WHITE PAPER

A Novel Method for Detecting Terminal Glycans Using SDS-PAGE

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ABSTRACT

We have developed a novel technique capable of probing terminal monosaccharides on proteins using SDS-PAGE. This technique does not require expensive equipment and is more specific than comparable methods. Additionally, it offers any laboratory the ability to gain a greater understanding of the glycan profiles of their protein of interest without significant investments.

- **SENSITIVE.** Labeling is through covalent bonding via click chemistry
- **INEXPENSIVE.** Does not require mass spectrometry or HPLC
- **DIRECT.** No requirement for releasing glycans from the protein
- **SIMPLE.** Only common laboratory equipment used for Western blot is needed
- **FAST.** Results can be obtained in a day

INTRODUCTION

Glycosylation has significant effects on protein structure, function, and protein-protein interactions. At the cellular level it can modulate adhesion and migration, proliferation, and differentiation. Glycans can also act as receptors for pathogens, and they can regulate the development and progression of cancer. Despite these important roles, glycosylation remains difficult to study. The most widely used techniques, such as HPLC and mass spectrometry, yield detailed information about glycan composition and structure. However, these methods often require glycan removal and labeling, and can be expensive and time-consuming. Other techniques, such as the use of lectins or antibodies, suffer from low affinity and/or lack of specificity.

We have developed a simple, yet sensitive, method that allows for the rapid detection of monosaccharides on either N-linked or O-linked glycans. This technique does not require removal of the glycan from the protein of interest, nor does it require use of specialized equipment. It combines the specificity of glycoenzymes with traditional click chemistry-based labeling, allowing for a remarkably specific assessment of terminal glycans.

In this method, azido-nucleotide-sugars are used as glycosyl donors in glycosyltransferase-catalyzed reactions (Table 1). Once the azido-sugar is incorporated into open acceptor sites, a biotinylated alkyne is added and conjugated to the target glycans in a classical click chemistry (Figure 1A) reaction. The labeled glycoprotein can then be assessed using SDS-PAGE and chemiluminescence-based detection in a manner similar to a Western blot (Figure 1B). By combining the use of glycosidases and the specificity of glycosyltransferases, researchers can quickly and inexpensively acquire information about the presence or absence of specific terminal glycans.

Table 1. Activated Nucleotide-Azido-Sugars

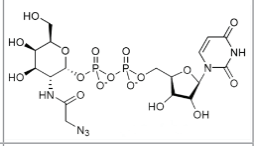
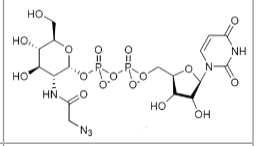
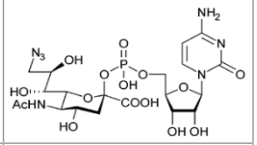
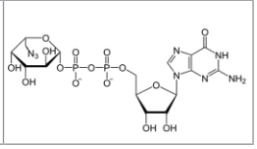
Enzymes Used	Activated Nucleotide-Azido-Sugar	Structure
GalNAc Transferases	UDP-Azido-GalNAc (Catalog # ES103)	
GlcNAc Transferases	UDP-Azido-GlcNAc (Catalog # ES104)	
Sialyltransferases	CMP-Azido-Sialic Acid (Catalog # ES102)	
Fucosyltransferases	GDP-Azido-Fucose (Catalog # ES101)	

