Supporting information for

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

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Materials. Biotinylated LacNAc (LacNAc-biotin) was purchased from Glycotech (02-022) and comprised LacNAc attached to biotin through a spacer (sp), -O$(CH_2)_3$NHCO$(CH_2)_5$NH-. CMP-sialic acid synthetase from *Neisseria meningitidis* group B (NmCSS) and α2-3-sialyltransferase from *Pasteurella multocida* (Pm2-3ST) were purchased from Sigma. The N-acetylneuraminic acid (Neu5Ac) aldolase was purchased from Toyobo. Sodium pyruvate (P2256), cytidine 5'-triphosphate disodium salt (C1506), $MgCl_2$ and ManNAc (A8176) were purchased from Sigma. Ac_4ManNAc was prepared as described.[1] ManNDAz(2me), ManNDAz(4me), and the peracetylated forms Ac_4ManNDAz(2me) and Ac_4ManNDAz(4me) were synthesized as previously described.[1-3] Sialidases were acquired as follows: *Streptococcus pneumoniae* NanA, NanB, and NanC were expressed recombinantly, as described below. *Arthrobacter ureafaciens* neuraminidase (AUNA) was purchased from Prozyme (gk80040) and *Salmonella typhimurium* neuraminidase (STNA) was purchased from New England Biolabs (P0728). NEU2 and NEU3 were expressed in *E. coli* as N-terminal MBP-fusion proteins and NEU4 as a GST-fusion protein and purified as described.[4-6] Anti-sLe^X antibody (CD15s) was purchased from BD Biosciences (551344) and anti-endoglin antibody was purchased from Abcam (ab169545).

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 OD_{600} ~ 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 x 30 s on ice. Lysates were centrifuged at 20,000*g* for 1 h at 4 °C. To bind protein, the supernatant was incubated with 100 µL 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 µmol min^{-1} mg^{-1} or 18 units mg^{-1}; NanB, 14 µmol min^{-1} mg^{-1} or 14 units mg^{-1}; NanC, 3 µmol min^{-1} mg^{-1} or 3 units mg^{-1}. A unit is defined as the amount of enzyme required to release 1 µmol of 2'-(4-methylumbelliferone)-α-D-N-acetylneuraminic acid (4-MU NANA) per minute.

Cell culture. hCMEC/D3 cells[7] were cultured in EBM-2 (Lonza) supplemented with 5 % (v/v) FBS, 1 % (v/v) penicillin/streptomycin, 1.4 µM hydrocortisone (Sigma), 5 µg mL^{-1} ascorbic acid, 1X chemically defined lipid concentrate (Life Technologies), 10 mM HEPES (Sigma), and

1 ng mL^{-1} human basic fibroblast growth factor (Sigma). Colo205 and BJAB K20 cells were cultured in RPMI (Life Technologies) supplemented with 10 % (v/v) FBS and 1 % (v/v) penicillin/streptomycin. For serum starving conditions, BJAB K20 cells were cultured in RPMI supplemented with 1 % (v/v) Nutridoma-SP (Roche) and 0.5 % (v/v) penicillin/streptomycin. All cells were cultured at 37 °C in 5 % CO_2. For sugar incorporation and crosslinking experiments, cells were cultured in media containing a final concentration of 100 μM Ac_4ManNAc, Ac_4ManNDAz(2me), Ac_4ManNDAz(4me), or an equal volume of ethanol for 72 h.

One-pot chemoenzymatic synthesis. One-pot chemoenzymatic synthesis of sialylated and SiaDAz-ylated LacNAc-biotin compounds was modeled on previously reported protocols.[8] Reaction mixtures contained 100 mM Tris-HCl pH 8.8, 20 mM $MgCl_2$, 7.5 mM ManNAc (or diazirine-containing analog, ManNDAz(2me) or ManNDAz(4me)), 7.5 mM CTP, 40 mM sodium pyruvate, 0.2–0.4 mM LacNAc-biotin, 2.3 mg mL^{-1} Neu5Ac aldolase, 0.4–0.8 mU NmCSS, and 0.2–0.4 mU Pm2-3ST in a final volume of 50–100 μL. Reactions were allowed to incubate overnight at 37 °C. Products were either used in the cell-free microwell plate assay or further purified for mass analysis. For further purification, reactions were stopped by addition of an equal volume of 95 % ethanol (pre-chilled on ice), which was then incubated for 30 min on ice. Samples were centrifuged for 30 min at 5000*g* and solvent was removed in a vacuum concentrator. The resulting residue was resuspended in water and purified by HPLC for downstream LC-MS analysis.

HPLC separation of sialylated and SiaDAz-ylated LacNAc-biotin. Reactions from the one-pot chemoenzymatic synthesis were injected onto a Dionex Acclaim® Polar Advantage C16 5 μm, 4.6 x 250 mm column attached to a Dionex Ultimate 3000 HPLC with UV detector. Samples were separated using an 11–50 % gradient of acetonitrile in H_2O and detected at 205 nm using UV. Peaks were collected and submitted for mass spectrometry analysis (Shimadzu Center for Advanced Analytical Chemistry, UT Arlington).

LC-MS analysis of one-pot reactions. Samples were injected onto a high resolution mass spectrometer following liquid chromatography (LCMS IT-TOF (Shimadzu)) using a Halo C18 2.1 x 100 mm column (Perfinity) with a flow rate of 0.18 mL min^{-1}. Mode of ionization for sample analysis was ESI. The solvent system consisted of 95/5 % H_2O/ACN (solvent A) and 100 % ACN (solvent B) with the following gradient of solvent B: 0–2 min, 0 %; 2–30 min, 15 %; 30–50 min, 40 %; 50–58 min, 70 %; 58–65 min, 0 %.

DMB derivatization of sialic acids. hCMEC/D3 or Colo205 cells were cultured with ethanol or Ac_4ManNDAz(2me) in ethanol (to achieve a final concentration of 100 μM Ac_4ManNDAz(2me)) for 72 h. Cells were then fractionated as follows: cells were harvested and counted before resuspension in hypotonic lysis buffer (10 mM Tris-HCl, pH 7.3, 10 mM $MgCl_2$, 1 mM EDTA, and 1 mM EGTA). Cells were allowed to swell for 15 min on ice and then were passaged through a 25 gauge needle for 5 min. Once homogenized, lysates were centrifuged two times at 1000*g* for 15 min in a refrigerated centrifuge to isolate the post nuclear supernatant. Lysates were then centrifuged at 100,000*g* for 1 h, after which the supernatant was removed and the pellet was washed twice in hypotonic lysis buffer followed by a 1 h, 100,000*g* centrifugation each time to isolate the membrane fraction. Membrane fractions were then dried in a vacuum concentrator.

