of metabolic oligosaccharide engineering to detect azide labeled carbohydrates

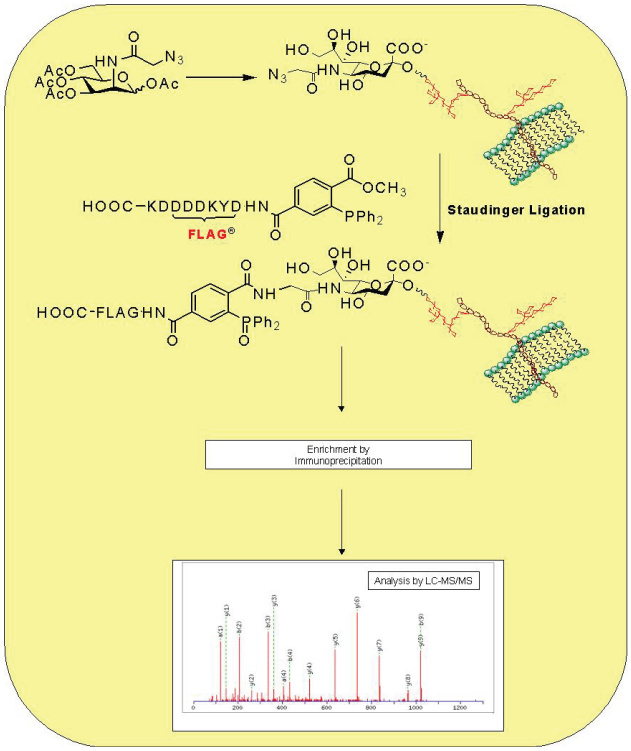


**Figure 2**

## Methods

**Incorporation of unnatural sugars:**
The $Ac_4$ManNAz and $Ac_4$GlcNAz (600 µL) were dissolved in ethanol and added to T75 flasks. A control flask (ethanol only) was also prepared. After allowing the ethanol to dry, each flask was seeded with $2.0\times10^6$ HeLa cells. The $Ac_4$ManNAz flasks were incubated for 3 days and the $Ac_4$GlcNAz for 24 hours, both at 37 °C in a $CO_2$ incubator. The cells were harvested using cell dissociation solution. The control sample was incubated with 0.2 mL of 250 µM FLAG-Phosphine (dissolved in Phosphate Buffered Saline(PBS)) and the pooled $Ac_4$ManNAz sample was incubated with 1.8 mL of 250 mM FLAG-Phosphine solution overnight at 2–8 °C with constant rotation. After incorporation, the $Ac_4$ManNAz cells were washed using PBS and lysed with RIPA buffer. The $Ac_4$GlcNAz cells were lysed In PBS with protease inhibitors by sonication on ice. After lysis, the cells were also incubated with FLAG-Phosphine solution overnight at 2–8 °C.

**Immunoprecipitation of FLAG-labeled glycoproteins:**
Cell lysate containing FLAG-labeled cell surface glycoprotein was denatured, reduced and alkylated and incubated with ANTI-FLAG BioM2 conjugate (300 µg) overnight. This in turn was incubated with Straptavidin–Agarose for purification of the glycoprotein-biotin conjugate. After several washes with PBS and water, the protein was eluted using 0.1 M Acetic Acid, pH 2.0. A dot blot analysis **(Figure 2B)** was performed to assess the immunoprecipitation efficiency. The purified protein was tryptically digested and the NeuNAz containing glycoproteins were deglycosylated using PNGase F prior to MS analysis. The dot blot was performed by spotting the samples onto nitrocellulose (1 µL/square). Both the dot blot and western blot were probed with Monoclonal ANTI-FLAG M2-Peroxidase and visualized using CPS3.

**MS analysis of glycoproteins enriched by immunoprecipitation:**
The LC/MS/MS system for the $Ac_4$ManNAz consisted of an Agilent 1100 series LC system coupled to a Thermo LTQ mass spectrometer equipped with an electrospray (ESI) interface in the positive ion mode. The electrospray needle was operated with a voltage differential of 4.5 kV. The chromatographic conditions were as follows: Column: 2.1 mm × 150 mm (Supelco Discovery HS $C_{18}$, 5 mm), flow: 100 µL/min, Mobile Phase: (A) 0.1% formic in water, and (B) 0.1% formic in Acetonitrile. The RP column was eluted into the mass spectrometer with a linear gradient of 0 to 15% B for 5 min followed by 15 to 60% B for 50 min at 100 µL/min.

