Selective Oxidation of *N*-Glycolylneuraminic Acid Using an Engineered Galactose Oxidase Variant

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ABSTRACT: *N*-Glycolylneuraminic acid (Neu5Gc) is a common cell surface ligand in animals which is not biosynthesized in humans, but can be acquired in human tissue from dietary sources such as red meat. It is important to understand the relevance of this potentially immunogenic glycan on human health, and selective detection methods are needed that can distinguish Neu5Gc from its biosynthetic precursor common in humans, i.e. *N*-acetylneuraminic acid (Neu5Ac). Here, we demonstrate that Neu5Gc can be selectively oxidized by an engineered variant of galactose oxidase without any reaction towards Neu5Ac. Oxidation of Neu5Gc itself allowed for the full spectroscopic characterization of the aldehyde product. In addition, we show that Neu5Gc is also oxidized when part of a typical animal oligosaccharide motif and when attached to a protein-linked *N*-glycan. Oxidation of Neu5Gc introduces bioorthogonal functionality that can be exclusively labelled. We demonstrate that in combination with sialidase mediated hydrolysis, this two-enzyme system can provide a useful tool for the selective detection of Neu5Gc in complex biological samples such as the biopharmaceutical alpha acid glycoprotein.

KEYWORDS: Neu5Gc, Galactose Oxidase, Selective Oxidation, Protein Modification, Glycoprotein

N-Glycolylneuraminic acid (Neu5Gc) (**1**) is produced in most mammals *via* hydroxylation of *N*-acetylneuraminic acid (Neu5Ac) (**2**) by the enzyme cytidine monophosphate-N-acetyl neuraminic acid hydroxylase. Humans have a mutation in the encoding gene,[1] which in combination with a lack of alternative synthetic pathways[2] leaves them unable to synthesize Neu5Gc (**1**) and leaves the human immune system prone to generate antibodies against this foreign glycan. Notably, small quantities of Neu5Gc (**1**) have been detected in epithelial and endothelial cells as well as some human carcinomas.[2–4] Neu5Gc (**1**) is derived from the human diet such as red meat and directly incorporated metabolically into human cells and tissue, making Neu5Gc (**1**) the first known human xeno-autoantigen.[5] Subsequent response of human anti-Neu5Gc antibodies promotes inflammation and has been implicated in the progression of a range of cancers.[6] Direct metabolic incorporation of Neu5Gc (**1**) also requires monitoring in biopharmaceuticals produced in non-human cell lines or serum, such as the anticancer drug cetuximab.[7]

A particular challenge is to detect Neu5Gc (**1**) in complex biomolecules in the presence of its structurally similar precursor Neu5Ac (**2**) (Figure 1), which is often more abundant. Current techniques rely on anti-Neu5Gc antibodies, either monoclonal, which only recognize a limited number of Neu5Gc (**1**) containing epitopes, or polyclonal, able to detect Neu5Gc (**1**) in a range of contexts but suffering from cross-reactivity that needs to be remedied *via* several affinity purification steps.[3,8] Several methods have been employed for quantifying Neu5Gc in bulk samples,[9] but need extensive cleavage, derivatization and workup, particularly in food.[10,11] Here we propose a novel strategy for selectively labelling terminal Neu5Gc (**1**) over Neu5Ac (**2**) *via* selective oxidation using an engineered galactose oxidase variant.

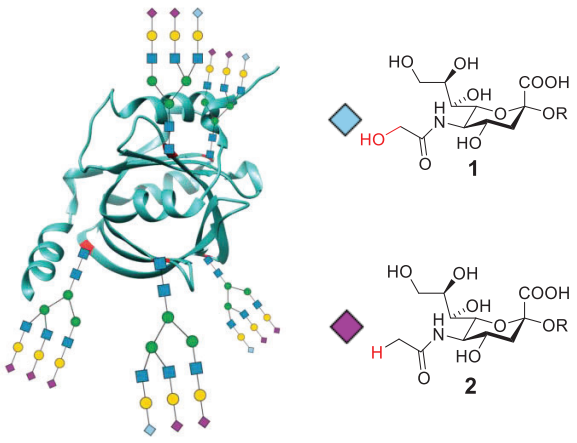
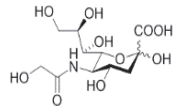
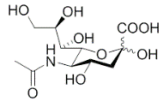
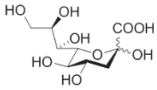


Figure 1: Structure of α-1-acid glycoprotein (AGP) containing typical sialic acids terminated *N*-glycans. Neu5Gc (**1**) and Neu5Ac (**2**) differ by only a single hydroxyl group indicated in red.