For DMB labeling, sialic acids were first released from samples by adding 50 µL 2.0 M acetic acid to membrane fractions, and incubating for 2 h at 80 °C. Samples were then cooled to room temperature before addition of 40 µL 1,2-diamino-4,5-methylenedioxybenzene (DMB) reaction solution (7.0 mM DMB, 1.4 M acetic acid, 750 mM 2-mercaptoethanol, 18 mM $Na_2S_2O_3$). Samples were incubated 2 h at 50 °C. After cooling to room temperature, 10 µL 0.2 M NaOH was added to each sample, and samples were centrifuged for 5 min at 10,000*g*, room temperature. Finally, samples were filtered through a 10 kDa MWCO filter and filtrate was diluted into water and run on an HPLC for analysis. Samples were injected onto a Dionex Acclaim® Polar Advantage C16 5 µm, 4.6 x 250 mm column attached to a Dionex Ultimate 3000 HPLC with fluorescence detector. Separation was performed using a gradient of 12–25 % acetonitrile (diluted in H_2O) and detected by fluorescence (ex: 373 nm/em: 448 nm). Integrated areas under the peaks of DMB-Neu5Ac and DMB-SiaDAz(2me) were determined, from which percent SiaDAz(2me) making up total membrane sialic acid was calculated.[3]

Cell-free 96-well PAL assay. Sialylated or SiaDAz-ylated LacNAc-biotin from the one-pot chemoenzymatic synthesis was diluted to 3 µM in sialidase reaction buffer as follows: NanA, NanB, NanC, and NEU2 (100 mM NaOAc pH 5.6, with 100 mM NaCl), AUNA (50 mM sodium phosphate pH 6.0), STNA (50 mM sodium citrate pH 6.0, with 100 mM NaCl and 100 µg mL^{-1} BSA), NEU3 and NEU4 (100 mM NaOAc pH 4.2). Glycan solutions were aliquoted into individual wells of a 96-well plate and treated with sialidase at three concentrations as follows: NanA (0.93, 2.8, or 8.4 mU), NanB (3.3, 10, or 30 mU), NanC (0.67, 2, or 6 mU), AUNA (0.56, 1.7, or 5 mU), STNA (5.6, 16.7, or 50 U), NEU2 (0.4, 1.2, or 3.6 mU), NEU3 (0.56, 1.7, or 5 mU), and NEU4 (0.2, 0.6, or 0.8 mU). Plates were incubated at 37 °C for 1 h before transferring to a 96-well streptavidin-coated plate (15503, Pierce) and incubating for 2 h at 4 °C to allow binding of biotinylated glycans to the plate. After incubation, wells were washed three times in PBS containing 0.05 % (v/v) Tween 20 (PBST). For PAL detection, chemoselective oxidation was induced by incubating wells in 100 µL 0.1 mM $NaIO_4$ in PBS for 30 min on ice. Oxidation was quenched by addition of 1 mM glycerol for 10 min on ice. Wells were emptied, washed one time in 1 mM glycerol, and three times in PBST before reaction with 10 mM aniline and 10 µM aminooxy-Alexa Fluor 488 (Invitrogen) in 100 mM NaOAc, pH 4.5. Ligation was allowed to occur for 2 h at 4 °C. Wells were washed six times in PBST and imaged on a fluorescence plate reader (ex: 488 nm/em: 515 nm). Three independent experiments were performed; within each experiment, each treatment was performed in triplicate.

Cell-based sialidase activity assay. BJAB K20 cells were cultured for 72 h with Ac_4ManNAc, Ac_4ManDAz(2me), or Ac_4ManDAz(4me) to achieve a final concentration of 100 µM . Cells were washed in PBS and treated with sialidase for 1 h in a 37 °C water bath in the appropriate activity buffer (above) as follows: NanA, 25.2 mU; NanB, 42 mU; NanC, 3 mU; AUNA, 10 mU; STNA, 100 U; NEU2, 1.4 mU; NEU3, 1 mU; and NEU4, 0.36 mU. Units are defined as the amount of sialidase needed to hydrolyze 1 µmol 4-MU NANA per min at 37 °C.

Flow cytometry analysis. For detection of α2-3-linked sialic acids, BJAB K20 cells cultured with Ac_4ManNAc or its diazirine derivatives and treated with sialidase as above were incubated with 50 µL of 10 µM MAL II-biotin (Vector Labs) for 1 h on ice. Cells were washed twice in ice-cold PBS containing 0.1 % (w/v) BSA. For detection of MAL II, cells were then incubated

with 50 µL of 7.7 µg mL^{-1} DTAF-streptavidin (Jackson Immunoresearch) for 1 h on ice in the dark. Cells were washed three times in PBS containing 0.1 % (w/v) BSA and analyzed on a FACSCalibur using the 488 nm laser line. Percent sialylation remaining was determined using the median fluorescence intensities for each treatment and subtracting background (no MAL II) before dividing by the value from the sample corresponding to 100 % sialylation remaining (*i.e.* no sialidase treatment). Averages and standard deviation were calculated for three trials.

For detection of sLe^X, hCMEC/D3 or Colo205 cells were treated with no sialidase, STNA, or NanB in the appropriate activity buffer for 1 h at 37 °C. Cells were then washed in PBS before incubation with 100 µL of 5 µg mL^{-1} anti-sLe^X antibody (CD15s, BD Biosciences) for 1 h at 4 °C. Cells were washed three times in PBS containing 0.1 % (w/v) BSA, then incubated with 100 µL 5 µg mL^{-1} goat anti-mouse IgG/IgM conjugated to FITC (BD Biosciences) for 1 h at 4 °C. Cells were washed three times in PBS containing 0.1 % (w/v) BSA and then analyzed on a FACSCalibur using the 488 nm laser line.

To determine the extent of STNA activity at 4 °C, hCMEC/D3 cells were incubated with 10 mU STNA for 1 h a 37 °C or on ice, washed, and labeled using MAL II-biotin as above.

Immunoblot of PVDF membranes. For immunoblot analysis, membranes were blocked with 5 % (w/v) BSA for 1 h, then incubated with 1:2000 dilution of anti-IgM antibody in 5 % (w/v) BSA in PBS containing 0.1 % (v/v) Tween20 (PBST) for 2 h at room temperature. Membranes were washed three times in PBST and imaged on a ChemiDoc™ MP Imaging System (Bio-Rad). For actin loading control, membranes were stripped (200 mM glycine, 0.1 % (w/v) SDS, 1 % (v/v) Tween20, pH 2.2) for 45 min at 37 °C, washed three times in PBST, and re-blocked in 5 % (w/v) BSA. Membranes were incubated with an anti-actin antibody (1:2500, Abcam) for 1 h at room temperature in PBST containing 5 % (w/v) BSA. Membranes were washed and then incubated with a goat anti-Rabbit antibody conjugated to HRP (1:5000, Life Technologies) in PBST containing 5 % (w/v) BSA. After washing, membranes were imaged on a ChemiDoc™ MP Imaging System (Bio-Rad).