The $Ac_4$GlcNAz sample was loaded into a LCQ ion trap mass spectrometer coupled with a Magic 2002 (Michrom BioResources) LC system and an electrospray interface (Proxeon). Reverse phase HPLC was performed with a capillary column (75 µm ID) packed with Vydac C18. Solvent A contained 5% ACN, 0.1% acetic acid, and 0.005% HFBA. Solvent B contained 95% ACN, 0.1% acetic acid, and 0.005% HFBA. Flow rate was maintained at 200 nL/min. Total gradient was 35 min. Capillary voltage was set at 28.5 V. LCQ was set in a information-dependent acquisition mode with 3 MS/MS followed by one full survey scan.

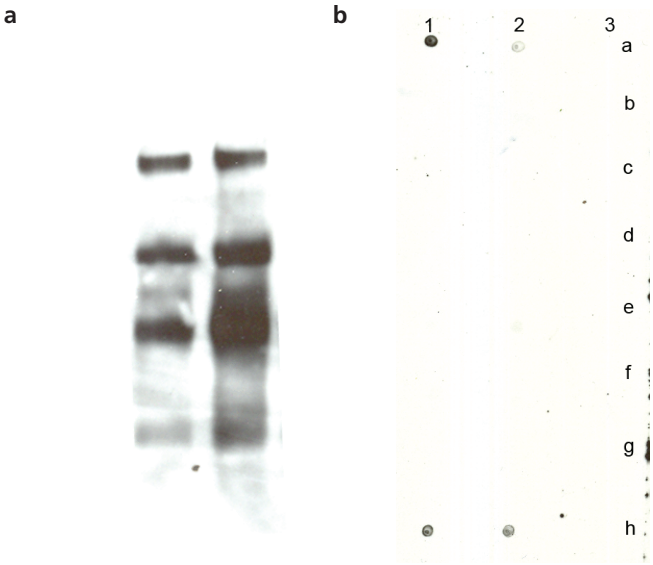


**Figure 3:** a) Western blot of ManNAz containing proteins. b) Dot blot of Immunoprecipitation of ManNAz containing protein (1 and 2) and control (3). The blot shows the load (a), after Ab incubation (b), unbound fraction (c), washes (d-g) and elution(h).

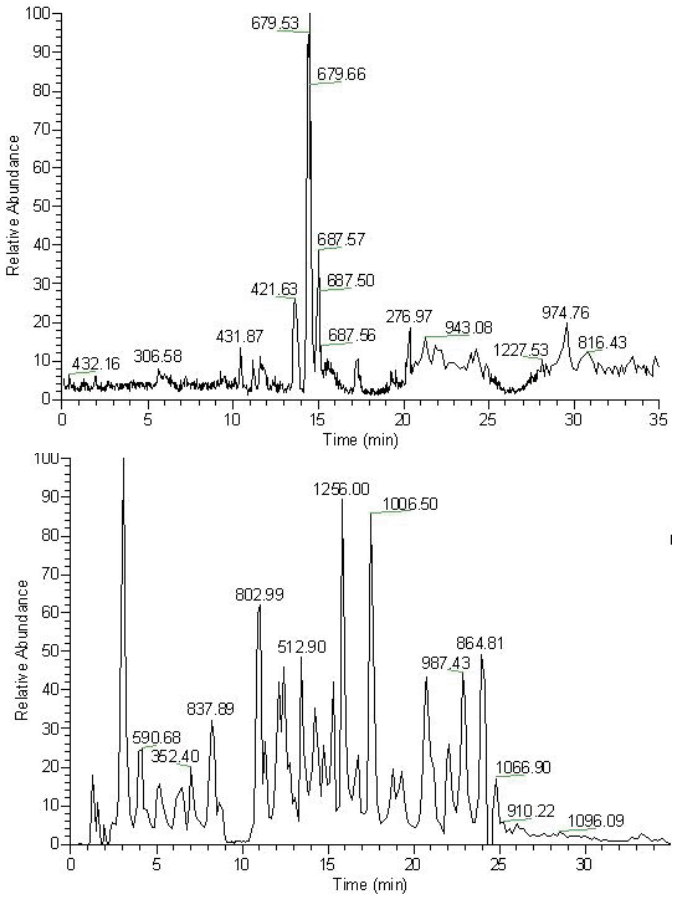


**Figure 4:** TIC profile of the peptides generated from samples obtained from the immunoprecipitation of GlcNAz-labeled cell surface glycoproteins top) control, bottom) sample