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Figure 1

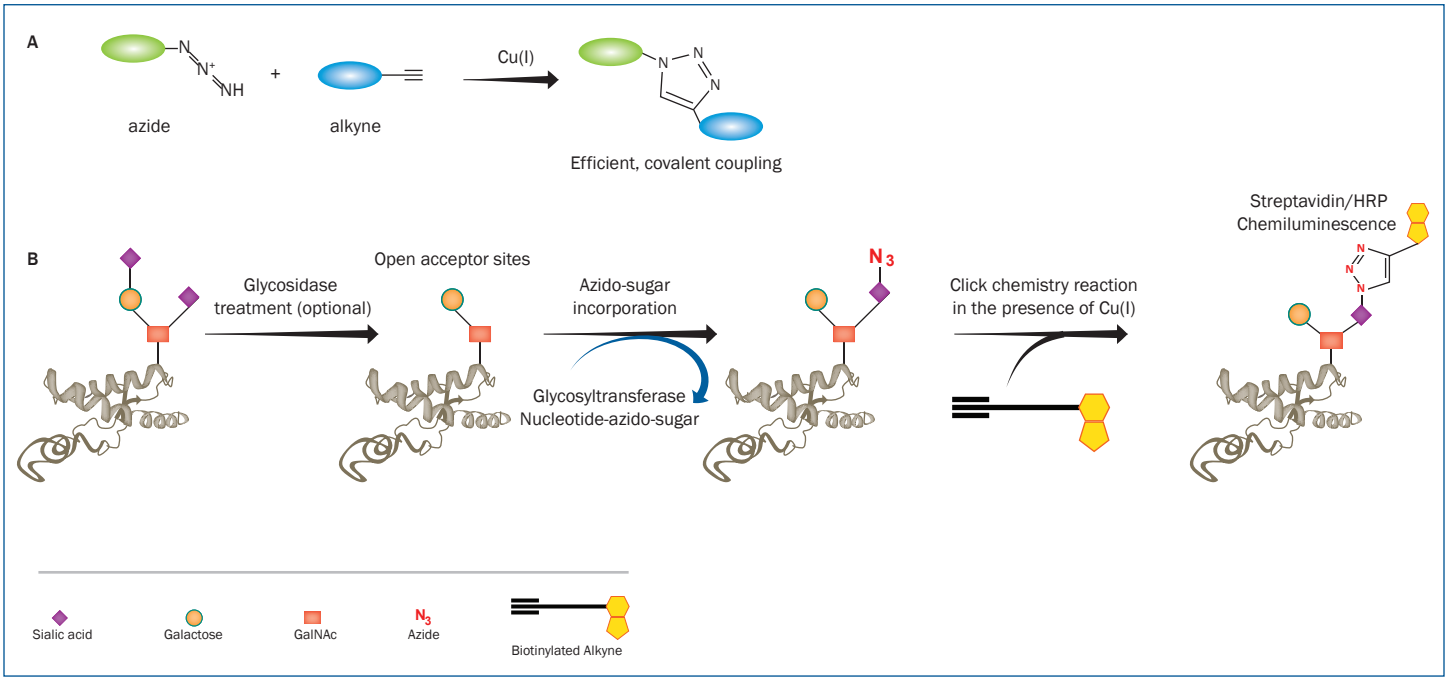

Figure 1. General Principle for Probing Glycans in a Glycoprotein. A. In classical click chemistry, copper catalyzes the reaction between an azide and an alkyne, forming a triazole moiety. This covalent reaction can be used for conjugation or labeling studies. **B.** We have developed Nucleotide-Azido-Sugars that act as substrates for glycosyltransferase reactions. The Azido-Sugar can be directly incorporated into a glycan structure or incorporated after first removing specific glycans with a glycosidase. The specificity of glycosyltransferases adds the Azido-Sugar to suitable acceptor sites and a biotinylated alkyne is used as a probe. Similar to a Western blot, the technique utilizes SDS-PAGE and chemiluminescence as a readout.

METHODS

Materials

Recombinant Human ST3GAL1, Recombinant Human ST6GAL1, Recombinant Human MGAT1, Recombinant *C. perfringens* Neuraminidase, Monoclonal Mouse anti-Human IL-1β Antibody, UDP-Azido-GlcNAc, CMP-Azido-Sialic Acid, Streptavidin-Conjugated HRP (Strep-HRP), and Biotinylated Alkyne were from R&D Systems. Bovine Fetuin and RNase B were from Sigma Aldrich. Desialylated Fetuin (D-Fetuin) was prepared by treating Fetuin with Recombinant *C. perfringens* Neuraminidase.

Glycosyltransferase Reactions

An enzyme reaction buffer was made containing Tris (25 mM), $MnCl_2$ (10 mM), and NaCl (150 mM) at pH 7.5. To probe for sialic acid on Fetuin or D-Fetuin (10 µg), CMP-Azido-Sialic Acid (1 nmol) and variable amounts of ST3GAL1 were mixed in 50 µL of the reaction buffer. To probe for sialic acid on the IgG heavy chain of anti-human IL-1β (10 µg), CMP-Azido-Sialic Acid (1 nmol) and of ST6GAL1 (2 µg) were mixed in 50 µL of the reaction buffer. For the GlcNAc transferase reaction, the glycoproteins (10 µg) were mixed with UDP-Azido-GlcNAc (1 nmol) and Recombinant Human MGAT1 (2 µg) in 50 µL of the reaction buffer. In all cases, the reactions were incubated at 37 °C for a minimum of 20 minutes.