Biotinylation of anti-sLe^X antibody. A 150 µg aliquot of anti-sLe^X antibody (CD15s, BD Biosciences) was dialyzed in 500 mL PBS two times for 3 h each. Once dialyzed, a 50-fold molar excess of Sulfo-NHS-Biotin (Thermo Scientific) was added for two hours, on ice. After biotinylation, the antibody was dialyzed in 500 mL PBS for 3 h followed by an overnight dialysis into PBS.

Proteomics analysis of anti-sLe^X crosslinked complex. hCMEC/D3 cells were grown in media containing 100 µM Ac_4ManNDAz(2me) for 72 h. Cells were harvested with trypsin, washed in PBS, and incubated with no sialidase (no UV control and plus UV) or with STNA for 1 h in a 37 °C water bath. Cells were then washed in PBS containing 0.1 % (w/v) BSA, and incubated with 5 µg mL^{-1} biotinylated anti-sLe^X antibody for 1 h at 4 °C with rotation. After antibody incubation, cells were placed in 6 cm cell culture dishes and crosslinked under UV (365 nm, 20 W) for 15 min. Cells were then harvested at 1000*g*, washed in PBS containing 0.1 % (w/v) BSA, and lysed ten minutes on ice in 50 mM ammonium bicarbonate buffer containing 0.2 % (w/v) RapiGest SF (Waters) and protease inhibitor (Roche). Cells were sonicated 15 s on, 15 s off at 20 % power for 45 s total sonication time. Lysates were then centrifuged at 20,000g for 10 min

at 4 °C. Proteins were quantified by BCA assay kit (Pierce) and 500 μg of protein was incubated with 25 μL streptavidin agarose and rotated overnight at 4 °C. Beads were pelleted and washed three times in lysis buffer, four times in PBS containing 2 M urea, and 4 times in PBS. For trypsin digest, beads were first resuspended in digestion buffer (6 M urea, 100 mM Tris (pH 8.0), 2M thiourea, 50 % (v/v) trifluoroethanol, and 0.1 % (w/v) RapiGest SF) and incubated at 37 °C for 30 min. Tris(2-carboxyethyl)phosphine (TCEP) was then added to a final concentration of 10 mM and beads were incubated at 37 °C for another 30 min. Next, iodoacetamide was added to a final concentration of 12 mM and beads were incubated at room temperature in the dark for 30 min. Finally, beads were diluted 1:10 in 100 mM Tris, pH 8.0 with 10 mM $CaCl_2$ and sequence grade modified trypsin (Promega) was added to a final concentration of 10 μg mL^{-1}. Trypsin digest was allowed to occur overnight in a shaking 37 °C incubator. After incubation, an additional 10 mg mL^{-1} trypsin was added to digests and allowed to proceed for another 2 h at 37 °C with shaking. Supernatants were then removed from the beads, and reactions were stopped by addition of trifluoroacetic acid to pH < 2 (~ 0.5 % (v/v) of total supernatant volume). Supernatants were incubated 45 min at 37 °C, then centrifuged at 20,000*g* for 10 min at room temperature. Samples were submitted to the UTSW Proteomics Core for analysis, as follows.

Digested samples were cleaned using solid-phase extraction with Oasis HLB plates (Waters) and were then analyzed by LC/MS/MS, using a Q Exactive mass spectrometer (Thermo Electron) coupled to an Ultimate 3000 RSLC-Nano liquid chromatography system (Dionex). Samples were injected onto a 180 μm i.d., 15-cm long column packed in-house with a reverse-phase material, ReproSil-Pur C18-AQ, 3 μm resin (Dr. Maisch GmbH, Ammerbuch-Entringen, Germany), and eluted with a gradient from 1-28 % buffer B over 40 min. Buffer A contained 2 % (v/v) acetonitrile (ACN) and 0.1 % formic acid in water, and buffer B contained 80 % (v/v) ACN, 10 % (v/v) trifluoroethanol, and 0.08 % formic acid in water. The mass spectrometer operated in positive ion mode with a source voltage of 2.2 kV, capillary temperature of 275 °C, and S-lens RF level at 50.0 %. MS scans were acquired at 70,000 resolution and up to 20 MS/MS spectra were obtained for each full spectrum acquired at 17,500 resolution using higher-energy collisional dissociation (HCD) for ions with charge ≥2. Dynamic exclusion was set for 7s.

Raw MS data files were converted to a peak list format and analyzed using the central proteomics facilities pipeline (CPFP), version 2.0.3.[9,10] Peptide identification was performed using the X!Tandem[11] and open MS search algorithm (OMSSA) [12] search engines against the human protein database from UniProt, with common contaminants and reversed decoy sequences appended.[13] Fragment and precursor tolerances of 20 ppm and 0.1 Da were specified, and three miscleavages were allowed. Carbamidomethylation of Cys was set as a fixed modification and oxidation of Met was set as a variable modification. Label-free quantitation of proteins across samples was performed using SINQ normalized spectral index software.[14]

For analysis, membrane proteins from each of three trials were determined using UniProt search engine. Data presented in Supplementary Table 2 represent all membrane proteins with spectral counts greater than 3 in either STNA-treated or untreated crosslinked samples. For these proteins, ratios of the normalized spectral index (MIC Sin) in crosslinked versus uncrosslinked control samples were determined. Proteins with ratios greater than 2 were compared for relative abundance in STNA-treated versus untreated crosslinked samples. Values higher than 1 were considered an enrichment in these samples.

Table S1. Observed and predicted *m/z* ratios for LacNAc compounds sialylated and SiaDAz-ylated in one-pot reaction.

Glycan	Observed mass	Predicted mass $[M+H]^+$	Predicted mass $[M+Na]^+$	Predicted mass $[M+K]^+$
Neu5Ac α2-3-LacNAc-biotin	1093.45	1070.46	1093.45 **(+0.00)**	
	1109.42			1109.42 **(+0.00)**
SiaDAz(2me) α2-3-LacNAc-biotin	1161.49	1138.50	1161.48 **(-0.01)**	
	1177.46			1177.46 **(+0.00)**
SiaDAz(4me) α2-3-LacNAc-biotin	1189.52	1166.53	1189.52 **(+0.00)**	
	1205.49			1205.49 **(+0.00)**

Table S2. Summary of sLe^X crosslinking LC-MS/MS results. SiaDAz(2me)-expressing cells were crosslinked to biotinylated anti-sLe^X antibody, purified on streptavidin agarose, trypsin digested, and analyzed by LC-MS/MS. Results from three trials are summarized below. Membrane proteins with ≥ 3 spectral counts in either the STNA-treated (STNA) or untreated, but crosslinked, samples (UV) were compared to uncrosslinked (No UV) samples for relative abundance. Those with a ratio greater than 2 were considered enriched in crosslinking, and the abundance of protein in STNA versus UV was then compared (STNA/UV). Proteins whose abundance compared to No UV is less than 2 are shaded in grey. N/A indicates no numerator for ratio analysis. ∞ indicates no denominator (i.e., protein was only seen in STNA or UV samples).

