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Enhanced crosslinking of diazirine-modified sialylated glycoproteins enabled through profiling of sialidase specificities

Janet E. McCombs[†], **Chunxia Zou**[§], **Randy B. Parker**[†], **Christopher W. Cairo**[§], and **Jennifer J. Kohler**[†,*]

[†]Department of Biochemistry, The University of Texas Southwestern Medical Center, Dallas, TX 75390-9038, USA

[§]Alberta Glycomics Centre, Department of Chemistry, University of Alberta, Edmonton, Alberta T6G 2G2, Canada

Abstract

Sialic acid-mediated interactions play critical roles on the cell surface, providing impetus for the development of methods to study this important monosaccharide. In particular, photocrosslinking sialic acids incorporated onto cell surfaces have allowed covalent capture of transient interactions between sialic acids and sialic acid-recognizing proteins via crosslinking. However, natural sialic acids also present on the cell surface compete with photocrosslinking sialic acids in binding events, limiting crosslinking yields. In order to improve the utility of one such photocrosslinking sialic acid, SiaDAz, we examined a number of sialidases, enzymes that remove sialic acids from glycoconjugates, to find one that would cleave natural sialic acids but remain inactive toward SiaDAz. Using this sialidase, we improved SiaDAz-mediated crosslinking of an anti-sialyl Lewis X antibody and of endoglin. This protocol can be applied generally to sialic acid-mediated interactions and will facilitate identification of sialic acid binding partners.

Sialic acids are involved in the regulation of a multitude of interactions.[1] Despite these essential roles, identifying sialic acid-dependent interactions remains difficult due to their transient nature. As a solution, we and others have reported use of photocrosslinking sialic acid analogs that can be used to covalently capture sialic acid-dependent interactions.[2-4] These analogs are metabolically incorporated into cellular glycoconjugates where they can be used to study sialic acid-dependent interactions in a native setting. To introduce sialic acid bearing the diazirine photocrosslinking group on the N-acyl side chain, we culture cells with a corresponding cell-permeable N-acyl-modified N-acetyl-D-mannosamine (ManNAc) analog. Previously, we showed that mammalian cells can metabolize a cell-permeable, diazirine-modified ManNAc analog, Ac_4ManNDAz(2me), to diazirine-modified sialic acid, SiaDAz(2me), and add SiaDAz(2me) to glycoconjugates destined for the cell surface.[3,5,6]

*jennifer.kohler@utsouthwestern.edu.

Supporting Information

Detailed descriptions of methods, characterization of chemoenzymatically synthesized sialylated and SiaDAz-ylated glycans, controls for labeling in cell-free and cell-based assays, characterization of cells used in crosslinking experiments, experiments performed in a second cell line showing enhanced SiaDAz-mediated crosslinking upon STNA treatment, and LC-MS/MS analysis of crosslinking anti-sLe^X. This material is available free of charge via the Internet at http://pubs.acs.org.

Similarly, cells can metabolize a mannosamine with a longer linker separating the pyranose and the diazirine, Ac_4ManNDAz(4me), but this process is less efficient and little SiaDAz(4me) appears on the surface of cells.[6] Even though production of SiaDAz(2me) is more efficient, SiaDAz(2me) does not replace all of the natural sialic acid, Neu5Ac, on the surface of mammalian cells. We have observed a range of incorporation efficiencies, from favorable cases, where about 65 % of cell surface Neu5Ac is replaced by SiaDAz(2me), to several cell lines where cell surface SiaDAz(2me) is undetectable.[7] Natural Neu5Ac competes for binding to sialic acid-recognizing proteins, which may reduce the overall efficiency of SiaDAz-mediated crosslinking. A method to selectively remove cell surface Neu5Ac while leaving SiaDAz-modified glycoconjugates intact would be predicted to enhance production of SiaDAz-crosslinked complexes.

Sialidases, also known as neuraminidases, are enzymes that remove sialic acids from glycoconjugates. Both bacteria and viruses produce extracellular sialidases that can remove sialic acids from mammalian host cells,[8,9] and the human genome also encodes at least four sialidases,[10] with a range of substrate specificities.[11] Previous studies have shown that substitutions on the N-acyl side chain of sialic acid can affect sialidase activity *in vitro*,[12-19] although these effects are isoenzyme specific. In addition, while sialic acid analogs bearing larger N-acyl groups are generally poor substrates for purified sialidases, some secreted sialidases appear to be more tolerant of these substitutions in metabolically labeled cells.[20] Thus, addition of diazirine to the N-acyl side chain of sialic acid could result in altered activity of sialidases, such that certain sialidases may be able to selectively cleave Neu5Ac, but not SiaDAz(2me) or SiaDAz(4me), from glycoconjugates.

In this work, we screened a panel of both human and bacterial sialidases in a cell-free microwell plate assay[16] to identify sialidases that are active toward Neu5Ac, but not SiaDAz(2me) or SiaDAz(4me). Using a cell-based assay, we found that *Salmonella typhimurium* neuraminidase (STNA) can remove Neu5Ac from cell surfaces, while leaving SiaDAz(2me)-modified glycoconjugates intact. Finally, we demonstrated the utility of this discriminating sialidase by treating cells with STNA, which enhanced SiaDAz(2me)-dependent crosslinking.

