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Glycan specificity of neuraminidases determined in microarray format

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Abstract

Neuraminidases hydrolytically remove sialic acids from glycoconjugates. Neuraminidases are produced by both humans and their pathogens, and function in normal physiology and in pathological events. Identification of neuraminidase substrates is needed to reveal their mechanism of action, but high-throughput methods to determine glycan specificity of neuraminidases do not exist. Here we use two glycan labeling reactions to monitor neuraminidase activity toward glycan substrates. While both periodate oxidation and aniline-catalyzed oxime ligation (PAL) and galactose oxidase and aniline-catalyzed oxime ligation (GAL) can be used to monitor neuraminidase activity toward glycans in microtiter plates, only GAL accurately measured neuraminidase activity toward glycans displayed on a commercial glass slide microarray. Using GAL, we confirm known linkage specificities of three pneumococcal neuraminidases and obtain new information about underlying glycan specificity.

Graphical Abstract

Keywords

Sialic acids; neuraminidase; chemoenzymatic labeling; microarray

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1. Introduction

Sialic acids are a group of nine-carbon α -keto acids typically found at the non-reducing termini of glycoproteins and glycolipids.¹ N-acetylneuraminic acid (Neu5Ac) is the primary sialic acid found in human glycoconjugates, while N-glycolylneuraminic acid (Neu5Gc) and deaminoneuraminic acid (2-keto-3-deoxy-D-glycero-D-galactonononic acid; KDN) are produced by other species. Sialic acids can be removed from glycoconjugates through the action of neuraminidases, also known as sialidases. These enzymes hydrolyze the glycosidic bond between sialic acid and the underlying glycan. Desialylation of cell surface glycoconjugates can have dramatic effects on their activity, stability, and subcellular localization.² The human genome encodes at least four neuraminidases (NEU1–NEU4), which exhibit varying subcellular localizations, expression patterns, and glycan specificities.^{3,4} Neuraminidases are also produced by both viral and bacterial pathogens, where they often function as virulence factors.^{5,6} By desialylating host cell glycoconjugates, viral and bacterial neuraminidases can potentially affect host cell signaling events.^{7,8} The glycan specificity of neuraminidases determines which host glycoconjugates are desialylated, and thus dictates the effects of these enzymes on host cell biology.

A variety of approaches have been employed to examine the glycan specificity of neuraminidases.^{9–14} In early efforts, neuraminidase activity toward individual glycans was examined in a one-by-one fashion, while more recent approaches have used microtiter plate assays to simultaneously examine a wider array of glycans. Recently, glycan microarrays have emerged as an important tool enabling high-throughput assessment of the specificity of glycan-binding proteins.^{15–18} These successes suggest that glycan microarrays might also be used to examine the specificity of glycan-modifying enzymes, such as neuraminidases. Indeed, several groups have used glycan microarrays to evaluate specificity of parainfluenza and influenza neuraminidases. In these efforts, neuraminidase-induced changes in glycan structure were detected using fluorescently-labeled detection reagents, such as galactose-specific lectins or whole virus, to bind to desialylated glycans.^{19–22} Notwithstanding these important studies, glycan microarrays have not yet been fully harnessed for analysis of neuraminidase specificity. One limitation is that commonly used detection reagents exhibit their own glycan specificities, restricting the set of glycans that can be analyzed.

We previously reported that a chemoselective glycan labeling reaction - periodate oxidation and aniline-catalyzed oxime ligation (PAL)²³ - could be used to interrogate neuraminidase specificity in microtiter plate format.¹³ Here we examine the utility of PAL and a second chemoselective glycan labeling strategy - galactose oxidase and aniline-catalyzed oxime ligation (GAL)²⁴ - to evaluate neuraminidase specificity in microarray format. We find that GAL accurately reports on neuraminidase activity toward glycans displayed on microarrays. Thus, applying GAL to microarrays may offer a high-throughput approach to evaluate neuraminidase specificity through the use of emerging glycan microarray resources.

2. Results

We used our previously reported microtiter plate assay with PAL detection¹³ to determine the substrate specificities of three pneumococcal neuraminidases - NanA, NanB, and NanC.

The specificities of these neuraminidases for different sialic acid linkages are known: NanA is relatively non-selective, cleaving α 2-3-, α 2-6-, and α 2-8-linked sialic acids, whereas NanB and NanC exhibit preferences for α 2-3-linked sialic acids.^{25,26} However, assessment of the effects of underlying glycan structures on the activities of these neuraminidases has been more limited. To determine the specificities of NanA, NanB, and NanC using the PAL microtiter plate assay, a set of biotinylated glycans (Figure 1) were treated with neuraminidase or not, before incubation in individual wells of a streptavidin-coated 96-well plate. After binding glycans in each well, remaining sialic acids were labeled using PAL and fluorescence was measured.