							Spectral counts			MIC Sin					
	Accession #	Gene	Description	Trial	# Seqs	% Coverage	No UV	STNA	UV	No UV	STNA	UV	STNA/No UV	UV/No UV	STNA/UV
1	P05023	ATP1A1	Sodium/potassium-transporting ATPase subunit alpha-1	1	6	11.30	0	5	0	0	2.9E-08	0	∞	N/A	∞
2	Q01650	SLC7A5	Large neutral amino acids transporter small subunit 1	1	3	6.90	0	3	0	0	5.5E-08	0	∞	N/A	∞
3	P43121	MCAM	Cell surface glycoprotein MUC18	1	3	5.90	0	3	1	0	3.9E-08	2.5E-09	∞	∞	15.49
4	P35613	BSG	Basigin	1	6	21.90	0	10	2	0	5.7E-07	1.1E-07	∞	∞	4.94
5	P11166	SLC2A1	Solute carrier family 2, facilitated glucose transporter member 1	1	2	5.10	0	3	1	0	9.8E-08	2.1E-08	∞	∞	4.56
6	P05556	ITGB1	Integrin beta-1	1	4	4.70	0	5	2	0	4.1E-08	1.0E-08	∞	∞	3.98
7	P08195	SLC3A2	4F2 cell-surface antigen heavy chain	3	6	11.00	2	5	4	6.0E-08	4.1E-07	1.2E-07	6.80	2.07	3.29
8	Q02413	DSG1	Desmoglein-1	1	4	4.20	5	6	2	3.4E-08	3.9E-08	1.2E-08	1.15	0.36	3.23
9	P08195-2	SLC3A2	Isoform 2 of 4F2 cell-surface antigen heavy chain	1	11	24.60	0	14	8	0	5.0E-07	1.6E-07	N/A	N/A	3.03
10	O00592	PODXL	Podocalyxin	1	7	13.90	1	9	5	7.6E-09	4.0E-07	1.4E-07	52.84	18.84	2.81
11	P01892	HLA-A	HLA class I histocompatibility antigen, A-2 alpha chain	1	5	18.10	1	5	2	1.5E-08	9.4E-08	3.6E-08	6.18	2.36	2.62
12	Q9ULI3	HEG1	Protein HEG homolog 1	1	6	4.90	6	8	4	5.4E-08	7.7E-08	2.9E-08	1.42	0.55	2.60
13	Q13740	ALCAM	CD166 antigen	1	3	5.50	0	3	2	0	3.6E-08	1.4E-08	N/A	N/A	2.54
14	P13987	CD59	CD59 glycoprotein	1	2	18.50	1	3	1	2.8E-07	5.6E-07	2.4E-07	2.01	0.87	2.30
15	O15427	SLC16A3	Monocarboxylate transporter 4	3	3	8.20	3	3	2	2.0E-07	1.6E-07	1.2E-07	0.79	0.57	1.38
16	Q8WUH6	C12orf23	UPF0444 transmembrane protein C12orf23	2	5	15.40	5	5	6	3.6E-07	6.3E-07	5.7E-07	1.77	1.61	1.10
17	O15427	SLC16A3	Monocarboxylate transporter 4	2	19	30.30	41	39	42	5.7E-06	8.1E-06	8.5E-06	1.42	1.49	0.96
18	P08195	SLC3A2	4F2 cell-surface antigen heavy chain	2	25	41.40	22	23	28	3.1E-06	4.7E-06	5.5E-06	1.53	1.78	0.86
19	Q13443	ADAM9	Disintegrin and metalloproteinase domain-containing protein 9	2	4	4.60	7	2	4	4.6E-07	2.2E-07	2.8E-07	0.48	0.61	0.78
20	Q01628	IFITM3	Interferon-induced transmembrane protein 3	2	3	48.90	3	2	4	2.9E-06	2.4E-06	3.2E-06	0.84	1.10	0.76
21	P21796	VDAC1	Voltage-dependent anion-selective channel protein 1	2	9	45.20	14	10	15	8.8E-06	7.0E-06	1.2E-05	0.79	1.41	0.56
22	Q8WUH6	C12orf23	UPF0444 transmembrane protein C12orf23	1	7	79.30	27	22	32	6.2E-06	5.4E-06	1.1E-05	0.87	1.70	0.51
23	P11166	SLC2A1	Solute carrier family 2, facilitated glucose transporter member 1	2	2	3.70	2	2	3	7.1E-07	4.8E-07	1.2E-06	0.67	1.64	0.41
24	P30414	NKTR	NK-tumor recognition protein	1	24	20.00	21	3	15	1.3E-07	1.1E-08	9.3E-08	0.09	0.74	0.12