Galactose oxidases (GOases) catalyze the oxidation of the C6-OH of terminal D-galactosides at the non-reducing end to their corresponding aldehydes using molecular oxygen. These enzymes are members of the copper radical oxidase AA5 family as classified in the carbohydrate active enzyme database.[12] GOases generally are strictly stereo-specific, highlighted by a 10^6-times higher activity towards D-galactose than to D-glucose.[13,14] GOase has previously been engineered to enable oxidation of a range of mono and disaccharides.[15,16] A range of common cell surface sugars with primary alcohol functionalities were successfully oxidized with the F_2 variant, as shown for glucose, mannose, talose and corresponding 2-NHAc derivatives. However, the oxidation of Neu5Ac (**2**) has never been detected, despite the presence of the primary C-9 hydroxyl group. Here we demonstrate the first example of the enzymatic oxidation of the sialic acid Neu5Gc (**1**). Oxidation generates a unique glyoxal group, which allows for bioorthogonal reductive amination using previously established methodologies.[15,17] These GOase-mediated oxidation strategies are complementary to bioorthogonal metabolic labelling of sialic acids in living and cancerous tissue.[18,19]

To identify a suitable biocatalyst for the oxidation of Neu5Gc (**1**), GOase variants were screened for activity towards parent sialic acids using a fast colorimetric assay that had been described previously.[15] The M_1, M_3 and F_2 variants, each containing multiple mutation sites compared to wild-type enzyme, had previously been shown to have broader substrate range and were expressed and purified together as described previously.[20] These variants were tested with three common cell surface sialic acids, i.e. Neu5Gc (**1**), Neu5Ac (**2**) and 2-keto-3-deoxynononic acid (Kdn) (**3**). Among all reactions tested, F_2 was the sole variant to show activity towards Neu5Gc (**1**) (Table 1, Figure 2), with no activity observed for the other sialic acids. Interestingly, all three variants showed activity towards glycolamide (**4**). Activity towards **4** and lack of activity towards **2** and **3** suggested that GOase F_2 oxidizes Neu5Gc (**1**) on the glycolyl OH group, rather than at the C-9 position (Figure 3).

[a]**Table 1**: Activity of GOase variants M_1, M_3 and F_2 against substrates **1-4**.

	Substrate	Specific activities (μmol min^{-1} mg^{-1})		
		M_1	M_3	F_2
1		-	-	0.47(±0.03)
2		-	-	-
3		-	-	-
4		5.3(±0.2)	18.8(±0.4)	22.5(±0.2)

[a] Specific activities were determined at 30°C in pH 7.4 phosphate buffer with 25 mM substrate concentration.

To confirm the selectivity of Neu5Gc (**1**) oxidation, the reaction was scaled up (Figure 2) and the product **5** was isolated and characterized by HRMS and ^1H and ^{13}C NMR. These data confirmed that GOase F_2 oxidized **1** selectively to the aldehyde **5**. HRMS analysis of oxidation product **5** showed a peak at *m/z* 346.07 (supported by MALDI-TOF analysis) that corresponded to the sodiated adduct of **5** (supporting information). NMR analysis showed a mixture of products generated by hydration of the aldehyde to the *gem*-diol (**6**) (Figure 2) and further isomerization. No starting material (**1**) could be detected by MS or NMR analysis, which indicated complete oxidation. The GOase F_2 variant was also incubated with Neu5Ac (**2**) under the same conditions; both NMR and HRMS analysis of reaction products showed no oxidation of Neu5Ac (**2**) (Supporting figures S3 and S10), thus confirming the enzyme's exquisite specificity towards Neu5Gc (**1**) over Neu5Ac (**2**). From 2D-NMR analysis it became apparent that the glycolyl aldehyde and the C7/C8 hydroxy groups formed intramolecular hemiacetals leading to the formation of a number of side products (Supporting figure S12), however the aldehyde is still freely available in solution for modification (Figure **4B**).