RESULTS AND DISCUSSION

Chemoenzymatic synthesis of SiaDAz-labeled glycans

To test sialidase specificity against SiaDAz(2me) and SiaDAz(4me) in our cell-free microwell plate assay, we first synthesized SiaDAz-labeled glycans to use as sialidase substrates. We chose to perform an established one-pot chemoenzymatic reaction that has proved useful for synthesis of diverse glycans with a variety of natural and unnatural sialic acids.[21] In this method, the sialic acid biosynthetic precursor ManNAc or a ManNAc analog is incubated with a non-sialylated acceptor glycan and the enzymes Neu5Ac aldolase, CMP-sialic acid synthetase, and an α2-3-sialyltransferase, in order to produce the desired sialylated glycan product (Figure 1). We chose biotinylated N-acetyl-D-lactosamine (LacNAc-biotin) as our acceptor glycan, and used ManNAc or a diazirine-containing analog, ManNDAz(2me) or ManNDAz(4me), to produce LacNAc-biotin modified with α2-3-linked Neu5Ac, SiaDAz(2me), or SiaDAz(4me). (Although SiaDAz(4me) is not efficiently

incorporated into cell surface glycoconjugates,[6] we thought that examining the SiaDAz(4me)-LacNAc-biotin substrate in our cell-free assay could provide additional information about the molecular basis of sialidase specificity.) The glycan products were separated by HPLC to identify non-sialylated, sialylated and SiaDAz-ylated glycans (Supplementary Figure 1), which were isolated and characterized by mass spectrometry. Observed *m/z* ratios were consistent with expected products (Supplementary Table 1; Supplementary Figures 2–4), indicating that the one-pot chemoenzymatic approach is suitable for synthesis of both SiaDAz(2me)- and SiaDAz(4me)-containing glycans. While the reaction using the natural sialic acid precursor ManNAc was nearly complete (~97 % sialylated LacNAc-biotin), use of ManNDAz(2me) or ManNDAz(4me) yielded ~9 % and 20 % SiaDAz-ylated product, respectively, suggesting the diazirine modification resulted in impaired activity of at least one enzyme in the one-pot reaction. Nonetheless, yields were sufficient for subsequent sialidase assays.

Specificity of sialidases against SiaDAz species

SiaDAz-ylated glycans in hand, we measured the activity of a panel of bacterial and mammalian sialidases against LacNAc-biotin modified with Neu5Ac, SiaDAz(2me), or SiaDAz(4me). We utilized an assay we reported previously,[16] in which biotinylated and sialylated glycans are treated with sialidase before immobilization in individual wells of streptavidin-coated 96-well plates. Remaining sialic acids are then labeled with Alexa Fluor 488 using the chemoselective periodate oxidation and aniline-catalyzed oxime ligation (PAL).[22] For the panel, we chose five bacterial enzymes, including three pneumococcal neuraminidases (NanA, NanB, and NanC),[23] an *Arthrobacter ureafaciens* neuraminidase (AUNA),[24] and a *Salmonella typhimurium* LT2 neuraminidase (STNA).[25] In addition, we examined three human sialidases: NEU2,[26] NEU3[27] and NEU4.[28]

We confirmed that PAL could label SiaDAz-containing glycans, as upon conjugation to aminooxy-Alexa Fluor 488, SiaDAz(2me)-LacNAc-biotin and SiaDAz(4me)-LacNAc-biotin yielded higher fluorescence values than unsialylated LacNAc-biotin (Supplementary Figure 5). Next, we used our cell-free microwell plate assay to measure the activity of sialidases toward α2-3-linked Neu5Ac. As expected,[12,23,29,30] we found that all five bacterial enzymes tested were active against α2-3-linked Neu5Ac (Figure 2a). Two of the mammalian sialidases, NEU2 and NEU4, showed good to moderate activity against Neu5Ac, respectively, but NEU3 showed no activity against Neu5Ac in this cell-free assay (Figure 2a). The lack of detectable activity for NEU3 is consistent with its reported preference for ganglioside-like substrates.[14,31] We then examined the ability of the panel of sialidases to remove diazirine-containing sialic acid analogs, SiaDAz(2me) (Figure 2b) and SiaDAz(4me) (Figure 2c). Two of the sialidases from pneumococcus, NanB and NanC, were able to cleave diazirine-containing sialic acids independent of linker length. While NEU2 was also able to cleave SiaDAz(2me) from LacNAc-biotin, it showed little to no activity against SiaDAz(4me), suggesting the longer linker may interfere with its catalytic activity. Conversely, while NanA showed no activity against SiaDAz(2me), it displayed moderate activity against SiaDAz(4me), suggesting that positioning the diazirine further from the pyranose ring may be more favorable for NanA enzyme activity. The remaining sialidases, AUNA, STNA, NEU3 and NEU4, showed no detectable activity against either

SiaDAz(2me) or SiaDAz(4me). We therefore predicted that these four sialidases along with NanA would be the most useful in enhancing crosslinking in cells through selective removal of Neu5Ac.

Activity of sialidases in the cellular environment

We next wanted to ensure that sialidases inactive toward SiaDAz would remain so in the complex cellular environment, which contains a wide array of sialylated glycans. For this analysis, we used BJAB K20 cells, which do not produce active UDP-GlcNAc 2-epimerase/ManNAc kinase (GNE). As a result, BJAB K20 cells cannot produce sialic acid in the absence of exogenously added ManNAc.[32] When grown in serum-free conditions where there is no sialic acid to scavenge, these cells are unable to synthesize sialylated glycoconjugates.[32] Supplementation of serum-free media with peracetylated ManNAc (Ac_4ManNAc), Ac_4ManNDAz(2me), or Ac_4ManNDAz(4me) enables incorporation of only the corresponding sialic acid (Neu5Ac, SiaDAz(2me), or SiaDAz(4me)) into glycoconjugates.[6] In this way, BJAB K20 cells provide an ideal platform in which to study sialidase specificity in the context of the cell. We tested the ability of BJAB K20 cells to metabolize Ac_4ManNDAz(4me), even though incorporation efficiencies are known to be poor,[6] to determine whether enough SiaDAz(4me) would be expressed to measure sialidase specificity. While addition of Ac_4ManNAc or Ac_4ManNDAz(2me) each produced considerable α2-3 sialylation on cell surfaces, as determined by binding of the α2-3-specific lectin MAL II, we were unable to detect SiaDAz(4me) on the cell surface (Supplementary Figure 6). This results suggests that incorporation of SiaDAz(4me) into cell surface glycans is inefficient, in line with our previous studies in Jurkat cells.[6] However, it is also possible that the longer linker length interferes with the ability of MAL II to detect SiaDAz(4me). Regardless, we were unable to further examine sialidase activity toward cells cultured with Ac_4ManNDAz(4me) due to the lack of MAL II binding. Importantly, for cells cultured with Ac_4ManNAc or Ac_4ManNDAz(2me), nearly 100 % of the α2-3-linked sialic acids on the cell surface were produced from exogenously added mannosamines (Figure 3a).