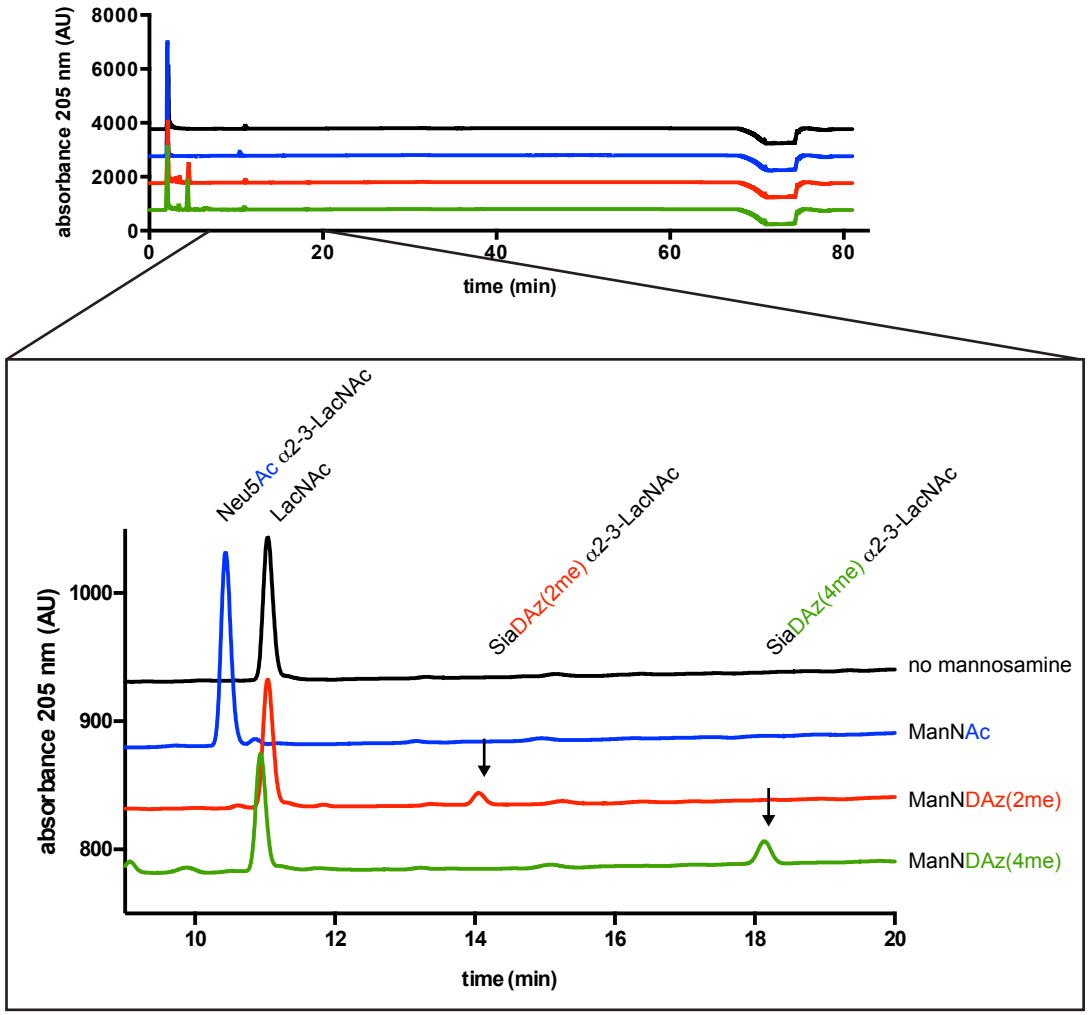

Figure S1. HPLC purification of sialylated and SiaDAz-ylated LacNAc-biotin. One-pot chemoenzymatic synthesis reactions from Figure 1 were separated by HPLC to purify sialylated LacNAc from unsialylated LacNAc. Reactions contained either no mannosamine (black), ManNAc (blue), ManNDAz(2me) (red), or ManNDAz(4me) (green) to produce the corresponding sialylated LacNAc-biotins – Neu5Ac α2-3-LacNAc-biotin (~10.5 min), SiaDAz(2me) α2-3-LacNAc-biotin (~ 14 min), and SiaDAz(4me) α2-3-LacNAc-biotin (~18.5 min). Unreacted LacNAc-biotin migrated at ~11 min. UV absorbance was measured at 205 nm. Full trace is shown at top, with the inset showing the region from 9 to 20 min.

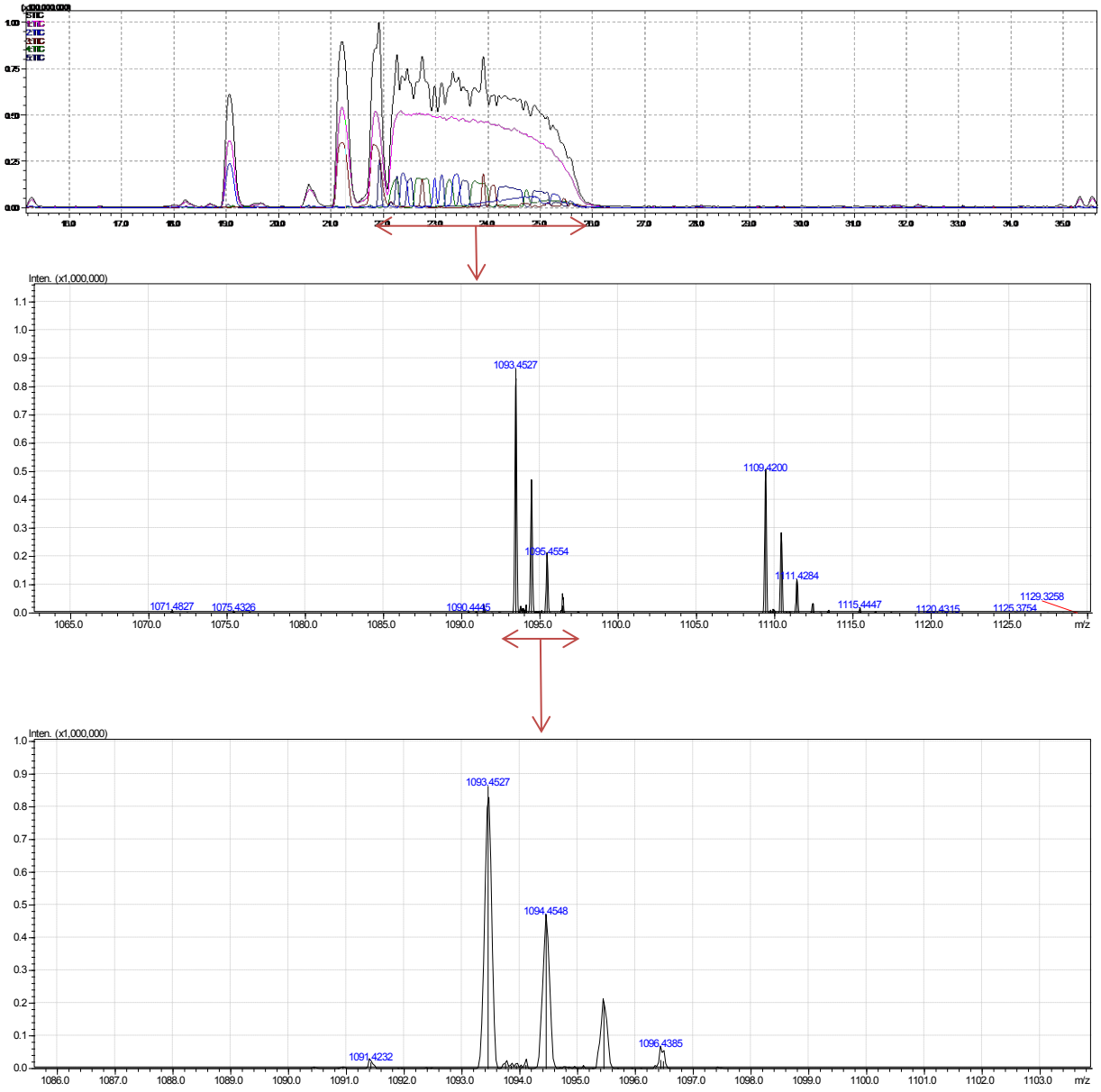

Figure S2. LC-MS identification of Neu5Ac α2-3-LacNAc-biotin.

