Communication

Two-step Chemoenzymatic Detecting N-Acetylneuraminic acid-#(2-3)-galactose Glycans

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Two-step Chemoenzymatic Detecting *N*-Acetylneuraminic acid-α(2-3)-galactose Glycans

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Supporting Information

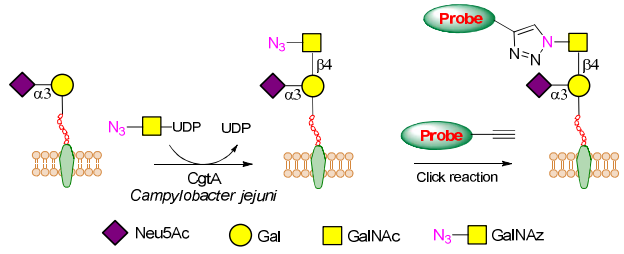
ABSTRACT: Sialic acids are typically linked α2-3 or α2-6 to the galactose that located at the non-reducing terminal end of glycans, playing important but distinct roles in a variety of biological and pathological processes. However, details about their respective roles are still largely unknown due to the lack of an effective analytical technique. Herein, a two-step chemoenzymatic approach for the rapid and sensitive detection of *N*-acetylneuraminic acid-α(2-3)-Galactose (Neu5Acα(2-3)Gal) glycans was described.

N-acetylneuraminic acid (Neu5Ac) is the most widespread form of sialic acid and almost the only form found in humans.[1] *N*-glycolylneuraminic acid (Neu5Gc) and ketodeoxynonulosonic acid (Kdn) are common in other vertebrates but rarely present in humans.[1] Neu5Ac is present essentially in all human tissues and always attaches to the galactose residue at the nonreducing terminal end of glycans through α2-3 or α2-6 linkage.[2] It is well established that Neu5Acα(2-3)Gal and Neu5Acα(2-6)Gal glycans play crucial but distinctive roles in diverse biological and pathological processes including immune responses, cell–cell and cell–pathogen interactions.[3] However, studies are hindered due to the lack of an effective method to analyze such glycans or glycoproteins.

Lectin binding has been the primary method to analyze sialylated glycans,[4] but lectins often suffer from weak binding affinity, limited specificity, and cross-reactivity. In recent years, the development of bioorthogonal chemistry provides a powerful tool for probing glycans, proteins and lipids.[5] Bioorthogonal functional groups (azide and alkyne) carried by *N*-acetylmannosamine or Neu5Ac analogues were metabolically incorporated into glycans, allowing the covalent conjugation by click chemistry reaction with either fluorescent tags for visualization, or affinity probes for enrichment of sialylated glycans and glycoproteins.[6] Chemical approach to tag sialylated glycans has also been suggested.[7] Nevertheless, these methods suffer from low detection sensitivity and efficiency, toxicity of labeling regents, and the inability to detect complicated glycan structures.

As a complementary strategy to remodel glycans with non-natural functionalities, chemoenzymatic labeling glycans relies on the substrate-specific glycosyltransferases, which transfer non-natural sugars that contain bioorthogonal functional groups onto target glycan *in vitro*.[8] Chemoenzymatic method does not rely on cell's biosynthetic machinery and therefore can be employed in any desired biological contexts where feeding cells with non-natural sugar analogs is not possible, such as human tissue extracts.[8b] As glycosyltransferase-mediated reaction and click chemistry reaction proceed high specificity and efficiency, chemoenzymatic labeling provides a higher sensitivity and selectivity compared to other analytical methods such as antibodies, lectins, and metabolic labeling. In this communication, we report a two-step chemoenzymatic method that takes advantage of the substrate promiscuity of a β-(1,4)-*N*-acetylgalactosaminyltransferase from *Campylobacter jejuni* (CgtA) and click chemistry reaction to rapidly and sensitively detect Neu5Acα(2-3)Gal glycans (Scheme 1).

Scheme 1. Two-step Chemoenzymatic detecting Neu5Acα(2-3)Gal glycans by CgtA with UDP-GalNAz.