As binding of MAL II to sialosides could be affected by the diazirine substituent, we used this lectin only for comparative analyses between cells cultured with the same precursor to assess whether different sialidases could cleave Neu5Ac and SiaDAz(2me) from the cell surfaces. Cells expressing the desired sialic acid species were treated with sialidase, labeled using MAL II, and analyzed by flow cytometry (Figure 3b and 3c). NanB and NanC were active against SiaDAz(2me), cleaving nearly all of Neu5Ac and SiaDAz(2me) from the cell surface, consistent with the cell-free assay. NEU2 also performed as expected, displaying activity against both Neu5Ac and SiaDAz(2me). NEU3, which had no activity in the cell-free assay, showed moderate activity against both Neu5Ac and SiaDAz(2me) on cell surfaces. NEU3 activity on cells may be a result of incorporation of SiaDAz(2me) into ganglioside substrates.[33,34] Interestingly, we found that NanA, AUNA, and NEU4, each of which showed no detectable activity toward SiaDAz(2me) in our cell-free assay, cleaved between 50 and 70 % of SiaDAz(2me) on cells surfaces. Of all the sialidases tested, only STNA was completely inactive toward SiaDAz(2me) on BJAB K20 cell surfaces, with 100 % of SiaDAz(2me) remaining after STNA treatment. We therefore predicted STNA would

be an ideal sialidase to remove naturally occurring sialic acids, but not SiaDAz(2me), from cell surfaces expressing a mixed population of sialic acids.

STNA amplifies SiaDAz-mediated crosslinking

To determine whether removing natural sialic acids from cell surfaces would enhance crosslinking, we first assessed crosslinking of cell surface sialylated proteins to an antibody that recognizes the α2-3-sialylated glycan epitope sialyl Lewis X (sLe^X) (Supplementary Figure 7a). We selected a brain endothelial cell line, hCMEC/D3,[35] and a colon epithelial cell line, Colo205, which incorporate SiaDAz into cell-surface glycans at ~ 56 % and ~ 4 % efficiency, respectively (Supplementary Figure 8). Using flow cytometry, we confirmed that the anti-sLe^X antibody binds to sialylated glycoconjugates on the surfaces of both hCMEC/D3 and Colo205 cells (Supplementary Figure 7b and c). Upon treatment with STNA or NanB, we saw a decrease in anti-sLe^X antibody binding for both cell types, indicating we could control cell surface sLe^X levels through sialidase treatment. Interestingly, Colo205 cells displayed a significant amount of binding of antibody after STNA treatment, suggesting STNA does not cleave all glycans recognized by anti-sLe^X antibody on Colo205 cells.

We hypothesized that STNA would remove Neu5Ac, but not SiaDAz(2me), from hCMEC/D3 and Colo205 cell surfaces, resulting in increased interactions between anti-sLe^X antibody and SiaDAz(2me) (Figure 4a). To test this idea, cells cultured with Ac_4ManNDAz(2me) for 72 h were treated with STNA or NanB before incubation with anti-sLe^X antibody and crosslinking under UV. We performed an immunoblot on the lysates from these cells using anti-IgM to recognize uncrosslinked and crosslinked anti-sLe^X antibody. Overall, we observed more reactivity in multiple regions of the blot when both Ac_4ManNDAz(2me) was added and UV light was applied, indicative of crosslinking between anti-sLe^X antibody and SiaDAz-modified glycoproteins. We focused attention on a high molecular weight band that was observed under crosslinking conditions, but absent from the control lanes (red asterisk in Figure 4b and Supplementary Figure 9a). Quantification of this band allowed us to determine that pretreatment with NanB essentially eliminated crosslinking in both cell lines, consistent with our observation that NanB can efficiently cleave SiaDAz(2me) from cell surfaces (Figure 4c and Supplementary Figure 8b). We also quantified the effect of STNA pre-treatment on the intensity of the high molecular weight band. For hCMEC/D3 cells, treatment with STNA resulted in a ~1.6-fold enhancement of the crosslinking band intensity when normalized to actin (Figure 4b and 4c). Similarly, Colo205 cells showed an STNA-dependent increase in anti-sLe^X antibody crosslinking (Supplementary Figure 9), though to a lesser extent than in hCMEC/D3 cells. We hypothesize that the less pronounced STNA effect in Colo205 cells is due to the inability of STNA to remove all antigens recognized by anti-sLe^X antibody (Supplementary Figure 7c). Together, these data indicate that crosslinking of SiaDAz-ylated molecules on cell surfaces can be enhanced by pre-treatment with a sialidase that removes Neu5Ac, but discriminates against SiaDAz.