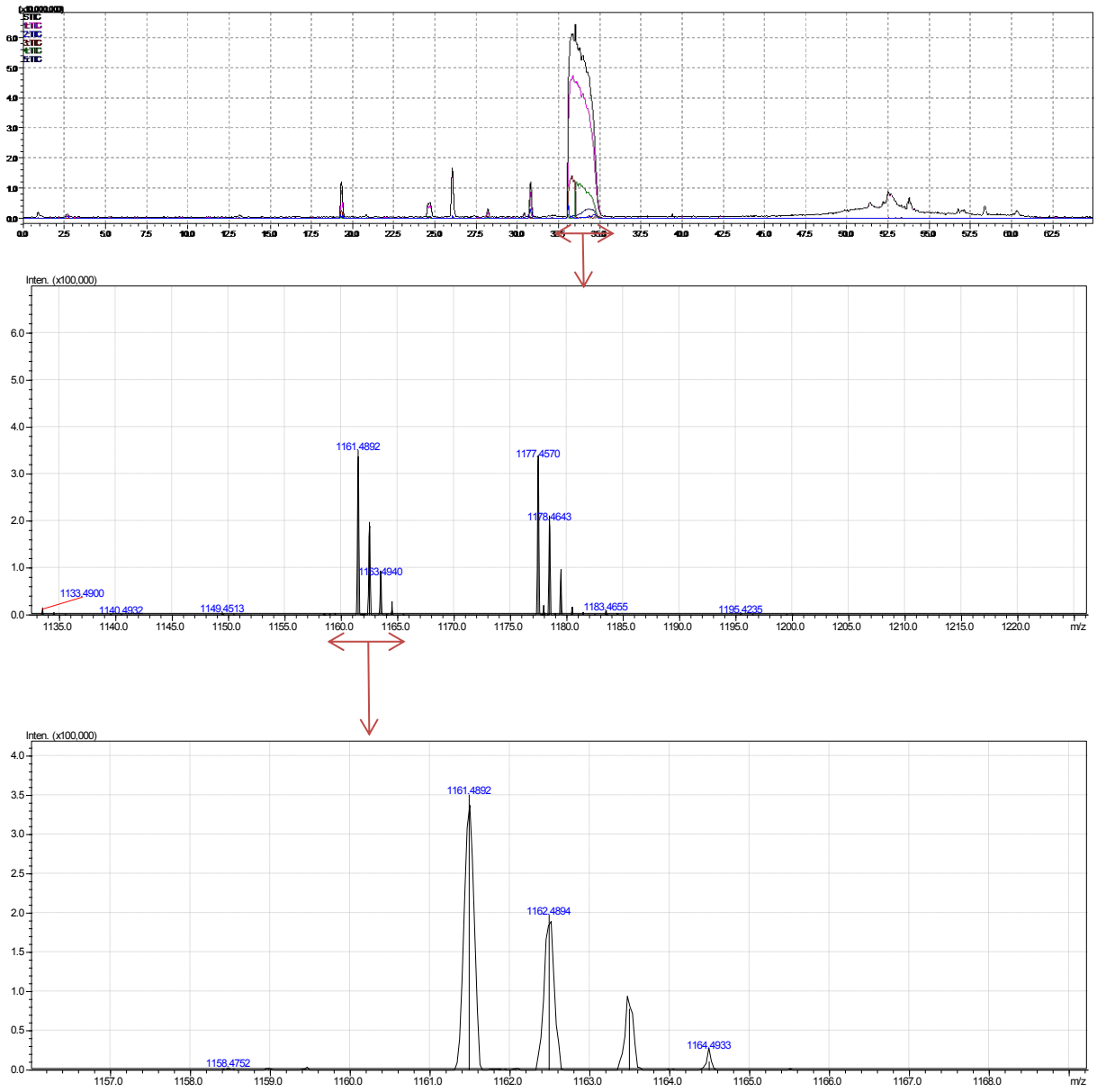

Figure S3. LC-MS identification of SiaDAz(2me) α2-3-LacNAc-biotin.

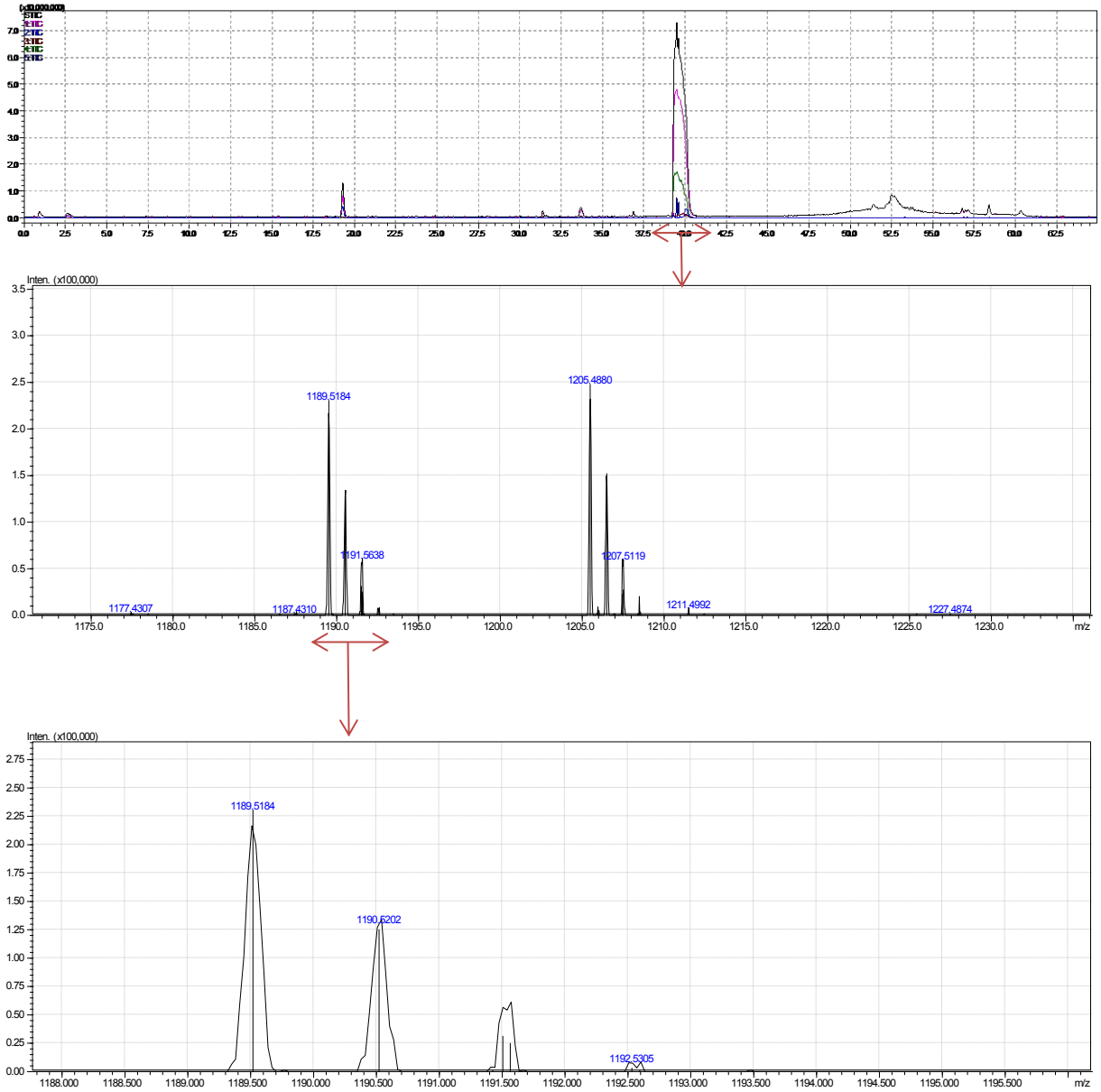

Figure S4. LC-MS identification of SiaDAz(4me) α2-3-LacNAc-biotin.