The Neu5Acα(2-3)Gal epitope localized on cell surface is well known to be the receptor of many infectious microbes such as the influenza virus.[2] Abnormal Neu5Acα(2-3)Gal expression has frequently been observed in many carcinomas.[3b] Traditionally, lectin binding using *Maackia amurensis* leukoagglutinin (MAL I) and hemagglutinin (MAH or MAL II) is the main method for Neu5Acα(2-3)Gal detection. However, MAL I only bind terminal Neu5Acα(2-3)Galβ(1-4)GlcNAc trisaccharide in *N*-glycans.[9] MAH binds preferentially trisaccharide Neu5Acα(2-3)Galβ(1-3)GalNAc in *O*-glycans.[10] They also bind some nonsialylated structures such as SO_4^{-3}-Galβ(1-3)GalNAc.[11] Moreover, it was reported that *Maackia amuren-*

sis lectins require a high minimum agglutinating concentration (up to 125 ug to 500 ug),[12] and therefore a long incubation time (typically overnight) is necessary for glycoprotein detection.[13] Thus, the development of an simple, rapid and sensitive method for detecting Neu5Acα(2-3)Gal glycans remains an unmet need.

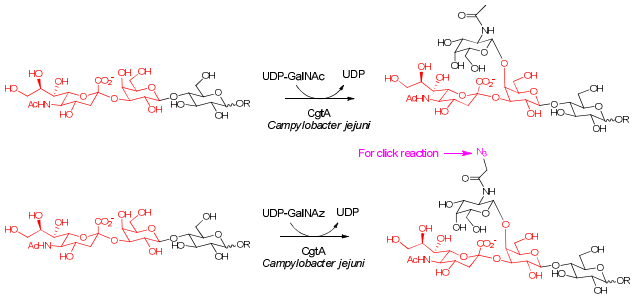


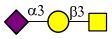
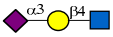
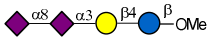
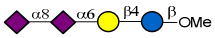
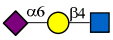
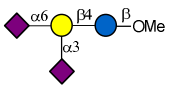
Figure 1. CgtA recognizes Neu5Acα(2-3)Gal with UDP-GalNAc or UDP-GalNAz

Neu5Acα(2-3)Gal disaccharide is the outer core component of many *Campylobacter jejuni* strains.[14] CgtA is responsible for the extension of Neu5Acα(2-3)Gal with GalNAc residue (Figure 1).[14] We reasoned that CgtA might tolerate substitution at the C-2 position of GalNAc, allowing for the introduction of an azide group for further click chemistry reaction (Figure 1). Substrate specificity study using Ganglio-oligosaccharide GM3 (entry 1, Table 1) shows that both UDP-GalNAz and UDP-GalNAc are efficient substrate of CgtA (Figure S1). Kinetic analysis revealed a k_{cat}/K_m value of 1.34 $nM^{-1}min^{-1}$ for UDP-GalNAc, and 1.51 $nM^{-1}min^{-1}$ for UDP-GalNAz. Indeed, treatment of GM3 with CgtA and UDP-GalNAz overnight led to a complete conversion of GM3. The product was confirmed by MALDI-TOF-MS and NMR (see supporting information).

Having demonstrated that CgtA accepts UDP-GalNAz as substrate, we next tested its substrate specificity towards sialylated oligosaccharides with UDP-GalNAz. Many sialylated oligosaccharides containing α2-3-, α2-6-, or α2-8-linked sialic acid (Table 1 and Table S1) were synthesized using the methods reported previously.[15] We found that CgtA requires only linear disaccharide structure of Neu5Acα(2-3)Gal (Table 1, entries 1 to 5) or Neu5Gcα(2-3)Gal (Table S1, entry 2) when using UDP-GalNAz as donor. Meanwhile, only very low relative activity towards the structure containing α2-6-linked sialic acid or without sialic acid was detected (Table 1, Entries 6 to 10; Table S1, entries 3 to 5). Indeed, no observable product or by-product (UDP) could be found on TLC after the incubation of these compounds with CgtA and UDP-GalNAz overnight. These findings indicated the potential of CgtA for application in selective labeling Neu5Acα(2-3)Gal glycans.