We hypothesized the enhanced anti-sLe^X crosslinking we observed with STNA treatment was due to reduced competition for antibody binding by Neu5Ac-containing sialosides. We

therefore predicted that the STNA-induced increase in crosslinking would be eliminated by adding excess anti-sLe^X antibody. To test this idea, we performed the anti-sLe^X crosslinking at five different antibody concentrations. As predicted, we found that the STNA-dependent increase in crosslinking was apparent at low antibody concentrations, but overcome at higher antibody concentration (Supplementary Figure 10). Thus, STNA treatment offers a useful approach when the amount of the sialic acid-recognizing protein is limited, such as an antibody in short supply.

Enhanced crosslinking increases MS spectral counts

Next, we examined whether the STNA-dependent increase in anti-sLe^X antibody crosslinking translated into improved detection of crosslinked proteins by mass spectrometry analysis. Similar to above, we performed crosslinking using a biotinylated anti-sLe^X antibody. hCMEC/D3 cells were then lysed and crosslinked complexes were isolated on streptavidin-agarose before analysis by LC-MS/MS. The relative abundance of specific glycoproteins in the two samples was determined by a label-free normalized spectral index quantitation method;[36] results are summarized in Supplementary Table 2. Abundance of proteins enriched in crosslinked samples was compared between STNA-treated cells and -untreated cells, and results are presented in Figure 4d. Detected levels of cell surface glycoproteins were consistently higher in samples treated with STNA, suggesting that STNA treatment has the potential to enhance the utility of SiaDAz in the identification of sialic acid-dependent binding interactions. The validity of the LC-MS/MS results is supported by the appearance of basigin (CD147), a protein known to be modified with sLe^X in endothelial cell types.[37] Similarly, CD59 appeared in our LC-MS/MS results and an antibody array screening method suggested that sLe^X is found on CD59 from human plasma.[38] We also identified proteins, such as podocalyxin (PODXL) and activated leukocyte cell adhesion molecule (ALCAM) not previously known to be modified by sLe^X, but whose biological activities are consistent with sLe^X modification. PODXL expression correlates with cancer aggressiveness,[39,40] and functions as an E- and L-selectin ligand through sialofucosylation modification on O-glycans,[41] while ALCAM is associated with tumor invasiveness.[42]

Improved crosslinking of endogenous proteins

The most significant potential application of the SiaDAz crosslinking method is to capture interactions among endogenously expressed proteins. In implementing this approach, it is important to recognize that using SiaDAz crosslinking in conjunction with STNA treatment could result in significant changes in cell surface glycoconjugate assemblies (i.e. the glycosynapse[43]) due to removal of natural sialic acids. Despite this caveat, information gained through this discovery method could provide initial insights into glycoprotein binding interactions, which could be further validated by other, less perturbing, methods. Therefore, we next examined whether STNA treatment could enhance crosslinking of endoglin, a cell surface glycoprotein endogenously expressed in hCMEC/D3 cells. Immunoblot analysis of SiaDAz crosslinked lysates using an anti-endoglin antibody revealed a distinct higher molecular weight band that was only present when Ac_4ManNDAz(2me) was included and UV was applied. The intensity of this SiaDAz-crosslinked endoglin was increased with STNA treatment (Figure 4e and f). Similar results

were observed when the experiment was performed at 4 °C (Supplementary Figure 11), suggesting that altered membrane trafficking upon sialidase treatment was not the dominant mechanism mediating the increase in crosslinking. Thus, STNA treatment can be used to enhance SiaDAz crosslinking of an endogenous protein, where manipulation of expression levels may be undesirable.

In summary, we report that crosslinking of SiaDAz(2me)-modified glycoconjugates can be enhanced by pre-treating cells with a commercially available sialidase, STNA, that removes Neu5Ac, but not SiaDAz(2me). While we have shown previously that SiaDAz(2me)-modified glycoproteins and glycolipids can be crosslinked to their binding partners, *de novo* identification of those binding partners remains a challenge because of difficulties associated with isolating sufficient crosslinked material for mass spectrometry-based proteomics analysis. For example, in experiments performed by Paulson and co-workers with a different photocrosslinking sialic acid analog, the inability to isolate sufficient crosslinked material necessitated use of a non-physiological scheme to perform the initial proteomics analysis.[44] In examination of cells cultured with Ac_4ManNDAz(2me), we have observed significant amounts of Neu5Ac on the cell surface; indeed, the amount of cell surface SiaDAz(2me) is near the lower limit of detection in some cell lines.[7] On the one hand, limited incorporation of SiaDAz(2me) is desirable, as this minimizes any perturbations caused by the unnatural substituent. But on the other hand, abundant cell surface Neu5Ac competes with cell surface SiaDAz(2me) for binding to sialic acid recognizing proteins, thereby limiting the maximal amount of crosslinking that can be achieved. Here we show that removal of cell surface Neu5Ac enhances the yield of SiaDAz(2me) crosslinked material in two cell lines with dramatically different levels of SiaDAz(2me) incorporation. We were pleased to observe that STNA treatment enhanced crosslinking even in hCMEC/D3 cells, which have robust SiaDAz(2me) incorporation. The sialidase specificity data reported here (Figs. 2 and 3) suggest that the STNA-dependent crosslinking enhancement might be due to the removal of competing Neu5Ac ligands, but do not exclude the possibility that additional mechanisms are at work. Indeed, it is important to note that STNA treatment could itself perturb endogenous interactions. Despite these caveats, we predict that this simple method could be applied to a variety of cell lines and interactions for preliminary identification of binding partners, to be confirmed by other methods.