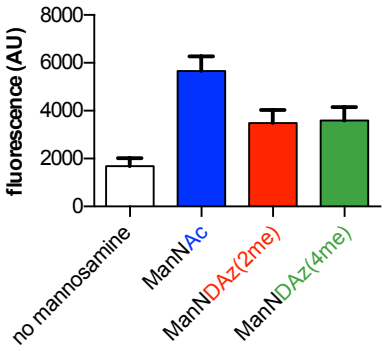

Figure S5. SiaDAz(2me) and SiaDAz(4me) are readily labeled by PAL. LacNAc-biotin left unsialylated, sialylated, or SiaDAz-ylated as in Figure 1 was immobilized on a streptavidin-coated plate and labeled using PAL with an aminooxy-Alexa Fluor 488. Sialylation or SiaDAz-ylation of LacNAc-biotin with Neu5Ac, SiaDAz(2me), or SiaDAz(4me) all resulted in a significant increase in fluorescence over unlabeled LacNAc-biotin, indicating PAL is able to label the diazirine-containing sialic acids. Error bars represent the standard deviation of five independent measurements.

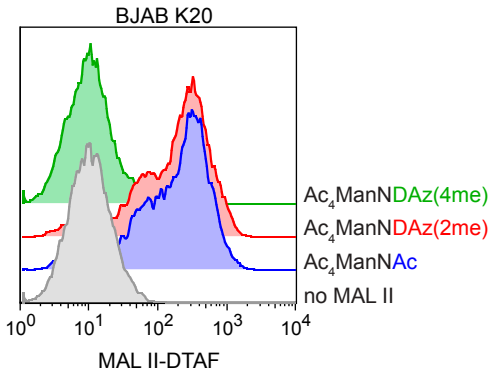

Figure S6. SiaDAz(2me), but not SiaDAz(4me), is detected on BJAB K20 cell surfaces. BJAB K20 cells cultured for 72 h with 100 μM Ac_4ManNAc, Ac_4ManNDAz(2me), or Ac_4ManNDAz(4me) were probed with MAL II-DTAF and analyzed by flow cytometry. Incubation with Ac_4ManNAc and Ac_4ManNDAz(2me), but not Ac_4ManNDAz(4me), resulted in an increase in fluorescence as compared to cells cultured without a sialic acid precursor.

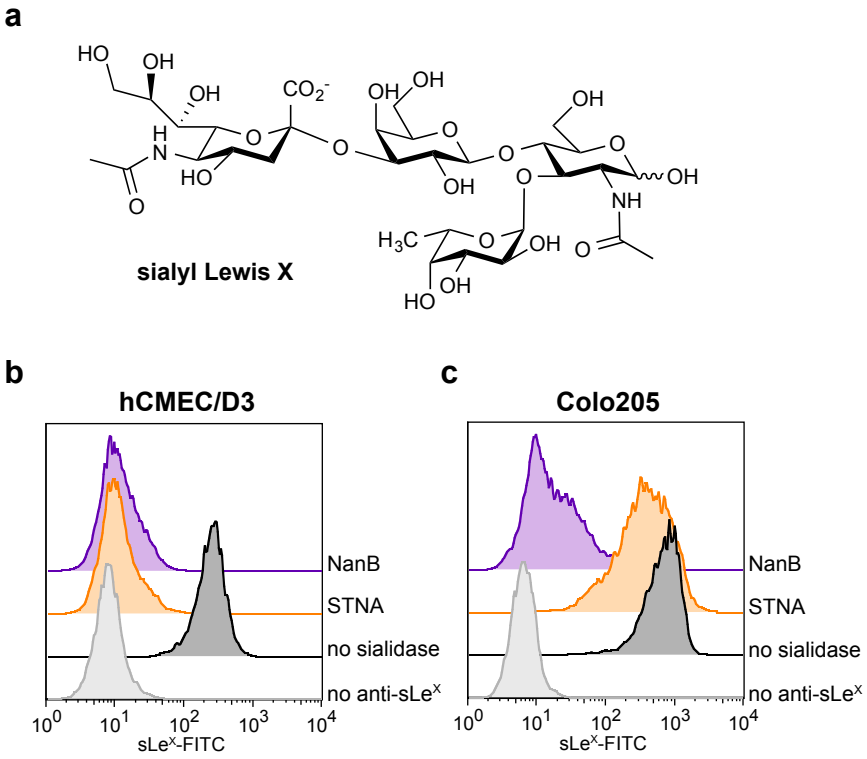

Figure S7. Anti-sLe^X antibody binds to hCMEC/D3 and Colo205 cells in a sialidase-dependent manner. (**a**) The sLe^X glycan epitope contains α2-3-linked Neu5Ac. (**b**) hCMEC/D3 or Colo205 cells were treated with NanB, STNA, or no sialidase, then probed with anti-sLe^X antibody followed by detection with a FITC-conjugated anti-IgM antibody. Fluorescence was measured by flow cytometry.

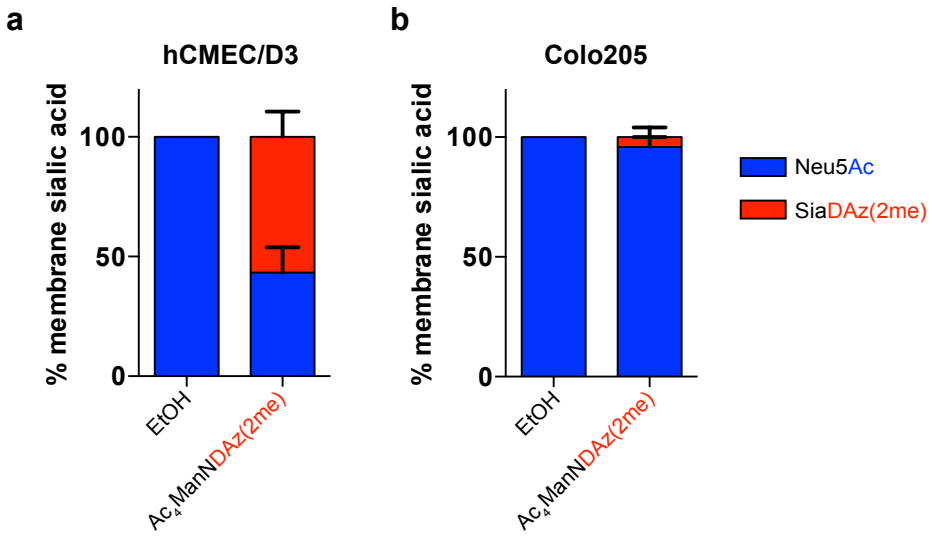

Figure S8. Membrane SiaDAz(2me) incorporation in hCMEC/D3 and Colo205 cells. Percentage of total membrane sialic acids that are either Neu5Ac or SiaDAz(2me) on (**a**) hCMEC/D3 and (**b**) Colo205 cell surfaces. Membrane fractions from cells cultured with Ac_4ManNDAz(2me) or ethanol (EtOH) were labeled through derivatization of sialic acids with DMB. Fluorescence was measured using HPLC (ex: 373 nm, em: 448 nm) and total amounts of DMB-Neu5Ac and DMB-SiaDAz(2me) were determined by quantifying area under the peaks. Error bars represent the standard deviation of two trials.