As expected, NanA was able to cleave α 2-3-linked N-acetylneuraminic acid (Neu5Ac). NanA displayed similar activities toward Neu5Ac α 2-3-linked to lactose (Lac) and to N-acetyl-D-lactosamine (LacNAc) (Figure 2A). NanA was also active against α 2-3-linked N-glycolylneuraminic acid (Neu5Gc), although cleavage of Neu5Gc appeared to be slightly less efficient than cleavage of Neu5Ac at similar enzyme concentrations. Interestingly, while NanA was highly active against sialyl Lewis X (sLe^X, Neu5Ac- α 2-3-Gal- β 1-4-(Fuc α 1-3)-GlcNAc-), it displayed slightly less activity toward sialyl Lewis a (sLe^a, Neu5Ac- α 2-3-Gal- β 1-3-(Fuc α 1-4)-GlcNAc-), a glycan in which the positions at which the sialylated galactose and fucose substituents are attached to GlcNAc are swapped relative to sLe^X (Figure 1). While the promiscuous NanA was also able to cleave α 2-6-linked Neu5Ac from both Lac and LacNAc, as expected, the enzyme showed slightly higher activity toward the LacNAc-containing glycan. Reports indicate that NanA collaborates with other glycosidases to cleave Neu5Ac- α 2-6-LacNAc from host cells surfaces during infection,²⁷ suggesting that pneumococcus may have evolved to achieve efficient recognition of this important glycan. Surprisingly, NanA did not appear to be active against Neu5Gc α 2-6-linked to LacNAc, even at the highest enzyme concentration. Taken together with results from α 2-3-linked Neu5Gc, pneumococcal NanA appears to prefer Neu5Ac over Neu5Gc. Finally, NanA displayed no activity against the GM1 oligosaccharide headgroup or a glycan containing KDN, a sialic acid species that occurs mainly in bacteria and lower vertebrates. Overall, these results emphasize the importance of the underlying glycan, as well as the sialic acid species, in determining sialidase specificity.

Activity of NanB against Neu5Ac α 2-3-linked to Lac or LacNAc was high, as expected (Figure 2B). While NanB was also able to cleave Neu5Gc α 2-3-linked to both Lac and LacNAc, it displayed a strong preference for Neu5Ac-containing glycans. In addition, NanB activity toward α 2-3-linked-Neu5Gc was greater when the underlying glycan was LacNAc compared to Lac, as was the case for NanA. Similar to NanA, NanB also displayed no activity against a KDN-containing glycan or the GM1 oligosaccharide headgroup. While NanB was able to cleave sialic acid from the fucosylated glycan sLe^X, it had no activity against sLe^a, indicating that placement of the fucose residue can have dramatic impact on neuraminidase activity. Finally, and consistent with previous reports,^{25,26} NanB was unreactive toward α 2-6-linked sialic acids.

Previously, we used this assay to determine the glycan specificity of NanC, and the results are reproduced here to enable comparison.¹³ Briefly, NanC displayed similar specificity as NanB, with activity toward α 2-3-linked sialic acids and not α 2-6-linked sialic acids (Figure

2C). Indeed, NanC efficiently cleaved α 2-3-linked Neu5Ac from both Lac and LacNAc, while displaying less activity toward Neu5Gc on the same underlying glycans. Like NanA and NanB, NanC showed greater activity toward Neu5Gc- α 2-3-LacNAc than Neu5Gc- α 2-3-Lac. In addition, NanC had no activity against a KDN-containing glycan, the GM1 oligosaccharide headgroup, or sLe^a. Interestingly, and unlike NanA or NanB, NanC was also inactive toward sLe^X. Results from the microtiter plate assay indicate the pneumococcal neuraminidases exhibit unique substrate specificities, which may be important during pneumococcal infection.

Interestingly, all three pneumococcal neuraminidases preferred Neu5Ac over Neu5Gc, with NanB and NanC discriminating more strongly than NanA. Neu5Gc differs from Neu5Ac by the addition of an -OH group on the N-acyl side chain (Figure 3A). Importantly, Neu5Gc cannot be synthesized by humans due to inactivation of the gene encoding CMP-N-acetylneuraminic acid hydroxylase,²⁸ but can be incorporated into human glycoconjugates through dietary sources.²⁹ However, Neu5Gc is largely excluded from incorporation into brain glycoconjugates.³⁰ Previously, we found that NanC preferentially hydrolyzes Neu5Ac, exhibiting reduced activity toward Neu5Gc.¹³ To further assess the specificity of NanA and NanB for Neu5Ac versus Neu5Gc, we treated 2-O-(*para*-nitrophenyl)- α -Neu5Ac (Neu5Ac- α -pNP) and 2-O-(*para*-nitrophenyl)- α -Neu5Gc (Neu5Gc- α -pNP) with NanA or NanB, and measured the amount of pNP released upon treatment with neuraminidase. While NanA readily hydrolyzed both Neu5Gc and Neu5Ac substrates, NanB behaved like NanC, displaying higher activity toward Neu5Ac-pNP as compared to Neu5Gc-pNP (Figure 3B), and confirming the results from the microtiter plate assay. The preference for Neu5Ac over Neu5Gc is consistent with pneumococcus being primarily a human pathogen, and with roles for the neuraminidases in meningitis infections, where little to no Neu5Gc would be encountered by the pathogen.