Labeling and Detection

Ascorbic acid (2 mM), $CuCl_2$ (0.1 mM) and Biotinylated Alkyne (0.1 mM) were directly added to the glycosyltransferase reaction. The reaction was incubated at room temperature for a minimum of 30 minutes. The samples were separated on a 12% SDS-PAGE gel and the proteins visualized with UV in the presence of trichlorethanol (TCE staining). The proteins were transferred to nitrocellulose, followed by blocking with 10% fat-free milk for 10 minutes, and washing with a TBST buffer containing Tris (25 mM), pH 7.6, NaCl (137 mM), and 0.01% Tween. The blots were probed with strep-HRP and visualized with enhanced chemiluminescence (ECL) peroxidase substrate in a manner similar to a Western blot.

RESULTS

Specific Labeling of the IgG Heavy Chain

IgG antibodies contain an N-linked glycosylation site on Asn-297 of the Fc region of the IgG heavy chain (Figure 2A). To test the specificity of the technique we assessed sialic acid incorporation into the glycan present in an anti-IL-1β monoclonal antibody. We incubated the sample with CMP-Azido-Sialic and ST6GAL1, a sialyltransferase that catalyzes 2,6-sialylation of Gal β1,4-GlcNAc structures on N-glycans. This was followed by incubation with Biotinylated Alkyne to label the incorporated glycan via click chemistry. The sample was separated by SDS-PAGE and the heavy and light chains were evident in protein staining of the gel (Figure 2B). The separated samples were then transferred to nitrocellulose and probed using a Strep-HRP and chemiluminescence. Highlighting the specificity, the labeling is only observed in the heavy chain containing the N-linked glycosylation site.

Detection of Sialic Acid on Fetuin

Sialoglycans can be probed by either direct incorporation of the azido-sugar using sialyltransferases or using a replacement approach by first using a neuraminidase to remove existing sialic acid. Fetuin and D-Fetuin samples were first incubated with CMP-Azido-Sialic Acid and ST3GAL1, a sialyltransferase that catalyzes the transfer of sialic acid to the galactose residues of core 1 O-glycans (Galβ1-3GalNAc-R). Staining for protein content indicates similar protein levels in all samples (Figure 3A). No azido-sialic acid incorporation is observed in Fetuin, while obvious incorporation of the azido sugar is observed in the desialylated sample (Figure 3B). The combination of the specific enzymes used suggests that the original Fetuin sample was fully sialylated on the core 1 galactose of O-linked sugars.

Detection of High Mannose

MGAT1 is a glycosyltransferase specific for transferring GlcNAc to high mannose and hybrid N-glycans, glycoforms common to viral proteins (Figure 4A). The enzyme was used to probe for the presence of mannose on the 1918 influenza viral Neuraminidase, bovine RNase B, Fetuin, and D-Fetuin. The protein content was measured using TCE staining. The samples were then separated using SDS-PAGE and the incorporated azido-GlcNAc was labeled using click chemistry and detected using Strep-HRP and chemiluminescence. The results indicate incorporation of GlcNAc into open sites on high mannose or hybrid structures of viral Neuraminidase and bovine RNase B. In contrast, no incorporation is observed in Fetuin or D-Fetuin, indicating a lack of high mannose on these proteins (Figure 4B).