To test the practicality of the described strategy on protein labeling, we used fetal bovine fetuin as an example. Fetal bovine fetuin is a well-studied model protein for sialylated glycans analysis and commercially available. It contains three *N*-glycosylation and three *O*-glycosylation sites, on which Neu5Ac attached to the terminal galactose residues through α2-3- or α2-6-linkage.[16] To perform a control, fetal bovine fetuin was treated with a sialidase (NanC), which specifically

Table 1. Substrate specificity of CgtA with UDP-GalNAz towards sialylated oligosaccharides

Entry	Substrate	RA (%)[a]
1		100
2		104.3
3		100.6
4		113.4
5		96.5
6		0.08
7		0.28
8		0.31
9		0.41
10		0.25

[a]RA: relative activity. See SI for experimental details. ● Glc ■ GlcNAc

hydrolyzes α2-3-linked Neu5Ac.[17] After the treatment of NanC, a slight migration change compare to native feutin was observed on SDS-PAGE gel (Figure 2A). The α2-6-linked Neu5Ac was confirmed by biotinylated *Sambucus nigra* agglutinin (B-SNA) (Figure 2A). Native fetuin or NanC-treated fetuin was labeled by CgtA with UDP-GalNAz at 37°C for 1 hour, while other control groups were performed in parallel. Following copper-free click reaction (DIBO-alkyne, 10 uM), the proteins were analyzed by western blot using streptavidin-linked horseradish peroxidase (S-HRP). Strong fluorescence in labeling group was observed, while all the control groups failed to be labeled (Figure 2A), demonstrating that the designed scheme could be used to specifically label Neu5Acα(2-3)Gal glycans on glycoprotein. The labeled fetuin was further treated with peptide *N*-glycosidase F (PNGF), which remove *N*-Glycans from glycoprotein, and detected by S-HRP. Fluorescence labeling in the PNGF-treated sample still can be observed (Figure 2A, bottom graph), indicating that both *N*-glycans and *O*-glycans were labeled. Meanwhile, the probe of same amount of fetuin with biotinylated MAL II is unsuccessful. Thus, our chemoenzymatic approach provides a more credible detection strategy for Neu5Acα(2-3)Gal glycans and enable the highly sensitive detection of glycoproteins.