Having evaluated neuraminidase specificity in the microtiter PAL plate assay, we next determined whether a complementary chemoselective labeling strategy would provide an additional measure of neuraminidase activity. For this, we turned to galactose oxidase and aniline-catalyzed oxime ligation (GAL).²⁴ In this method, if neuraminidase removes sialic acids, then GAL is able to oxidize and label the underlying galactose (Gal) or N-acetylgalactosamine (GalNAc) residue. In this approach, an increase in fluorescence is indicative of removal of sialic acids by neuraminidase, providing signal that is inverse to that observed in the PAL method. To determine whether GAL could report on neuraminidase activity in microtiter plate format, we treated biotinylated Lac, Neu5Ac- α 2-3-Lac, and Neu5Ac- α 2-6-Lac with neuraminidase or not before adhering glycans to a streptavidin-coated plate. Immobilized glycans were labeled with GAL using aminooxy-conjugated Alexa Fluor 488, then fluorescence was measured. Treatment of Neu5Ac- α 2-3-Lac and Neu5Ac- α 2-6-Lac with NanA resulted in complete desialylation of each glycan (Figure 4A), consistent with results from the PAL microtiter plate assay. NanB and NanC also cleaved Neu5Ac- α 2-3-Lac, but were unreactive toward Neu5Ac- α 2-6-Lac, as expected (Figure 4B and C). We were therefore satisfied that both PAL and GAL could provide desired, and complementary, reports of neuraminidase activity.

To expand the neuraminidase activity assay to a microarray format, we first needed to determine whether the chemoselective labeling strategies could be applied to glycans displayed on glass slides. To do this, Lac functionalized with an arylamine (4-aminophenyl β -D-lactopyranoside; LacAP) was sialylated with Neu5Ac in either α 2-3 or α 2-6 linkage using a one-pot, chemoenzymatic reaction. The resulting Neu5Ac- α 2-3-LacAP and Neu5Ac- α 2-6-LacAP were then printed onto N-hydroxysuccinimide (NHS)-activated glass slides.¹⁶ Once dry, slides were treated with either a nonspecific neuraminidase from *Arthrobacter ureafaciens* (AUNA), which cleaves both α 2-3- and α 2-6-linked sialic acids, or an α 2-3-linkage specific neuraminidase from *Salmonella typhimurium* (STNA). After neuraminidase treatment, slides were labeled with aminooxy Alexa Fluor 647 using PAL (Figure 5A). Upon measuring fluorescence using a microarray scanner, the fluorescence intensities of Neu5Ac- α 2-3-LacAP and Neu5Ac- α 2-6-LacAP on slides treated with AUNA were decreased compared to slides not treated with neuraminidase (Figure 5B), as expected for the nonspecific neuraminidase. In contrast, treatment with STNA greatly reduced fluorescent labeling of Neu5Ac- α 2-3-LacAP, but had less effect on fluorescent labeling of Neu5Ac- α 2-6-LacAP (Figure 5B). Thus, chemoselective labeling strategies are suited for labeling glycans on glass slides, and for reporting on neuraminidase activity.

Next, we evaluated whether chemoselective labeling could be used to detect sialylated glycans on a microarray. We chose to use a commercial glycan array available from RayBiotech, where each slide is printed with 4 replicates of 100 different sialylated and non-sialylated glycans (Figure 6A). To ensure we could accurately identify sialic acid-terminating and Gal/GalNAc-terminating glycans using PAL and GAL, respectively, glycans were biotinylated using PAL or GAL followed by conjugation to a streptavidin-labeled fluorophore. To our disappointment, upon labeling slides with PAL (Figure 6B), sialylated glycans were indistinguishable from unsialylated glycans (Figure 6C, sialylated glycans shown in purple). Indeed, the fluorescence signal appeared to correlate more strongly with glycan position in the array than with sialylation status, suggesting that reagent accessibility might be driving the kinetics of labeling. In optimizing use of PAL in the microtiter plate assay, we had found that reducing the periodate concentration 10-fold (to 0.1 mM) improved the specificity of detection of sialylated glycans,¹³ but lowering the periodate concentration used for PAL on glass slides to 0.1 mM yielded no improvement in discrimination between sialylated and unsialylated glycans. Unfortunately, the indiscriminate labeling observed with PAL made it impossible to use this method to determine substrates of neuraminidases. Labeling glycans on the slide using GAL proved more promising (Figure 6D). Fluorescent labeling of Gal and GalNAc-terminating glycans was generally higher than other glycans on the slide (Figure 6E, Gal/GalNAc-terminating glycans shown in orange), although branched and fucosylated glycans terminating in Gal/GalNAc (represented by orange bars with black lines) were not labeled by GAL as efficiently. Glycan branching is known to interfere with the ability of the galactose oxidase to oxidize the Gal and GalNAc residues,³¹ which would be expected to render labeling through GAL less efficient. Even so, the high fluorescence signals that we observed for most non-sialylated glycans suggested that GAL labeling of glycans on neuraminidase-treated slides should provide information on neuraminidase activity. We therefore focused on GAL labeling for microarray analyses.