Figure 2

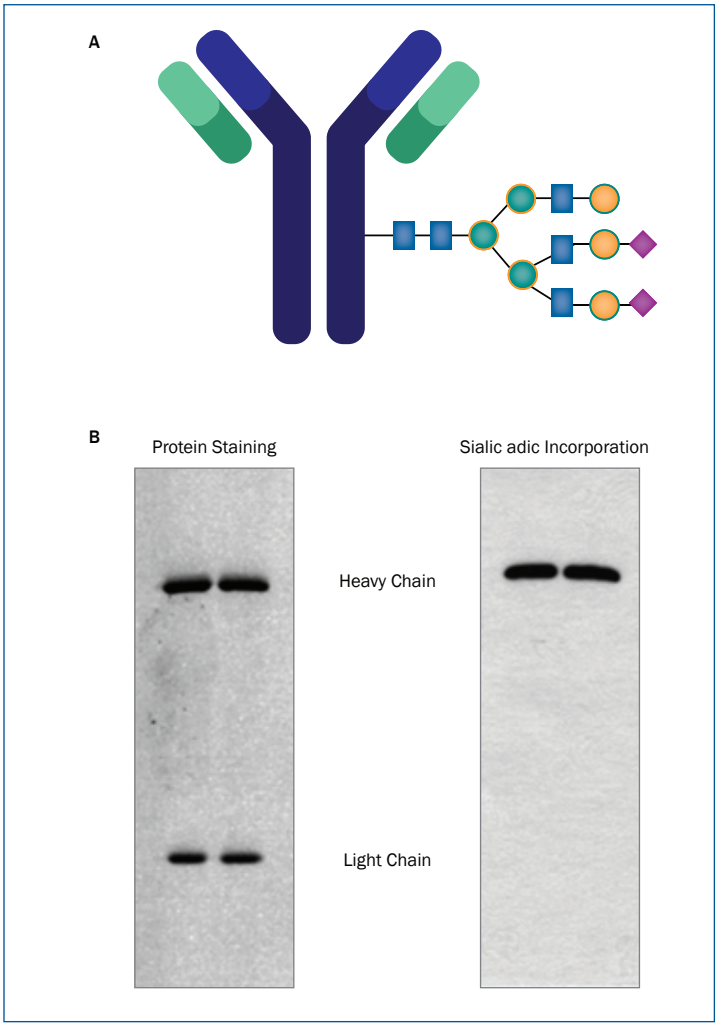

Figure 2. Sialic Acid Incorporation into the IgG Heavy Chain. The Fc region of the IgG heavy chain contains an N-linked glycosylation site at Asn-297. **B.** Sialic acid was transferred to galactose on an anti-Human IL-1β monoclonal antibody using CMP-Azido-Sialic Acid and the sialyltransferase Recombinant Human ST6GAL1. The incorporated Azido-Sialic Acid was conjugated to Biotinylated Alkyne via click chemistry and separated by SDS-PAGE. The heavy and light chains are easily resolvable by protein staining (left panel). Transfer to nitrocellulose and chemiluminescence detection reveals sialic acid incorporation only in the heavy chain containing the N-linked glycosylation site.

Figure 3

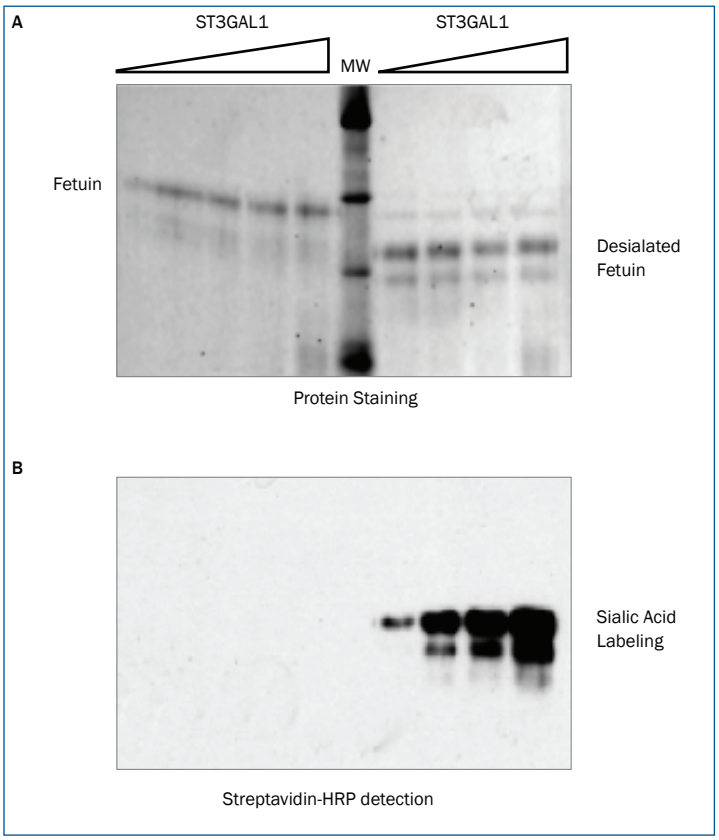

Figure 3. Labeling and Detection of Sialic Acid on O-glycans of Bovine Fetuin. Bovine Fetuin (left side of the molecular marker; MW) and D-Fetuin (right side of the molecular marker) were incubated with increasing amount of Recombinant Human ST3GAL1 (5, 25, 50, 250 ng) and CMP-Azido-Sialic Acid. The reactions were then conjugated with Biotinylated Alkyne and separated by SDS-PAGE. The separated proteins were then transferred to a nitrocellulose membrane and detected with conventional Streptavidin-HRP chemiluminescence reagents. **A**. Total protein staining. **B**. Streptavidin-HRP detection of Azido-Sialic Acid incorporation. No incorporation of Azido-Sialic Acid is detected in Fetuin, while strong labeling is observed on D-Fetuin.