In the course of these experiments, we gained insight into the specificity of sialidases. Using our cell-free assay, we were able to observe that sialidases exhibit a range of abilities to discriminate between Neu5Ac and sialic acid modified with unnatural N-acyl substituents. Indeed, our observation that NanB can remove SiaDAz(2me) and SiaDAz(4me) while STNA cannot is consistent with previous findings for these same enzymes acting on other unnatural N-acyl-modified sialic acids.[13] Other sialidases distinguished between the two SiaDAz analogs based on their linker length: NEU2 was only able to remove the smaller analog, SiaDAz(2me), while NanA preferred the analog SiaDAz(4me) with a longer linker separating the diazirine from the pyranose ring. Interestingly, sialidases with broader linkage specificity (*e.g.* NanA and AUNA) exhibited more restricted tolerance of N-acyl substitutions. The results presented here suggest that sialidases are surprisingly sensitive to the exact structure of unnatural substituents, suggesting that sialidase specificity against

novel unnatural sialic acids should always be tested experimentally, and not assumed based on comparison to related structures. Overall, different sialidases exhibit distinct abilities to discriminate among subtle changes in N-acyl-substituted sialic acid analogs.

Methods

Crosslinking in cells

hCMEC/D3 cells or Colo205 cells were cultured for 72 h in media containing ethanol or a final concentration of 100 μM Ac_4ManNDAz(2me). Cells were treated with STNA, NanB, or buffer for 1 h at 37 °C or 4 °C as indicated. For antibody crosslinking, cells were then incubated with 5 μg mL^{-1} dilution of anti-sLe^X antibody (CD15s, BD Biosciences) in PBS containing 0.1 % (w/v) BSA for 1 h, rotating at 4 °C. In titration experiments, the antibody concentration used was 20, 10, 5, 2.5, and 1.25 μg mL^{-1}. For both antibody and endoglin crosslinking, cells were irradiated with UV (365 nm, 20 W) for 30 min and 15 min, respectively, on ice to induce crosslinking. Cells were harvested at 1000*g* for 3 min, washed, and resuspended in RIPA lysis buffer (50 mM Tris HCl, pH 8.0, 150 mM NaCl, 1 % NP-40, 0.5 % sodium deoxycholate, 0.1 % SDS) containing protease inhibitor cocktail added just before use. Concentration of supernatant was determined by BCA assay (Pierce). A total of 10 μg protein was loaded onto a 7.5 % gel and transferred to a PVDF membrane at 87 mA for 16 h at 4 °C.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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References

(1). Varki A. Sialic acids in human health and disease. Trends Mol. Med. 2008; 14:351–360. [PubMed: 18606570]

(2). Han S, Collins BE, Bengtson P, Paulson JC. Homomultimeric complexes of CD22 in B cells revealed by protein-glycan cross-linking. Nat. Chem. Biol. 2005; 1:93–97. [PubMed: 16408005]

(3). Tanaka Y, Kohler JJ. Photoactivatable crosslinking sugars for capturing glycoprotein interactions. J. Am. Chem. Soc. 2008; 130:3278–3279. [PubMed: 18293988]

(4). Feng L, Hong S, Rong J, You Q, Dai P, Huang R, Tan Y, Hong W, Xie C, Zhao J, Chen X. Bifunctional unnatural sialic acids for dual metabolic labeling of cell-surface sialylated glycans. J. Am. Chem. Soc. 2013; 135:9244–9247. [PubMed: 23725545]

(5). Bond MR, Zhang H, Vu PD, Kohler JJ. Photocrosslinking of glycoconjugates using metabolically incorporated diazirine-containing sugars. Nat. Protoc. 2009; 4:1044–1063. [PubMed: 19536272]

(6). Bond MR, Zhang H, Kim J, Yu S-H, Yang F, Patrie SM, Kohler JJ. Metabolism of diazirine-modified N-acetylmannosamine analogues to photo-cross-linking sialosides. Bioconjug. Chem. 2011; 22:1811–1823. [PubMed: 21838313]

(7). Pham ND, Fermaintt CS, Rodriguez AC, McCombs JE, Nischan N, Kohler JJ. Cellular metabolism of unnatural sialic acid precursors. Glycoconj. J. 2015; 32:515–529. [PubMed: 25957566]

(8). Vimr ER, Kalivoda KA, Deszo EL, Steenbergen SM. Diversity of microbial sialic acid metabolism. Microbiol. Mol. Biol. Rev. 2004; 68:132–153. [PubMed: 15007099]

(9). Itzstein, von M. The war against influenza: discovery and development of sialidase inhibitors. Nat. Rev. Drug Discov. 2007; 6:967–974. [PubMed: 18049471]

(10). Miyagi T, Yamaguchi K. Mammalian sialidases: physiological and pathological roles in cellular functions. Glycobiology. 2012; 22:880–896. [PubMed: 22377912]

(11). Smutova V, Albohy A, Pan X, Korchagina E, Miyagi T, Bovin N, Cairo CW, Pshezhetsky AV. Structural basis for substrate specificity of mammalian neuraminidases. PLoS ONE. 2014; 9:e106320. [PubMed: 25222608]

(12). Chokhawala HA, Yu H, Chen X. High-throughput substrate specificity studies of sialidases by using chemoenzymatically synthesized sialoside libraries. Chembiochem. 2007; 8:194–201. [PubMed: 17195254]

(13). Cao H, Li Y, Lau K, Muthana S, Yu H, Cheng J, Chokhawala HA, Sugiarto G, Zhang L, Chen X. Sialidase substrate specificity studies using chemoenzymatically synthesized sialosides containing C5-modified sialic acids. Org. Biomol. Chem. 2009; 7:5137–5145. [PubMed: 20024109]

(14). Sandbhor MS, Soya N, Albohy A, Zheng RB, Cartmell J, Bundle DR, Klassen JS, Cairo CW. Substrate recognition of the membrane-associated sialidase NEU3 requires a hydrophobic aglycone. Biochemistry. 2011; 50:6753–6762. [PubMed: 21675735]