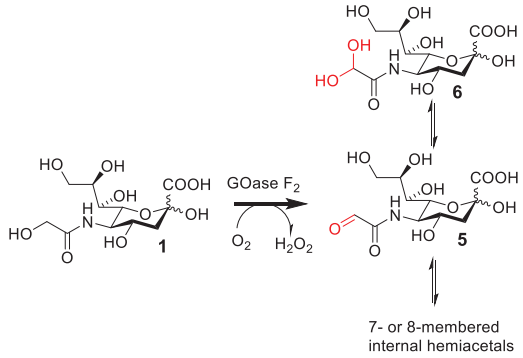


Figure 2: Oxidation of Neu5Gc (**1**) and subsequent formation of the *gem*-diol (**6**) observed by ESI-MS (*m/z* 364 [M+Na^+]) and NMR. 2D-NMR shows the formation of 7 or 8 membered hemiacetals (Supporting figure S12 for proposed structures).

^1H and ^{13}C NMR analysis of oxidized Neu5Gc showed the generation of a new peak at 5.3ppm corresponding to the α-proton of the *gem*diol on the glycolyl group (compound **6**) - the glycolyl aldehyde (**5**) was not observed in any of the NMR spectra. The oxidation at the glycolyl position was also supported by the presence of 2 doublets at ppm 3.75 corresponding to the C9 protons, as this signal remained unchanged when comparing oxidized (**5, 6**) and non-oxidized Neu5Gc (**1**) (Supporting information figure S7). Together, the analytical data confirms the selective enzymatic oxidation of Neu5Gc (1) at the glycolyl and not the C-9 hydroxyl group. The biocatalytic oxidation of Neu5Gc has a number of advantages over conventional organic chemistry that requires the use of toxic periodate based methods which cannot distinguish between sialic acids.[21] GOase on the other hand displays excellent selectivity for Neu5Gc (**1**) over Neu5Ac (**2**) and Kdn (**3**).

Based on the known crystal structure of the GOase (PDB 1GOG)[22,23] from *Fusarium graminearum*, we could apply molecular docking simulations to gain insights into the functional binding of the glycolamide (4) moiety in the active site. Glycolamide (4) binds in the active site cavity with its hydroxy oxygen atom coordinating the catalytic copper coenzyme at a 2.4 Å distance, which is reasonable for oxidation (Figure 3).[23,24] Notably, the ligand orientation is in good agreement with an acetate-soaked GOase crystal structure (PDB 1GOF) displaying a distance of 2.3 Å between acetate oxygen and copper (Figure S16). The binding pose is stabilized by hydrogen bonds to R330 and polar interaction to the catalytically relevant residue Y272 with the ligand's carbonyl moiety. In addition, we performed docking of glycolamide in variant F_2, modelled on GOase variant E1 (PDB

2WQ8).[15] The binding pose resembles the positioning in the wild-type enzyme, but with an altered hydrogen bond network towards mutated residues R330K and Q406E, both of which are known for their involvement in substrate binding (Figure 3).[23–25] This extended hydrogen bonding might explain experimentally observed higher acitvity for variant F_2. The relevance of active site restructuring in F_2 is further underpinned by the finding that it was required to compute a functional binding mode for Neu5Gc, while this was unsuccesful using GOase wild-type as receptor (Figure S16- B). Several hydrogen bonds between the sugar and mutated active site residues G195E, R330K and Q406E position the glycolamide moiety towards the copper coenzyme. Hence, the docking offers a structural rationale for the experimentally determined selectivity towards Neu5Gc.

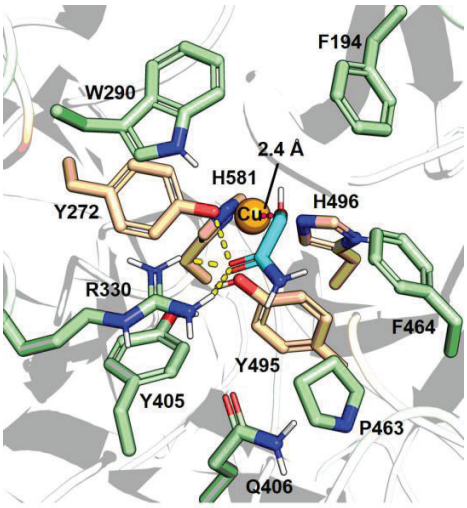


Figure 3: Molecular docking (AutoDock VINA as implemented in YASARA) of glycolamide (cyan) in GOase wild-type from *Fusarium graminearum* (PDB 1GOG). Copper (dark orange) coordinating residues are depicted as wheat-colored sticks and further active site residues surrounding bound ligand as green sticks. Ligand coordination via hydroxyl oxygen to the copper is indicated specifying the 2.4 Å distance (pink). Computed polar contacts (PyMOL) to the ligand are shown (yellow)