To measure neuraminidase activity on the glass slides, slides were treated with buffer alone or buffer containing NanA, NanB, or NanC. Because each slide contained four replicates of the same 100 glycans separated into four wells, one slide was used for each trial, and the experiment was performed in triplicate. In this way, variation occurring through the use of different slides and reagents was limited. Overall, we found treatment with NanA on the glass slide resulted in cleavage of both α 2-3- and α 2-6-linked sialic acids, as expected (Figure 7A). Indeed, consistent with results from the 96-well plate assay, NanA was able to cleave α 2-3- and α 2-6-linked Neu5Ac, as well as α 2-3- and α 2-6-linked Neu5Gc, from Lac glycans. Consistent with a previous report,²⁵ NanA was active toward α 2-8 linked sialic acids, cleaving as many as three consecutively-linked sialic acids, though a substrate with four sialic acids in tandem was less favored. NanA showed moderate reactivity toward KDN on the glycan microarray, unlike the results in the 96-well microtiter plate assay (Figure 2A). However, the linkage of KDN to the underlying glycan varied between assays, being α 2-3-linked in the plate assay and α 2-8-linked on the slide. NanA also displayed activity toward both sLe^X and sLe^a, consistent with the 96-well plate assay, as well as toward Neu5Gc-labeled sLe^a. Unsurprisingly, we were unable to measure differences in sialylation on branched glycans, likely due to inefficient labeling of terminal Gal and GalNAc residues on these glycans (Figure 6E).

Treating slides with NanB followed by labeling of glycans using GAL gave the expected result of cleaving α 2-3-linked sialic acids but not α 2-6-linked sialic acids (Figure 7B). Interestingly, the preference for Neu5Ac over Neu5Gc was also apparent on some, but not all, underlying glycans. Consistent with being a more selective neuraminidase, NanB displayed no activity against α 2-8-linked sialic acids. Unlike the results from the plate assay, NanB showed little to no activity in cleaving sialic acid from sLe^X, while displaying activity against the sialic acid in sLe^a modified with Neu5Ac. Substitution of this Neu5Ac with Neu5Gc in sLe^a eliminated this activity. This is likely due to preference by NanB for Neu5Ac over Neu5Gc (Figure 3). Similar to NanA, we were unable to measure NanB activity toward against sialic acids on branched glycans, due to inefficient GAL labeling.

Results for NanC were similar to those observed for NanB. In particular, NanC was specific for α 2-3-linked sialic acids, with no measurable activity against either α 2-6- or α 2-8-linked sialic acids. While NanC showed no activity against sLe^X, as observed in the plate assay, NanC did appear to cleave Neu5Ac, though not Neu5Gc, from sLe^a. As for NanA and NanB, NanC activity toward branched glycans could not be measured.

3. Discussion

Overall, results from the microarray assay for the three neuraminidases NanA, NanB, and NanC recapitulate and extend the specificity findings observed in the 96-well microtiter plate assay. We were able to expand the previously reported neuraminidase specificity assay¹³ using glass slides and an alternative chemoselective labeling strategy (GAL), opening up the possibility of analyzing sialylated glycans in a high throughput format to assess neuraminidase substrate specificity. The use of GAL labeling for detection makes it possible to screen a wider array of glycans than might be possible using a Gal/GalNAc-recognizing lectin to detect desialylation; however, the specificity of galactose oxidase³¹

does impose limits on which glycans can be analyzed. Further, although PAL labeling could distinguish sialylation status for the two glycans we printed ourselves (Figure 5), we were unable to identify conditions that enabled such discrimination on the larger commercial RayBiotech array. This result may be due to nonspecific oxidation that can occur in PAL labeling or could reflect differences in glycan printing density or chemistry in the different formats. Indeed, other glycan microarray formats might be more amenable to PAL detection.³²

Sialoglycan microarrays are now available.^{33,34} Indeed, by isolating cellular glycans from desired cells and enriching for sialylated species, it is feasible to design microarrays consisting of cell-specific sialylated glycans, which would be useful in studying how pathogenic neuraminidases interact with specific cells during infection. The method reported here has the potential to provide insight into neuraminidase specificities and how underlying glycans can contribute to that specificity, thereby increasing our understanding of how neuraminidase activity contributes to infection.