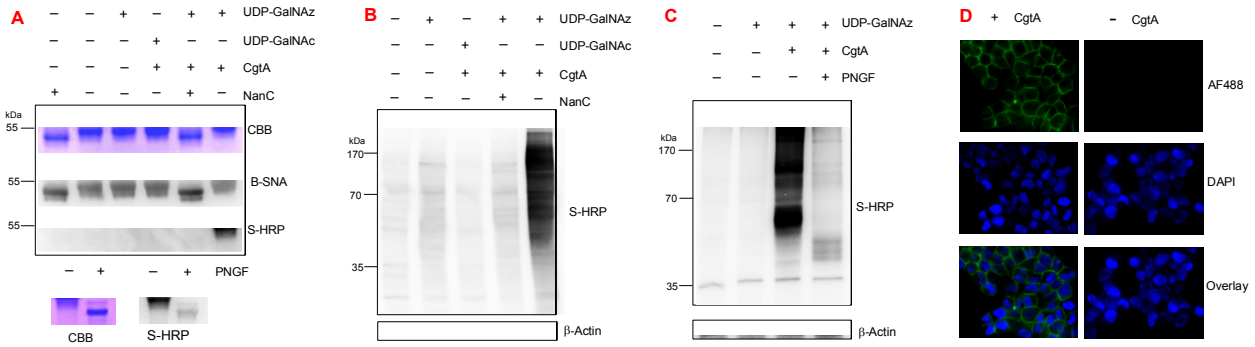


Figure 2. (A) Chemoenzymatic detection Neu5Acα(2-3)Gal glycans on fetal bovine fetuin. CBB: Coomassie brilliant blue staining. S-HRP: streptavidin-linked horseradish peroxidase. B-SNA: biotinylated SNA. NanC: The sample was treated with NanC before performing labeling reaction. PNGF: the labeled fetuin was further treated with PNGF (bottom graph). **(B)** Chemoenzymatic detection of Neu5Acα(2-3)Gal glycoproteins from HEK293T cell lysates. **(C)** Chemoenzymatic detection of Neu5Acα(2-3)Gal glycoproteins on cell surface of HEK 293T. **(D)** The imaging of cell-surface Neu5Acα(2-3)Gal glycans on live HeLa cells (Green) using fluorescence microscopy. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; blue). See SI for experimental details.

We next determined whether the approach could be used to track Neu5Acα(2-3)Gal glycoproteins in complex samples. Cell lysates from human embryonic kidney 293 (HEK293T) cells was incubated with CgtA and UDP-GalNAz at 37°C for 1 hour, while control groups were performed parallel. Following the Cu(I)-catalyzed azide–alkyne cycloaddition (CuAAC) reaction with diazo-biotin-alkyne, biotinylated cell lysates was detected by western blot using S-HRP (Figure 2B). Strong fluorescence labeling in cell lysates was observed, while only background level of nonspecific labeling in NanC-treated group and other control groups was observed (Figure 2B), highlighting further the specificity of the designed strategy towards Neu5Acα(2-3)Gal glycans. Cell surface Neu5Acα(2-3)Gal glycans were selectively labeled by incubating suspension HEK293T living cells with CgtA and UDP-GalNAz at 37°C for 30 min. Following the biotinylation by CuAAC reaction, strong fluorescence labeling was observed compared to control groups in western blot (Figure 2C). Then, several other randomly selected human cancer cell lines including A549, HeLA, and HepG2 were chemoenzymatically labeled using the same strategy (see supporting information). The labeled samples were further treated with PNGF, resulting in significant fluorescence reduction, indicating that Neu5Acα(2-3)Gal mainly attach to *N*-glycans in these cell lines.

We next investigated the potential application of the described strategy for Neu5Acα(2-3)Gal glycans imaging and quantification. The determination of the expression level of Neu5Acα(2-3)Gal glycans is very important to understand sialic-acid-related microbe infection and carcinogenesis.[18] Adherent HeLa cells were labeled by CgtA and UDP-GalNAz at 37°C for 30 min. After copper-free click reaction (DIBO-biotin, 30 uM), a fluorescent reporter was subsequently installed by incubation with streptavidin-linked Alexa Fluor 488 (10 ug/ml). Membrane-associated fluorescence was observed for cells treated with both CgtA and UDP-GalNAz, whereas no fluorescence labeling was detected for control cells labeled in the absence of CgtA, confirming the specificity of the *in situ* chemoenzymatic reaction (Figure 2D). The fluorescence intensity, which reflects the expression level of Neu5Acα(2-3)Gal glycans proportionally, was determined by flow cytometry (Figure S9).

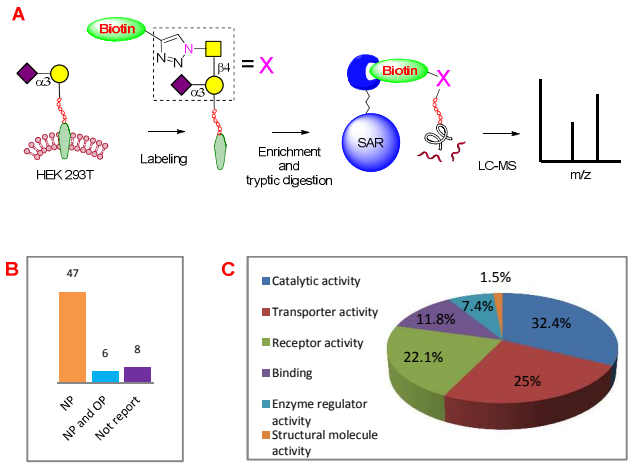


Figure 3. (A) Global identification of cell surface Neu5Acα(2-3)Gal glycoproteins from HEK293T. SAR: streptavidin agarose resin. (B)Total 61 proteins were probed. NP: *N*-glycosylation protein. OP: *O*-glycosylation protein. (C) Molecular function of the identified sialoglycoproteins.