## List of proteins identified from $Ac_4$ManNAz modified cells

| | |
|---|---|
| Protein from Ribosomal L29e family (1) | similar to 60S ribosomal protein L29 |
| Protein from Ribosomal L29e family (2) | Ion transport protein |
| 60S ribosomal protein L13 | Protein from serpin family |

**Table 1:** All proteins are known glycoproteins.

## List of proteins identified from $Ac_4$GlcNAz modified cells

| | |
|---|---|
| Glyceraldehyde-3-phosphate dehydrogenase** | Cleavage and polyadenylation specificity factor subunit 1 |
| Heat shock cognate 71 kDa protein | Heat shock 70 kDa protein 6 |
| 78 kDa glucose-regulated protein | Rho-associated protein kinase 2 |
| Ryanodine receptor 3 | Alpha-enolase |
| Aspartate aminotransferase, mitochondrial | Alpha-actin-2** |
| Elongation factor 1-alpha 1** | Probable G-protein coupled receptor 133 |
| Fructosamine-3-kinase | Protein FAM9B |
| Transcription initiation factor TFIID 210 kDa subunit | Contactin-associated protein-like 3 |

**Table 2:** **Known *O*-GlcNAcylated protein

## MS/MS Fragmentation of K.IGGIGTVPVGR.V

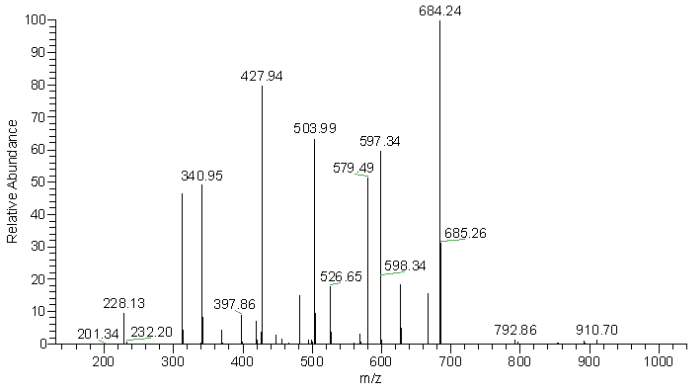


**Figure 5:** Elongation factor 1-alpha 1

## Results and Discussion

The results of this study clearly show the applicability of the azide incorporation and optimized immunoprecipitation. After incubation with $Ac_4$ManNAz, the NeuAz containing proteins were easily visualized using a western blot **(Figure 3A)**. The immunoprecipitation was extremely successful. This optimized procedure takes into account potential steric hindrances by first denaturing the protein and then using a combination of soluble ANTI-FLAG BioM2 conjugate and streptavidin coated agarose beads to pull down the labeled protein. This ANTI-FLAG BioM2/ streptavidin system allows for preferential (low pH) elution of the FLAG labeled glycoproteins, leaving the M2-Biotin attached to the resin. The progress of this optimized immunoprecipitation can be quickly and easily visualized by the use of a dot blot **(Figure 3B)**. Protein was visualized only in the load and the elution, demonstrating the effectiveness of the FLAG system immunoprecipitation.

This successful enrichment then allows for the proteins to be readily analyzed and identified using mass spectrometry. Using this technique several proteins were identified in the both the $Ac_4$ManNAz **(Table 1)** and $Ac_4$GlcNAz **(Table 2)** samples. The $Ac_4$GlcNAz identification was very successful with only one protein found in the negative control **(Figure 4)**. Several of the identified proteins are known to be *O*-GlcNAcylated, while others are apparently unique in identification.

## Conclusions

- Through the manipulation of this technology, a specific immunoprecipitation technique was developed.
- This specific enrichment strategy allows for azide containing proteins to be analyzed and identified with LC-MS/MS.
- This technology allowed for the identification of previously unknown *O*-GlcNAc proteins.

## Acknowledgements

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