4. Experimental

4.1. Materials

Neuraminidases NanA, NanB, and NanC from *Streptococcus pneumoniae* were expressed recombinantly, as described below. Galactose oxidase from *Dactylium dendroides* (50-592-347) was purchased from Worthington Biochemical Corporation, sodium periodate (311448), and aniline (A9880) were purchased from Sigma, and aminooxy-biotin (90113) was purchased from Biotium. Alexa Fluor® 488 C5-Aminooxyacetamide, bis(triethylammonium) salt (aminooxy Alexa Fluor 488; A-30629) was purchased from Thermo Fisher Scientific. Glycan Array 100 (GA-Glycan-100) was purchased from RayBiotech. NHS-coated glass slides for printing glycans, Nexterion® Slide H (1070936), were purchased from Schott. 2-O-(p-nitrophenyl)- α -d- N-acetylneuraminic acid (Neu5Ac-pNP; N502501) and 2-O-(p-nitrophenyl)- α -d-N-glycolylneuraminic Acid (Neu5Gc-pNP; N503725) were purchased from Toronto Chemicals. *Arthrobacter ureafaciens* neuraminidase (AUNA) was purchased from Prozyme (gk80040) and *Salmonella typhimurium* neuraminidase (STNA) was purchased from New England Biolabs (P0728). CMP-sialic acid synthetase from *Neisseria meningitidis* group B (NmCSS), α -2,3-sialyltransferase from *Pasteurella multocida* (Pm2-3ST; S1951), α -2,3-sialyltransferase from *Photobacterium damsela* (Pd2-6ST; S2076), sodium pyruvate (P2256), cytidine 5'-triphosphate disodium salt (C1506), MgCl₂ and ManNAc (A8176) were purchased from Sigma. The N-acetylneuraminic acid (Neu5Ac) aldolase was purchased from Toyobo. 4-Aminophenyl β -d-lactopyranoside (LacAP, EA04486) was purchased from Carbosynth. Ac₄ManNAc was prepared as described.³⁵

4.2. Description of biotinylated glycans

Biotinylated glycans were acquired from the Consortium for Functional Glycomics (CFG), Glycotect, or were synthesized using established protocols. Glycans acquired from the CFG were: Neu5Gc α 2-3Gal β 1-4Glc-Sp1-biotin, Neu5Gc α 2-3Gal β 1-4GlcNAc-Sp1-biotin, Neu5Gc α 2-6Gal β 1-4GlcNAc-Sp1-biotin, KDN α 2-3Gal β 1-4GlcNAc-Sp1-biotin,

Neu5Ac α 2-3Gal β 1-4[Fuca1-3]GlcNAc β -Sp1-biotin, Gal β 1-4[Fuca1-3]GlcNAc β -Sp1-biotin, Neu5Ac α 2-3Gal β 1-3[Fuca1-4]GlcNAc β -Sp1-biotin, Gal β 1-3[Fuca1-4]GlcNAc β -Sp1-biotin, Neu5Ac α 2-3[Gal β 1-3GalNAc β 1-4]Gal β 1-4Glc β -Sp1-biotin, where Sp1 = (CH₂)₂NHCO((CH₂)₅NH)₂. Glycans acquired from Glycotech were: Neu5Ac α 2-3Gal β 1-4Glc-Sp2-biotin, Neu5Ac α 2-6Gal β 1-4Glc-Sp2-biotin, Neu5Ac α 2-3Gal β 1-4GlcNAc-Sp2-biotin, Gal β 1-4Glc-Sp2-biotin, Gal β 1-4GlcNAc-Sp2-biotin where Sp2 = (CH₂)₃NHCO(CH₂)₅NH. Neu5Ac α 2-6Gal β 1-4GlcNAc-Sp2-biotin was prepared chemoenzymatically from Gal β 1-4GlcNAc-Sp2-biotin as described by others.³⁶ Glycan concentration was determined using FluoReporter Biotin Quantitation Kit (Invitrogen F30755) according to the manufacturer's instructions.