(15). Li Y, Cao H, Yu H, Chen Y, Lau K, Qu J, Thon V, Sugiarto G, Chen X. Identifying selective inhibitors against the human cytosolic sialidase NEU2 by substrate specificity studies. Mol. Biosyst. 2011; 7:1060–1072. [PubMed: 21206954]

(16). Parker RB, McCombs JE, Kohler JJ. Sialidase specificity determined by chemoselective modification of complex sialylated glycans. ACS Chem. Biol. 2012; 7:1509–1514. [PubMed: 22704707]

(17). Khedri Z, Muthana MM, Li Y, Muthana SM, Yu H, Cao H, Chen X. Probe sialidase substrate specificity using chemoenzymatically synthesized sialosides containing C9-modified sialic acid. Chem. Commun. (Camb.). 2012; 48:3357–3359. [PubMed: 22361713]

(18). Khedri Z, Li Y, Muthana S, Muthana MM, Hsiao C-W, Yu H, Chen X. Chemoenzymatic synthesis of sialosides containing C7-modified sialic acids and their application in sialidase substrate specificity studies. Carbohydr. Res. 2014; 389:100–111. [PubMed: 24680514]

(19). Zamora CY, d'Alarcao M, Kumar K. Fluorogenic sialic acid glycosides for quantification of sialidase activity upon unnatural substrates. Bioorg. Med. Chem. Lett. 2013; 23:3406–3410. [PubMed: 23639536]

(20). Zamora CY, Ryan MJ, d'Alarcao M, Kumar K. Sialidases as regulators of bioengineered cellular surfaces. Glycobiology. 2015; 25:784–791. [PubMed: 25795684]

(21). Yu H, Chokhawala HA, Huang S, Chen X. One-pot three-enzyme chemoenzymatic approach to the synthesis of sialosides containing natural and non-natural functionalities. Nat. Protoc. 2006; 1:2485–2492. [PubMed: 17406495]

(22). Zeng Y, Ramya TNC, Dirksen A, Dawson PE, Paulson JC. High-efficiency labeling of sialylated glycoproteins on living cells. Nat. Methods. 2009; 6:207–209. [PubMed: 19234450]

(23). Xu G, Kiefel MJ, Wilson JC, Andrew PW, Oggioni MR, Taylor GL. Three Streptococcus pneumoniae sialidases: Three different products. J. Am. Chem. Soc. 2011; 133:1718–1721. [PubMed: 21244006]

(24). Iwamori M, Kaido T, Iwamori Y, Ohta Y, Tsukamoto K, Kozaki S. Involvement of the C-terminal tail of Arthrobacter ureafaciens sialidase isoenzyme M in cleavage of the internal sialic acid of ganglioside GM1. J. Biochem. 2005; 138:327–334. [PubMed: 16169883]

(25). Hoyer LL, Roggentin P, Schauer R, Vimr ER. Purification and properties of cloned Salmonella typhimurium LT2 sialidase with virus-typical kinetic preference for sialyl alpha 2-3 linkages. J. Biochem. 1991; 110:462–467. [PubMed: 1769974]

(26). Monti E, Preti A, Rossi E, Ballabio A, Borsani G. Cloning and characterization of NEU2, a human gene homologous to rodent soluble sialidases. Genomics. 1999; 57:137–143. [PubMed: 10191093]

(27). Monti E, Bassi MT, Papini N, Riboni M, Manzoni M, Venerando B, Croci G, Preti A, Ballabio A, Tettamanti G, Borsani G. Identification and expression of NEU3, a novel human sialidase associated to the plasma membrane. Biochem. J. 2000; 349:343–351. [PubMed: 10861246]

(28). Monti E, Bassi MT, Bresciani R, Civini S, Croci GL, Papini N, Riboni M, Zanchetti G, Ballabio A, Preti A, Tettamanti G, Venerando B, Borsani G. Molecular cloning and characterization of NEU4, the fourth member of the human sialidase gene family. Genomics. 2004; 83:445–453. [PubMed: 14962670]

(29). Gut H, King SJ, Walsh MA. Structural and functional studies of Streptococcus pneumoniae neuraminidase B: An intramolecular trans-sialidase. FEBS Lett. 2008; 582:3348–3352. [PubMed: 18775704]

(30). Parker RB, Kohler JJ. Regulation of intracellular signaling by extracellular glycan remodeling. ACS Chem. Biol. 2010; 5:35–46. [PubMed: 19968325]

(31). Albohy A, Richards MR, Cairo CW. Mapping substrate interactions of the human membrane-associated neuraminidase, NEU3, using STD NMR. Glycobiology. 2015; 25:284–293. [PubMed: 25294388]

(32). Keppler OT, Hinderlich S, Langner J, Schwartz-Albiez R, Reutter W, Pawlita M. UDP-GlcNAc 2-epimerase: a regulator of cell surface sialylation. Science. 1999; 284:1372–1376. [PubMed: 10334995]

(33). Bond MR, Whitman CM, Kohler JJ. Metabolically incorporated photocrosslinking sialic acid covalently captures a ganglioside-protein complex. Mol. Biosyst. 2010; 6:1796–1799. [PubMed: 20625600]

(34). Whitman CM, Yang F, Kohler JJ. Modified GM3 gangliosides produced by metabolic oligosaccharide engineering. Bioorg. Med. Chem. Lett. 2011; 21:5006–5010. [PubMed: 21620696]

(35). Weksler BB, Subileau EA, Perrière N, Charneau P, Holloway K, Leveque M, Tricoire-Leignel H, Nicotra A, Bourdoulous S, Turowski P, Male DK, Roux F, Greenwood J, Romero IA, Couraud PO. Blood-brain barrier-specific properties of a human adult brain endothelial cell line. FASEB J. 2005; 19:1872–1874. [PubMed: 16141364]