Having established the selective oxidation of Neu5Gc (**1**) as a free monosaccharide, we explored activity towards Neu5Gc (**1**) as part of glycoconjugates, such as trisaccharide **7**. The trisaccharide Neu5Gc-α-2,3-Galβ-1,4-GlcNAc (**8**) is a common protein glycan and cell surface motif and was synthesized using a one pot multi enzyme system with a CMP-sialic acid synthase from *Neisseria meningitidis* and α-2,3-sialyltransferase from *Pasteurella dagmatis* (supporting information).[26] Trisaccharide **8** was incubated with GOase F_2 for 16 hrs (Figure 4), and analysis of the reaction product (**9**) by mass spectrometry confirmed formation of the aldehyde hydrate (Figures S17 and S19).

Modification of the generated aldehyde allows for access to a number of biologically relevant analogues. For example Cao and colleagues utilized the generated aldehyde on galactose moieties for use in biological probes. [27] After the successful oxidation of trisaccharide **7** we utilized previously established methodologies for the synthesis of chemically conjugated Neu5Gc glycans (**9**).[15,17]Aldehyde **8** was incubated with a 10 fold excess of 3-hydroxybenzoic acid hydrazide (3HBH) and aminooxy biotin respectively. Full conversion of the conjugated glycans (**9a b**) was achieved within 2 hours and was confirmed by mass spectrometry. The successful biotinylation of this glycan motif suggests the potential for the methodology to be used in whole cell labelling.

After demonstrating the ability of GOase F_2 to oxidize Neu5Gc (**1**) as a monosaccharide and as part of a glycoconjugate (**7**), we then investigated its ability to oxidize Neu5Gc when bound to a glycoprotein (**10**) and in the presence of Neu5Ac (**2**). In the first instance, GOase F_2 activity was assayed against bovine glycoprotein α-1-acid glycoprotein (AGP), which carries glycans with terminal sialic acids of both Neu5Ac and Neu5Gc.[28] AGP glycoprotein is an important pharmaceutical target as it is abundant in severe infections and is one of the four potential biomarkers for five year mortality.[29,30] AGP glycoprotein contains a significant percentage of Neu5Gc [31] and has been shown to be highly sialylated. The specific activity (determined using HRP-ABTS assay) of the GOase F_2 variant against AGP was 0.083 μmol min^{-1} mg^{-1} (±0.001) suggesting that F_2 is capable of oxidizing glycosidically bound Neu5Gc (**10**) on whole glycoproteins.

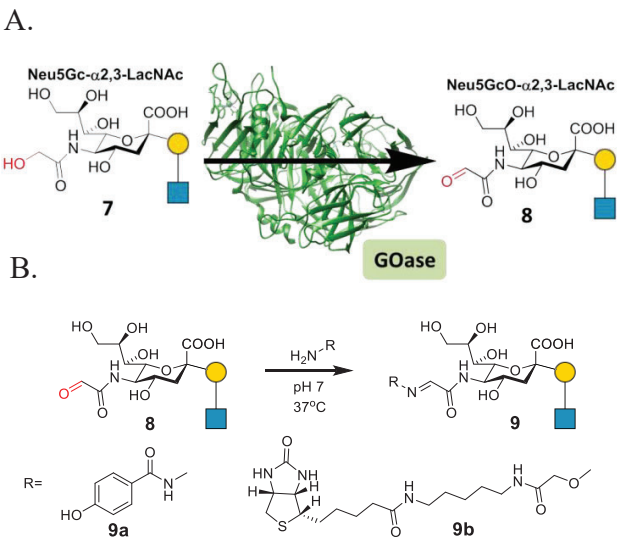


Figure 4: **A**. Oxidation of trisaccharide 8 with GOase F_2 **B.** Generation of conjugated trisaccharides through modification of **8** via hydrazide or oxime ligation.