4.3. Production of recombinant NanA, NanB, and NanC

NanA, NanB, and NanC were amplified from *Streptococcus pneumoniae* strain TIGR4 genomic DNA, purchased from ATCC (BAA-334D-5) and cloned into the pET28a vector between the NcoI and XhoI restriction sites upstream of the 6xHis tag. Coding gene sequences were confirmed by DNA sequencing. Each resulting plasmid was transformed into *E. coli* BL21(DE3). A 5 mL overnight culture from a single colony was added to 500 mL of kanamycin-containing LB and grown at 37 °C with shaking at 250 rpm to an OD₆₀₀ ~ 0.6 prior to induction with 0.5 mM IPTG for NanA and NanB, or 1 mM IPTG for NanC. Induction proceeded for 20 h at 18 °C with shaking at 250 rpm. After harvesting by centrifugation, cells were lysed in 20 mM sodium phosphate, pH 7.4, containing 500 mM NaCl, 2 mg mL⁻¹ lysozyme (Sigma), 20 mM imidazole (Sigma), and one Complete Protease Inhibitor Cocktail Tablet (Santa Cruz), followed by sonication for 3 × 30 s on ice. Lysates were centrifuged at 20,000g for 1 h at 4 °C. To bind protein, the supernatant was incubated with 100 μ L NiNTA agarose (Qiagen) at 4 °C for 1 h with rotation. After washing with 20 mM sodium phosphate, pH 7.4, containing 500 mM NaCl and 20 mM imidazole, proteins were eluted with 300 mM imidazole in 20 mM sodium phosphate, pH 7.4, and dialyzed into the following buffers: NanA, 100 mM Tris-HCl, pH 8.0, containing 150 mM NaCl; NanB and NanC, 20 mM sodium phosphate, pH 7.0, containing 150 mM NaCl. Protein purity was analyzed by SDS-PAGE followed by Coomassie stain. Specific activities were determined as follows: NanA, 18 μ mol min⁻¹ mg⁻¹ or 18 units mg⁻¹; NanB, 14 μ mol min⁻¹ mg⁻¹ or 14 units mg⁻¹; NanC, 3 μ mol min⁻¹ mg⁻¹ or 3 units mg⁻¹. A unit is defined as the amount of enzyme required to release 1 μ mol of 2'-(4-methylumbelliferone)- α -d-N-acetylneuraminic acid (4-MU NANA) per minute.

4.4. Cell-free 96-well PAL assay

Biotinylated glycans were diluted to 1.1 μ M in neuraminidase activity buffer (100 mM NaOAc, pH 5.6, 100 mM NaCl). Glycan solutions (90 μ L) were aliquoted into individual wells of a 96-well plate and treated with 10 μ L of sialidase at the following final concentrations: NanA (0.93, 2.8, or 8.4 mU), NanB (3.3, 10, or 30 mU), NanC (0.67, 2, or 6 mU). Reactions were incubated at 37 °C for 1 h before transferring reactants to a 96-well streptavidin-coated plate (15503, Pierce) and incubating for 1 h at 4 °C to allow binding of biotinylated glycans to the plate. After incubation, wells were washed three times in 100 μ L PBS containing 0.05 % (v/v) Tween 20 (PBST). For PAL detection, chemoselective oxidation was induced by incubating wells in 100 μ L 0.1 mM NaIO₄ in PBS for 30 min on

ice. Oxidation was quenched by addition of 100 μ L 1 mM glycerol for 10 min on ice. Wells were emptied, washed one time in 100 μ L 1 mM glycerol, and three times in 100 μ L PBST before reaction with a 100 μ L solution containing 10 mM aniline and 10 μ M aminooxy-Alexa Fluor 488 in 100 mM NaOAc, pH 4.5. Ligation was allowed to occur for 2 h at 4 °C. Wells were washed six times in 100 μ L PBST and imaged on a Synergy Neo (BioTek) fluorescence plate reader (ex: 485/20 nm, em: 516/20 nm). For GAL detection, chemoselective oxidation was induced by addition of 100 μ L of 50 U/mL galactose oxidase and incubation at 37 °C for 30 min. Wells were emptied, washed 3 times in 100 μ L PBST, and ligation was induced by addition of a 100 μ L solution containing 10 mM aniline and 10 μ M aminooxy Alexa Fluor 488 in 100 mM NaOAc, pH 4.5 for 2 h at 4 °C. Wells were emptied, washed six times in 100 μ L PBST, and imaged as for PAL labeling. All experiments were performed three times; within each experiment, each treatment was performed in triplicate.

4.5. pNP activity assay

Neu5Ac- α -pNP and Neu5Gc- α -pNP were diluted to a concentration of 1 mM in neuraminidase activity buffer (100 mM NaOAc, pH 5.6, 100 mM NaCl). Enzymes were diluted in activity buffer as follows: NanA (0.47, 0.93, and 1.9 mU), NanB (1.7, 3.3, 6.6 mU), NanC (0.34, 0.67, 1.3 mU). To each well of a 96-well plate was added 50 μ L of 1 mM Neu5Ac- α -pNP or Neu5Gc- α -pNP and 50 μ L of desired neuraminidase. Plates were then incubated at 37 °C for 1 h. Reactions were quenched with 100 μ L NaCO₃, pH 10.7, before reading absorbance on a SpectraMax M5 plate reader (Molecular Devices) at 405 nm. Amount of pNP released was determined using a standard curve of pNP at 1 mM, 0.8 mM, 0.6 mM, 0.4 mM, 0.2 mM, 0.1 mM, 0.05 mM, and 0 mM.