Figure 4

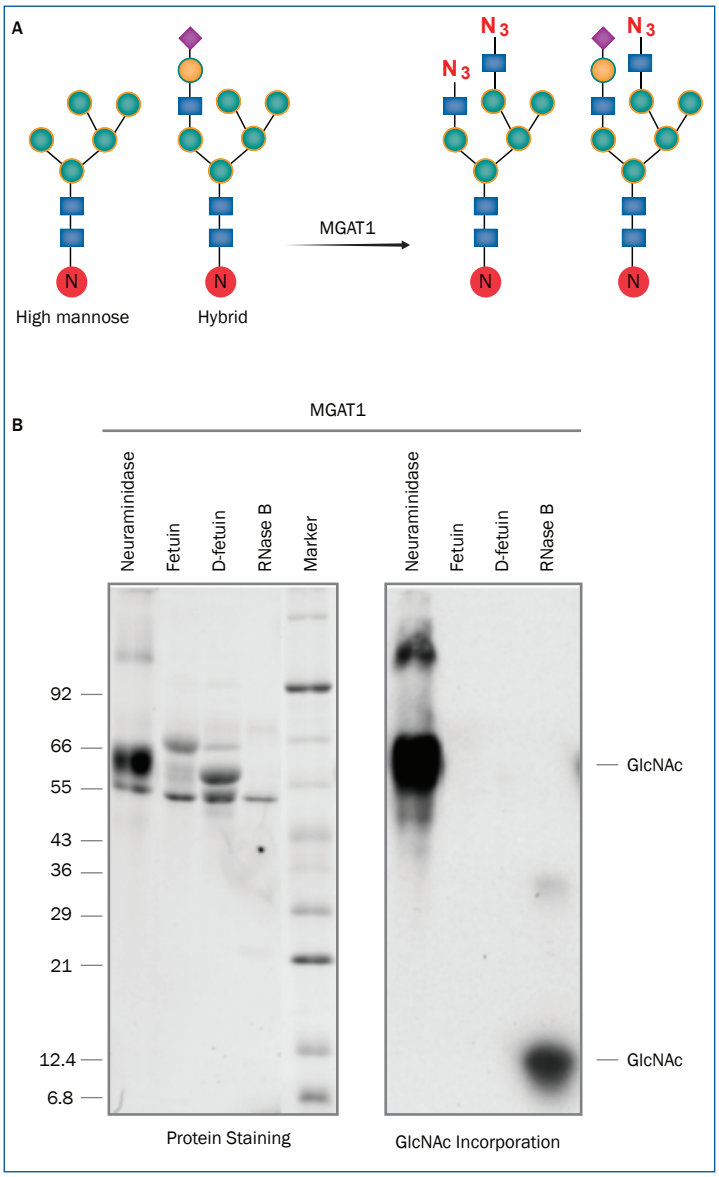

Figure 4. Probing for the Presence of High Mannose N-glycans. A. MGAT1 transfers GlcNAc to high mannose N-glycans. **B**. H1N1 influenza viral Neuraminidase, bovine Fetuin, D-Fetuin and RNAse B were treated with MGAT1 and UDP-Azido-GlcNAc. The reactions were then conjugated to Biotinylated Alkyne, separated by SDS-PAGE, and the protein content visualized (left panel). The samples were transferred to nitrocellulose and detected using Streptavidin-HRP chemiluminescence. High mannose N-glycans are only detected on the viral Neuraminidase and bovine RNAse B. Note: RNase B is not visible in the protein staining as it lacks a tryptophan residue required for detection. Adapted from Wu, Z.L. *et al.* (2016) Glycobiology **26**:334.

DISCUSSION

The glycan complement present on proteins can have significant effects on protein structure, activity, and protein-protein interactions. For example, Lewis-X structures play important roles in cell migration, polysialic acid can affect neuronal cell growth and maturation, and high mannose glycans on viral proteins can contribute to immune evasion. In addition, the glycan profile on antibodies can have significant effects on affinity and half-life, directly affecting the pharmacokinetics of the antibody therapeutics. For this reason regulating glycosylation patterns is a crucial part of biotherapeutic development and manufacturing.

We describe a novel technique for probing the glycan component of glycoproteins. To highlight this new technique, we have shown that glycosyltransferase-mediated labeling using azido-nucleotide-sugars can be utilized to probe for sialoglycans and high mannose structures. Although not shown here, we have also used it successfully to probe for fucose (including core fucose) and GalNAc on a number of substrates. Because labeling is achieved through enzymes that are known to be selective for substrate recognition, this method is extremely specific. Using this technique, any laboratory with equipment to run Western blots now has the ability to detect glycosylation patterns on proteins and gain a better understanding of the role glycans play in their biological system.