To confirm that the observed activity is due to the oxidation of the glycosidically bound Neu5Gc, all terminal sialic acids were released from the AGP glycans using a bacterial sialidase, followed by MS analysis (Figure 5). Interestingly, both substrates and oxidized sialic acids were released by the glycosidase to produce monosaccharides 1, 2 and 5 and aglycone 12. MALDI-Tof analysis clearly indicated the presence of oxidized product (5) and non-oxidized Neu5Ac (2) after the reaction with the F_2 variant, indicating selective and complete oxidation of the terminal Neu5Gc. Taken together, these results support our previous observations and demonstrate that GOase F_2 is capable of oxidizing Neu5Gc (1) in complex glycoconjugates even in the presence of other sialic acids/Neu5Ac. It should be noted that in complex systems AGP glycans might also contain terminal galactose moieties that are substrates for the F_2 variant. Such background activity will require one additional step of either pretreatment with wild-type GOase to cap terminal galactose residues, or pretreatment with sialidase to monitor any non-specific oxidations.

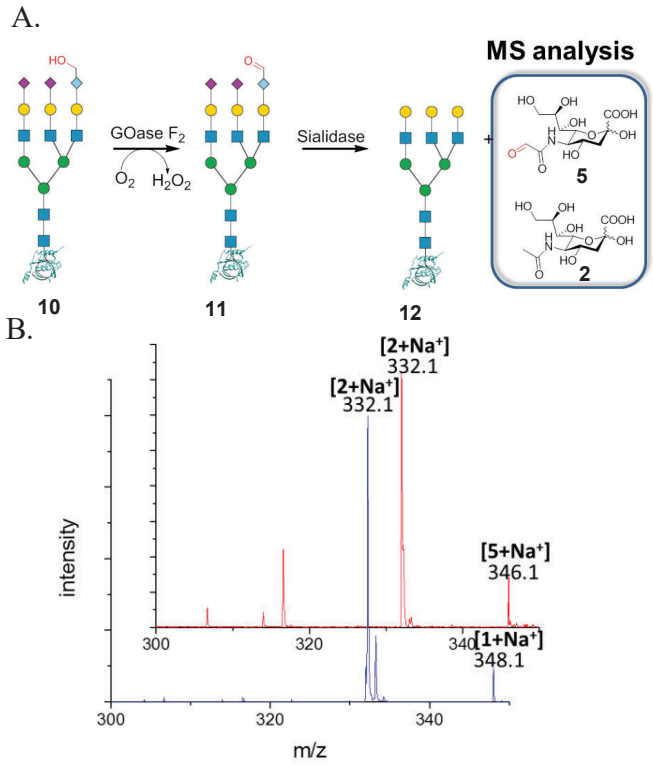


Figure 5: A. Oxidation of Neu5Gc (**1**) to (**5**) when bound to glycoprotein AGP. B. MALDI-ToF analysis of sialic acids released from AGP using sialidase before (blue) and after (red) incubation with 0.48 mg/mL GOase F_2.

In summary, we have shown that the GOase F_2 variant is able to oxidize Neu5Gc (**1**) as a monosaccharide and in glycoconjugates (such as **10**) with exquisite selectivity for the glycolyl hydroxyl of Neu5Gc and consequently does not show activity towards Kdn (**3**) or Neu5Ac (**2**) Spectroscopic analysis of the product revealed that the glycolyl group was selectively oxidized over other hydroxyl groups in Neu5Gc (**1**), including the primary C-9 hydroxyl group. The oxidation product could be released from the glycoconjugate using bacterial sialidases. This two enzyme system (Figure 5A) allows specific detection of oxidized Neu5Gc in complex glycan samples. Further to this, the oxidation introduces a bioorthogonal aldehyde into cell surface glycoconjugates containing Neu5Gc (**10**) in a highly selective manner which can be used for subsequent selective derivatization as previously demonstrated.[15,17] As such the present method is complementary to metabolic labelling which cannot distinguish between Neu5Ac and Neu5Gc. Selectivity for labelling of Neu5Gc (**1**) over other sialic acids (**2, 3**) should also be useful for the quality control in production of biopharmaceuticals, given the concern of Neu5Gc (**1**) as a potential immunogenic contaminant.[32]

ASSOCIATED CONTENT

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Notes

The authors declare no competing financial interests.

Supporting Information

Materials, experimental procedures, characterization of new compounds. This material is available free of charge *via* the internet at http://pubs.acs.org

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