(36). Trudgian DC, Ridlova G, Fischer R, Mackeen MM, Ternette N, Acuto O, Kessler BM, Thomas B. Comparative evaluation of label-free SINQ normalized spectral index quantitation in the central proteomics facilities pipeline. Proteomics. 2011; 11:2790–2797. [PubMed: 21656681]

(37). Kato N, Yuzawa Y, Kosugi T, Hobo A, Sato W, Miwa Y, Sakamoto K, Matsuo S, Kadomatsu K. The E-selectin ligand basigin/CD147 is responsible for neutrophil recruitment in renal ischemia/reperfusion. J. Am. Soc. Nephrol. 2009; 20:1565–1576. [PubMed: 19443639]

(38). Rho J-H, Mead JR, Wright WS, Brenner DE, Stave JW, Gildersleeve JC, Lampe PD. Discovery of sialyl Lewis A and Lewis X modified protein cancer biomarkers using high density antibody arrays. J. Proteomics. 2014; 96:291–299. [PubMed: 24185138]

(39). Casey G, Neville PJ, Liu X, Plummer SJ, Cicek MS, Krumroy LM, Curran AP, McGreevy MR, Catalona WJ, Klein EA, Witte JS. Podocalyxin variants and risk of prostate cancer and tumor aggressiveness. Hum. Mol. Genet. 2006; 15:735–741. [PubMed: 16434482]

(40). Somasiri A, Nielsen JS, Makretsov N, McCoy ML, Prentice L, Gilks CB, Chia SK, Gelmon KA, Kershaw DB, Huntsman DG, McNagny KM, Roskelley CD. Overexpression of the anti-adhesin podocalyxin is an independent predictor of breast cancer progression. Cancer Res. 2004; 64:5068–5073. [PubMed: 15289306]

(41). Thomas SN, Schnaar RL, Konstantopoulos K. Podocalyxin-like protein is an E-/L-selectin ligand on colon carcinoma cells: comparative biochemical properties of selectin ligands in host and tumor cells. Am. J. Physiol. Cell Physiol. 2009; 296:C505–13. [PubMed: 19118161]

(42). van Kempen LC, van den Oord JJ, van Muijen GN, Weidle UH, Bloemers HP, Swart GW. Activated leukocyte cell adhesion molecule/CD166, a marker of tumor progression in primary malignant melanoma of the skin. Am. J. Pathol. 2000; 156:769–774. [PubMed: 10702391]

(43). Hakomori S-I. The glycosynapse. Proc. Natl. Acad. Sci. U. S. A. 2002; 99:225–232. [PubMed: 11773621]

(44). Ramya TNC, Weerapana E, Liao L, Zeng Y, Tateno H, Liao L, Yates JR, Cravatt BF, Paulson JC. In situ trans ligands of CD22 identified by glycan-protein photocross-linking-enabled proteomics. Mol. Cell. Proteomics. 2010; 9:1339–1351. [PubMed: 20172905]

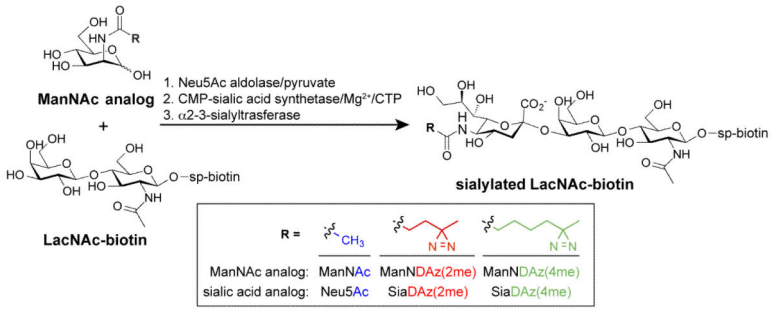

Figure 1. One-pot chemoenzymatic synthesis of SiaDAz-ylated LacNAc-biotin

ManNAc or a diazirine-modified ManNAc analog, ManNDAz(2me) or ManNDAz(4me), is incubated with biotinylated LacNAc, Mg^{2+}, CTP, pyruvate, CMP-sialic acid synthetase, Neu5Ac aldolase, and an α2-3-sialyltransferase at 37 °C overnight. The resulting products are glycans sialylated with the desired sialic acid species, Neu5Ac, SiaDAz(2me), or SiaDAz(4me). Spacer (sp) = -O$(CH_2)_3$NHCO$(CH_2)_5$NH-.

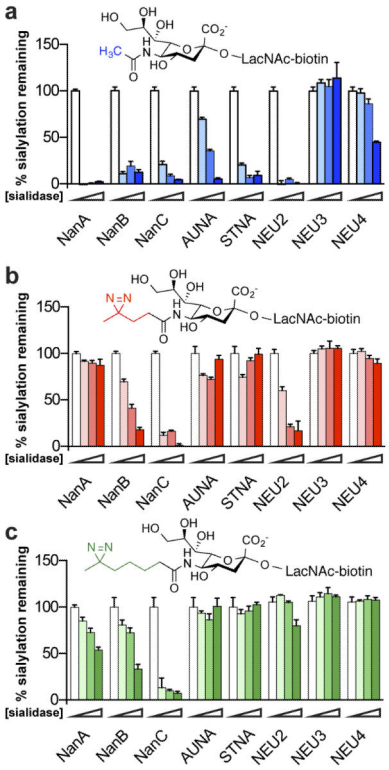

Figure 2. Sialidase specificity depends on SiaDAz linker length

LacNAc-biotin was sialylated with (**a**) Neu5Ac, (**b**) SiaDAz(2me), or (**c**) SiaDAz(4me) before treatment with the indicated sialidases. The percent sialylation remaining after sialidase treatment was determined using PAL labeling. Error bars represent the standard deviation of three trials.