Finally, Neu5Acα(2-3)Gal sialoglycoproteins that exist on the cell surface of HEK 293T were globally identified using the described strategy (Figure 3A). It is well established that cell surface glycoproteins containing Neu5Acα(2-3)Gal glycans play important roles in living cells, where these proteins are the potential therapeutic targets. However, there has been relatively little study of profiling such sialoglycoproteins due to the lack of an effective enrichment method. Although lectin affinity chromatography has been explored to enrich sialoglycoproteins,[19] this method was limited by the weak binding affinity and limited specificity of lectins. Alternatively, avidin-biotin complex is the strongest known non-covalent interaction between a protein and ligand (K_d = 10^{-14} ~10^{-15} M), making avidin-biotin binding to be an ideal system for protein enrichment.[20] In this work, the biotinylated cell surface proteins of HEK293T were captured using streptavidin agarose resin (SAR). After digestion with trypsin, the peptide fragments were analyzed by LS-MS. After filtering the non-specific binding proteins in control group, 61 pro-

teins were identified (Table S2). 53 probed proteins are the reported glycoproteins (Figure 3B), highlighting the feasibility of the described strategy. Among of these reported glycoproteins, 47 proteins contain *N*-glycans and six proteins contain both *N*- and *O*-glycans. This data is also well in accord with the western blot observation (Figure 2C). Molecular function analysis using the Protein ANalysis THrough Evolutionary Relationships (PANTHER) Classification System[21] was displayed in Figure 3C. Four main categories including catalytic activity (32.4%), transporter activity (25%), receptor activity (22.1%), and binding (11.8%) take up more than 90% of the total probed proteins. These are consistent with the well-known functions of cell surface sialoglycoproteins.[6f]

In conclusion, on the basis of a glycosyltransferase that could specifically recognize Neu5Acα(2-3)Gal with UDP-GalNAz and site-specific click chemistry reaction, we have developed the first strategy for the rapid and sensitive detecting Neu5Acα(2-3)Gal glycans. This method is far superior to the traditional lectin-based methods to detect Neu5Acα(2-3)Gal, which are limited by their inherent disadvantages. This method also allows that the global analysis of Neu5Acα(2-3)Gal glycoproteins is achievable, providing a powerful tool for sialic-acid-related research. Moreover, substrate specificity study indicated that the described strategy can be also used to probe Neu5Gcα(2-3)Gal glycans, which are currently detected by polyclonal monospecific antibody.[2] Future studies will enable the exploration of new glycosyltransferase for use in more glycans detecting.

ASSOCIATED CONTENT

The Supporting Information is available free of charge on the ACS Publications website at DOI:

Materials, experimental methods, and supporting figures and tables (PDF)

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Notes

The authors declare no competing financial interests.

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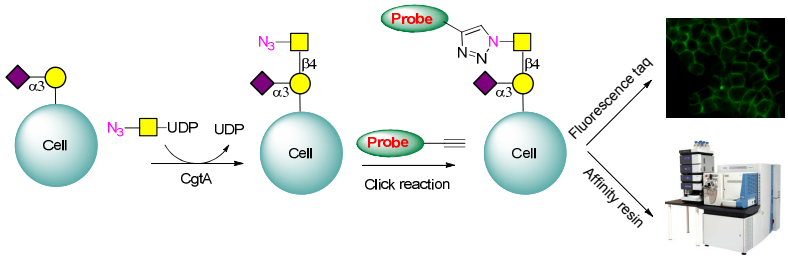
Cell
UDP
UDP
CgtA
Cell
Probe
Click reaction
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Fluorescence taq
Affinity resin