4.6. One-pot chemoenzymatic synthesis of sialylated LacAP

The one-pot chemoenzymatic synthesis of sialylated LacAP compounds was modeled on previously reported protocols.³⁷ Reaction mixtures contained 100 mM Tris-HCl pH 8.8, 20 mM MgCl₂, 7.5 mM ManNAc, 7.5 mM CTP, 40 mM sodium pyruvate, 0.2 mM amino-linked lactose, 2.3 mg mL⁻¹ Neu5Ac aldolase, 0.4 mU NmCSS, and 0.2 mU Pm2-3ST (for Neu5Ac- α -2-3-LacAP) or Pd2-6ST (for Neu5Ac- α -2-6-LacAP) in a final volume of 50 μ L. Reactions were allowed to incubate overnight at 37 °C. Products were purified using high performance anion exchange chromatography and verified by LC-MS/MS.

4.7. Printing of sialylated LacAP onto NHS slides

Glycans sialylated and purified as above were dissolved in 300 mM potassium phosphate buffer, pH 8, with 0.001 % (v/v) Tween 20 to a final concentration of 1 mM and 10 μ L was pipetted into a well of a 384-well source microplate. Glycans were printed onto 1" $\times$ 3" glass NHS-coated slides (Nexterion® Slide H) using an NanoPrint(TM) LM60 Microarrayer (ArrayIt) robotic printer equipped with 946MP3 pins (Telechem International, Inc.). Slides were printed at ambient humidity, typically 50 %. Sample dip time was 2 s and surface dwell time was 0.05 s. Each glycan was printed in a 5 $\times$ 5 square for 25 replicates per sample. After printing, slides were allowed to fully dry before storage at -80 °C until use.

4.8. Labeling of glycans on glass slides using PAL and GAL

For NHS-coated slides prepared as above, slides were allowed to equilibrate to room temperature before washing in 500 mL TBST for 5 min. Slides were then blocked in 50 mL TBST containing 50 mM ethanolamine for 1 h at room temperature. After rinsing slides in water, excess liquid was removed and 100 μ L 5 mU AUNA or 50 U STNA diluted into manufacturer supplied activity buffers were added to slides under coverslips. Slides were incubated at 37 °C for 1 h. After neuraminidase treatment, slides were rinsed in PBS and treated with 4 mL 1 mM NaIO₄ in PBS pH 7.4 for 30 min at 4 °C. Oxidation was quenched with 4 mL 1 mM glycerol in PBS then washed with PBS. Excess liquid was removed from the slide, and then the slide was incubated with 4 mL ligation solution (100 mM NaOAc, pH 4.5, 10 mM aniline, and 10 μ M aminooxy Alexa Fluor 647) for 2 h at 4 °C. Slides were then washed 5 min in 50 mL TBST twice, 10 min in 50 mL TBST once, 10 min in 50 mL TBS once, and once in 50 mL water for 20 min. Excess liquid was removed from slide and slide was imaged using a GenePix 4100A microarray scanner (Axon Instruments).

For PAL labeling of glycan microarrays purchased from RayBiotech, each well of the slides was blocked in 400 μ L manufacturer provided sample diluent for 30 min at room temperature. Excess liquid was removed, slides were rinsed in PBS, and then glycans were oxidized by addition of 400 μ L 1 mM NaIO₄ in PBS for 30 min at 4 °C. Reactions were quenched by addition of 400 μ L 1 mM glycerol for 5 min. Solutions were decanted from wells, and 400 μ L ligation buffer (100 mM NaOAc, pH 4.5, 10 mM aniline, and 20 μ M aminooxy-biotin) was added to each well for 2 h at 4 °C. Solutions were removed, and wells were washed in 800 μ L manufacturer provided Wash Buffer I five times for 5 min each followed by 800 μ L Wash Buffer II twice for 5 min each. After washing, each well was incubated with 400 μ L manufacturer provided Cy3-conjugated streptavidin for 1 h at room temperature in the dark. Wells were washed in 800 μ L Wash Buffer I as above, followed by removal of the gasket and a 15 min wash in 30 mL Wash Buffer I, a 5 min wash in 30 mL Wash Buffer II, and a 5 min wash in 30 mL water. Excess water was removed, and slides were allowed to dry before imaging on a GenePix 4100A microarray scanner (ex: 532, em: 575/35).

For GAL labeling of neuraminidase-treated and untreated glycan microarrays purchased from RayBiotech, slides were blocked in 400 μ L manufacturer provided sample diluent for 30 min at room temperature. Wells were washed twice in 800 μ L PBS followed treatment with 1.9 mU NanA, 6.6 mU NanB, 1.3 mU NanC, or nothing in 400 μ L 100 mM NaOAc, pH 5.6, 100 mM NaCl, and 0.1 % (v/v) BSA for 1 h at 37 °C. Slides were rinsed twice in PBS, pH 6.7, followed by incubation in 400 μ L of the GAL reaction (50 U/mL galactose oxidase, 10 mM aniline, 200 μ M aminooxy biotin in PBS pH 6.7), covered, for 30 min at 37 °C. Solutions were decanted, and slides were washed, conjugated with a Cy3 dye, and imaged as above for PAL labeled slides. Experiments were performed in triplicate.