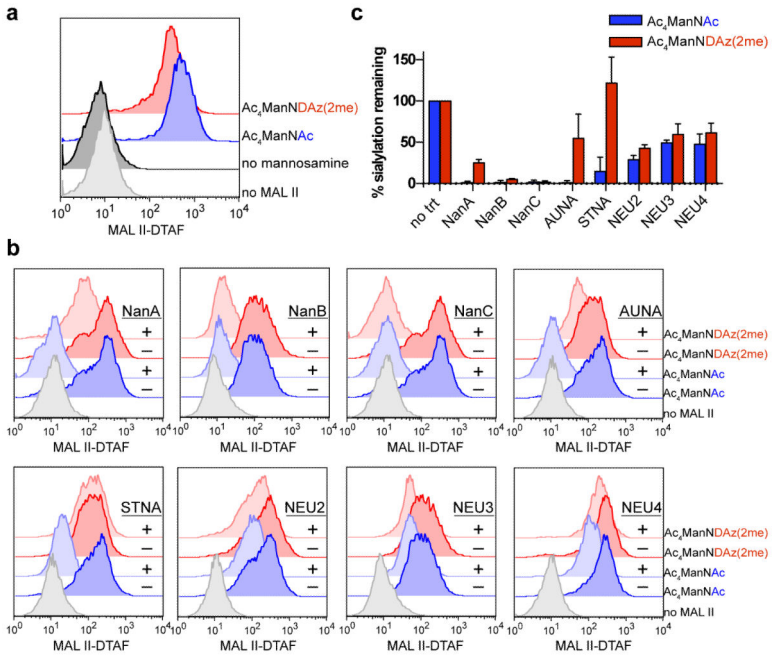

Figure 3. Activity of sialidases toward α2-3-linked sialic acids on BJAB K20 cell surfaces

(**a**) α2-3-linked sialic acids on the surface of BJAB K20 cells were detected by flow cytometry using MAL II lectin. Cells cultured with no mannosamine precursor do not produce α2-3-linked sialic acids. (**b**) BJAB K20 cells were cultured with Ac_4ManNAc, Ac_4ManNDAz(2me), or no mannosamine, then left untreated or treated with the indicated sialidase. Remaining α2-3-linked sialic acids on the cell surface were detected by flow cytometry using MAL II lectin. (**c**) Quantification of analyses shown in panel (**b**), representing the amount of α2-3-linked Neu5Ac or SiaDAz(2me) remaining on BJAB K20 cell surfaces after treatment with the indicated sialidase. Error bars represent the standard deviation of three trials.

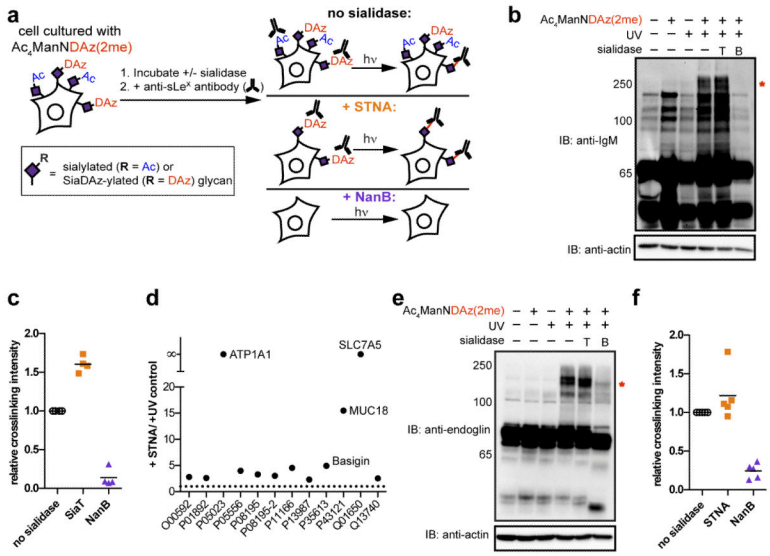

Figure 4. Removal of Neu5Ac from hCMEC/D3 cell surfaces results in enhanced crosslinking of SiaDAz-ylated glycoproteins

(**a**) Sialidase method for crosslinking enhancement. Cells cultured with Ac_4ManNDAz(2me) display a mixed population of sialic acids on their cell surfaces. Crosslinking is dependent on the proportion of Neu5Ac (Ac) and SiaDAz(2me) (DAz) remaining after treatment with buffer, STNA (which removes only Ac), or NanB (which removes both Ac and DAz). Upon exposure to UV, STNA-treated cells contain a higher proportion of crosslinked DAz than untreated or NanB-treated cells, enhancing crosslinking through DAz. (**b**) Immunoblot of crosslinked lysates. hCMEC/D3 cells cultured with or without Ac_4ManNDAz(2me) were treated with NanB (B), STNA (T), or no sialidase. Anti-sLe^X antibody was added and the cells were UV irradiated or not. (**c**) Quantification of normalized intensities of high molecular weight anti-sLe^X crosslinked bands (indicated by asterisk in panel **b**). (**d**) LC-MS/MS results. Proteins enriched in anti-sLe^X crosslinked samples are more abundant in cells pre-treated with STNA before crosslinking compared to untreated, but crosslinked, cells. Dotted line represents values expected if STNA had no effect (ratio = 1). (**e**) Immunoblot of endoglin crosslinking in hCMEC/D3 cells cultured with or without Ac_4ManNDAz(2me) and treated with NanB (B), STNA (T), or no sialidase. (**f**) Quantification of normalized intensities of high molecular weight crosslinked bands (indicated by asterisk in panel **e**).