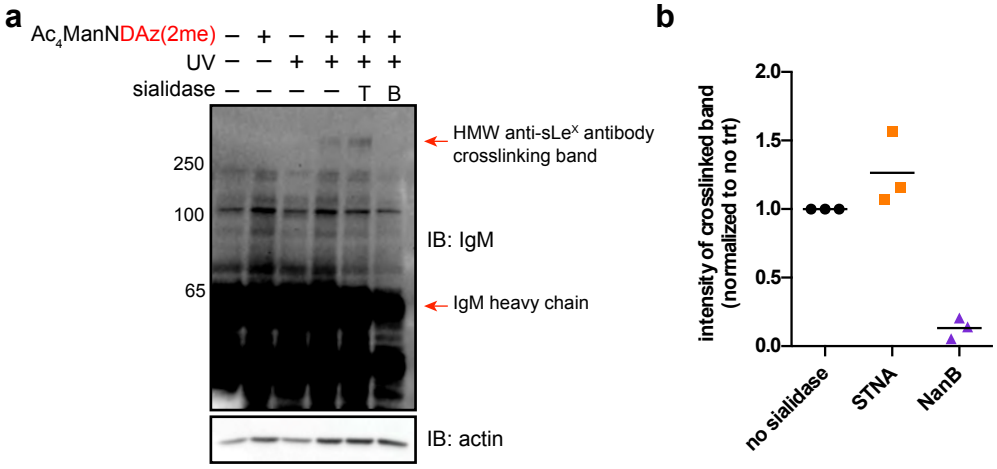

Figure S9. Removal of Neu5Ac from Colo205 cell surfaces results in enhanced crosslinking of SiaDAz-ylated glycoproteins to anti-sLe^X antibody. (**a**) Immunoblot of crosslinked lysates probed with anti-IgM to detect anti-sLe^X antibody. Colo205 cells were cultured with or without Ac_4ManNDAz(2me), then treated with no sialidase, STNA (T), or NanB (B). Anti-sLe^X antibody was added and the cells were either UV irradiated or left untreated. (**b**) Quantification of normalized intensities of high molecular weight (HMW) crosslinked bands (upper red arrow in panel **a**) from lysates of Colo205 cells cultured with Ac_4ManNDAz and exposed to UV. Comparison is between cells treated with no sialidase, STNA, or NanB prior to crosslinking.

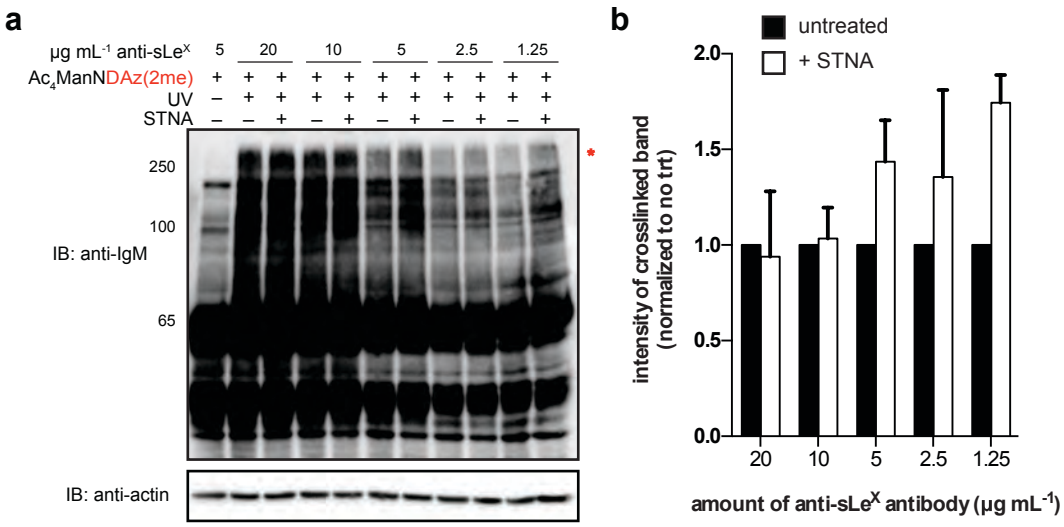

Figure S10. Anti-sLe^X antibody crosslinking enhancement is overcome by high antibody concentration. (**a**) Immunoblot of crosslinked lysates probed with anti-IgM antibody to detect anti-sLe^X antibody. hCMEC/D3 cells cultured with Ac_4ManNDAz(2me) were treated with buffer or STNA followed by incubation with the indicated amount of anti-sLeX antibody. Samples were then treated with UV or not. (**b**) Quantification of normalized intensities of high molecular weight (HMW) crosslinked bands (asterisk in panel **a**). Intensity of the high molecular weight band is compared for each anti-sLe^X antibody concentration in untreated (black bars) or STNA-treated (white bars) samples. Average and SD of three experiments is shown.

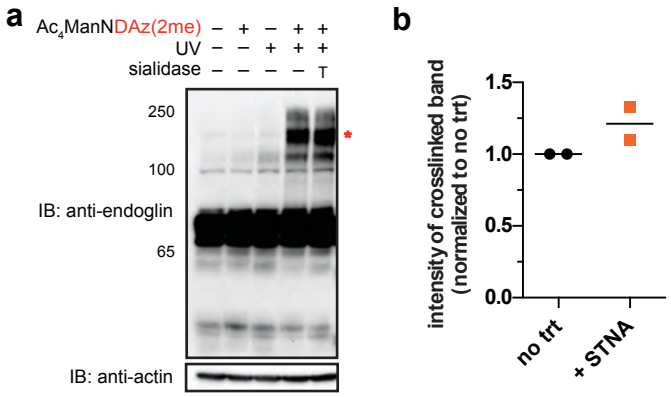

Figure S11. STNA treatment at 4 °C enhances SiaDAz-mediated crosslinking of endoglin. (**a**) Immunoblot of crosslinked lysates probed with anti-endoglin antibody. hCMEC/D3 cells were cultured with or without Ac_4ManNDAz(2me), then treated with no sialidase or STNA (T) at 4 °C before being UV irradiated or left untreated. (**b**) Quantification of normalized intensities of high molecular weight crosslinked bands (asterisk in panel **a**). Comparison is between cells treated with no sialidase or STNA at 4 °C prior to crosslinking.

Supporting Information References

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