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Highlights

- Desialylation of glycans by neuraminidases can be detected by chemoselective labeling.
- Chemoenzymatic labeling of unsialylated glycans can be performed on glass slides.
- Three pneumococcal neuraminidases exhibit distinct glycan specificities.

Figure 1.
Structures of glycans used in microtiter plate assay.

Figure 2. Substrate specificity of pneumococcal neuraminidases NanA, NanB, and NanC evaluated by PAL labeling of glycans in 96-well microtiter plates
Glycans were treated with NanA (A), NanB (B), or NanC (C) neuraminidase, then fluorescently labeled with PAL. Fluorescence was measured and compared to values for glycans not treated with neuraminidase. Data presented are percent sialylation remaining after neuraminidase treatment for increasing amounts of enzyme. Error bars represent the standard deviation of three replicates in one experiment. Experiments were performed in triplicate. Panel C is adapted with permission from RB Parker, JE McCombs, and JJ Kohler, “Sialidase specificity determined by chemoselective modification of complex sialylated glycans,” *ACS Chemical Biology*, 7:1509–14. Copyright 2012 American Chemical Society.

Figure 3. Relative activity of pneumococcal neuraminidases toward Neu5Ac and Neu5Gc
(A) Neu5Gc differs from Neu5Ac by the addition of an –OH group (indicated in red) on its N-acyl side chain. (B) Release of pNP from Neu5Ac-α-pNP (black bars) and Neu5Gc-α-pNP (white bars) treated with NanA, NanB, and NanC was measured. Error bars represent standard deviation of 3 replicates.

Figure 4. Pneumococcal neuraminidase specificity can be assessed by GAL labeling

Neu5Ac-α2-3-Lac and Neu5Ac-α2-6-Lac were treated with pneumococcal neuraminidases NanA (A), NanB (B), or NanC (C), then fluorescently labeled with PAL. Fluorescence was measured and compared to values for glycans not treated with neuraminidase. Data are the percent sialylation remaining after neuraminidase treatment for increasing amounts of enzyme. Error bars represent the standard deviation of three replicates in one experiment. Experiments were performed in triplicate.

Figure 5. Neuraminidase specificity detected using PAL labeling on glass slides

NHS glass slides printed with Neu5Ac α 2-3-LacAP and Neu5Gc α 2-3-LacAP were treated with no neuraminidase (NA), the non-specific AUNA, or the α 2-3-specific STNA. **(A)** Images of slides printed with glycans and treated as indicated. Background subtraction of images was performed in ImageJ. **(B)** Quantification of the amount of sialic acid remaining as determined by fluorescence intensity of PAL labeling after the indicated treatment. Error bars represent the standard deviation of 25 spots of glycan printed on the slide.

Figure 6. GAL labeling on glass slides identifies sialylated glycans more accurately than PAL labeling

(A) Schematic of the RayBiotech glass slide microarray used in this study. Glycans terminating in sialic acids are shaded in purple and glycans terminating in Gal or GalNAc residues are shaded in orange. Branched glycans that have both terminal sialic acid and Gal/GalNAc are shaded in red. Positive (+) and negative (-) controls printed on the slide are also shown. (B) Representative image of a slide labeled using PAL. Background subtraction of images was performed in ImageJ. (C) Fluorescence measured for each glycan spot of a PAL-labeled slide imaged as in (B). Sialylated glycans, expected to be labeled by PAL, are indicated in purple. Black lines above graph represent each row of printed glycans. Graph represents the results from one slide. Error bars are the standard deviation of four replicates of the indicated glycan. Experiment was performed at least three times. (D) Representative image of a slide labeled using GAL. Background subtraction of images was performed in ImageJ. (E) Fluorescence measured for each glycan spot of a GAL-labeled slide imaged as in (D). Linear glycans terminating in Gal or GalNAc residues are indicated in orange. Glycans terminating in Gal or GalNAc that have multiple branches are indicated by orange bars with black stripes. Graph represents the results from one slide. Error bars are the standard deviation of four replicates of the indicated glycan. Experiment was performed at least three times.

Figure 7. Pneumococcal neuraminidase glycan specificity measured by GAL labeling of glycan microarray

Glass slides printed with a glycan microarray were treated with a pneumococcal neuraminidase (colored bar) or not (white bar) and fluorescently labeled using GAL. Slides were imaged and fluorescence was measured and compared to the equivalent unsialylated glycan for each glycan spot. Data are presented as percent sialylation remaining after treatment with (A) NanA, (B) NanB, and (C) NanC. Glycans are indicated by number and labeled at the bottom of the figure. Error bars represent the standard deviation of four replicates of the indicated glycan on a single slide. Experiment was performed in triplicate.