Glycocalyx scaffolding with synthetic nanoscale glycomaterials

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SUPPORTING INFORMATION

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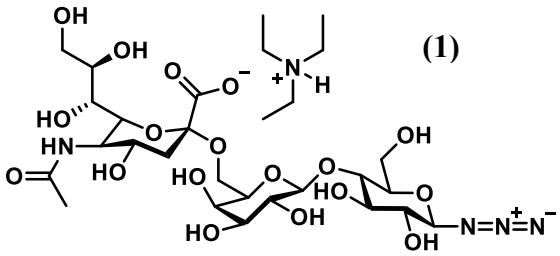
Materials.

6'-sialyllactose and 3'sialyllactose glycan were obtained from Carbosynth (San Diego, CA). Unless otherwise stated, all other chemicals were purchased from Sigma Aldrich. Purchased starting materials were used as received unless otherwise indicated. Size exclusion chromatography was performed on a Hitachi Chromaster system equipped with an RI detector and a 5 μm, mixed bed, 7.8 mm I.D. x 30 cm TSKgel column (Tosoh Bioscience). Polymers were analyzed using an isocratic method: 0.7 mL/min in DMF (0.2% w/v LiBr, 70 ^{o}C). AlexaFluor 488-C_5-malelimide was purchased from Molecular Probes (Cat # A10254). DSPE-PEG(2000)-DBCO was purchased from Avanti Lipids (Cat # 880229). PD-10 columns were purchased from GE Healthcare Life Sciences (Cat. # 17085101). Quick Spin Columns for radiolabeled DNA purification were purchased from Roche Diagnostics (Cat. # 11273913001).

Synthesis of 6'-sialyllactose-azide triethylammonium salt (1).

Installation of the reducing-end azide was achieved by using similar methods previously reported.[1,2] In 1 mL of 4:1 D_2O:CH_3CN, 6'-sialyllactose (28.4 mg, 0.0433 mmol) and triethylamine (30 μL, 0.2116 mmol) were mixed and stirred at 0 ^{o}C for 5 minutes. Freshly recrystallized ADMP (2-azido-1,3-dimethylimidazolinium hexafluorophosphate; 37 mg, 0.1300 mmol; previously synthesized as previously described[3]) was added to the stirred solution at 0 ^{o}C and allowed to react for 3h. NMR of the crude mixture after 3h showed loss of anomeric peaks from 6'-sialyllactose (65% conversion). The reaction mixture was then diluted with 4 mL of water containing 0.1% trifluoroacetic acid (TFA) and a 1.8 mL aliquot was purified by semipreparative HPLC (column, Jupiter Proteo C12, ϕ10 x 250 mm; eluent = 5:100:0.1 CH_3CN:H_2O:TFA; flowrate = 4 mL/min; column oven, 27 ^{o}C; detection, UV (214 nm). The collected fractions were pooled and lyophilized to yield clear colorless crystals (5.54 mg per aliquot).



(1)

(1) 6'-sialyllactose-azide triethylammonium salt – ^{1}H NMR (500 MHz, D_2O); δ (ppm) 5.34 (s, 1H) 4.67 (d, J = 7.88, 1H), 4.40 (d, J = 7.88 Hz, 1H), 4.04 (d, J = 7.88 Hz, 1H), 3.92 - 3.44 (m, 16H), 3.27 (t, J = 8.95 Hz, 1H), 3.13 (q, J = 7.30 Hz, 6H), 2.68 - 2.61 (m, 1H), 1.96 (s, 3H), 1.77 - 1.63 (m, 1H), 1.21 (t, J = 7.40 Hz, 9H). Mass Spectrometry (ESI-MS) calculated for $C_{29}H_{52}N_5O_{18}$ $[M\text{-}Et_3N]^{1-}$, m/z 657.21, found $[M\text{-}Et_3N]^{1-}$, m/z 658.31; $[M+H\text{-}Et_3N\text{-}N_3]^{1-}$, m/z 615.36.

Figure S1. ^{1}H NMR of 6'-sialyllactose azide (**1**).

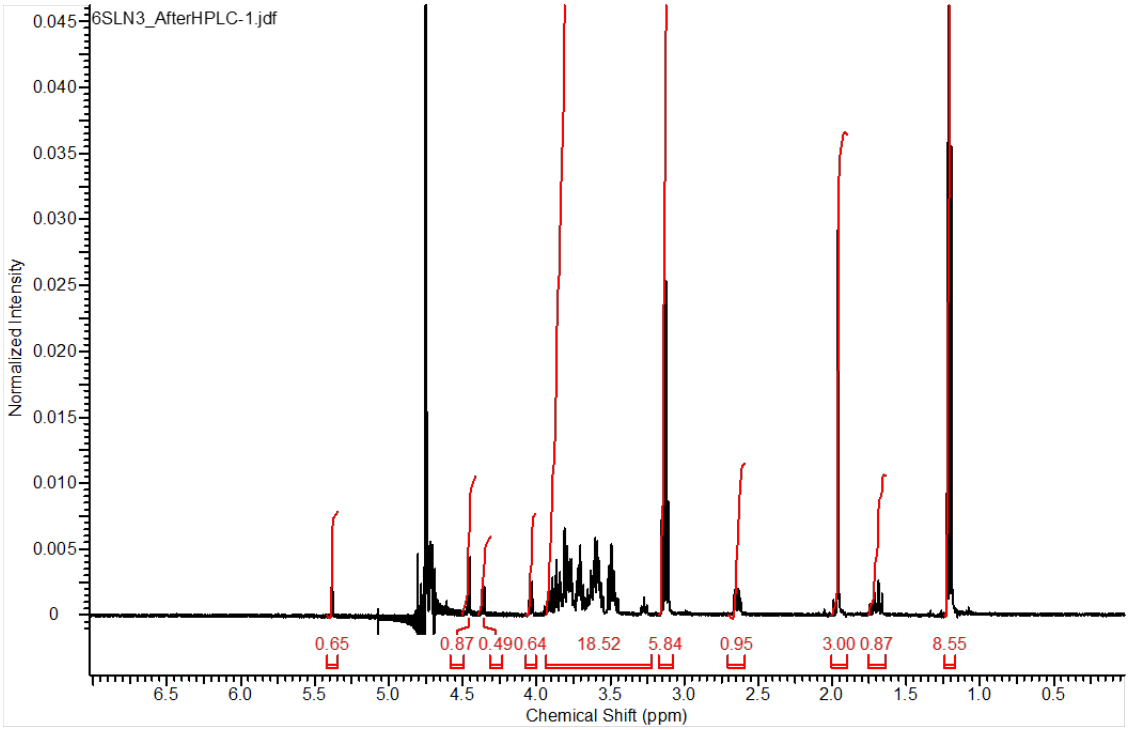


Figure S2. ^{13}C NMR of 6'-sialyllactose azide (**1**).

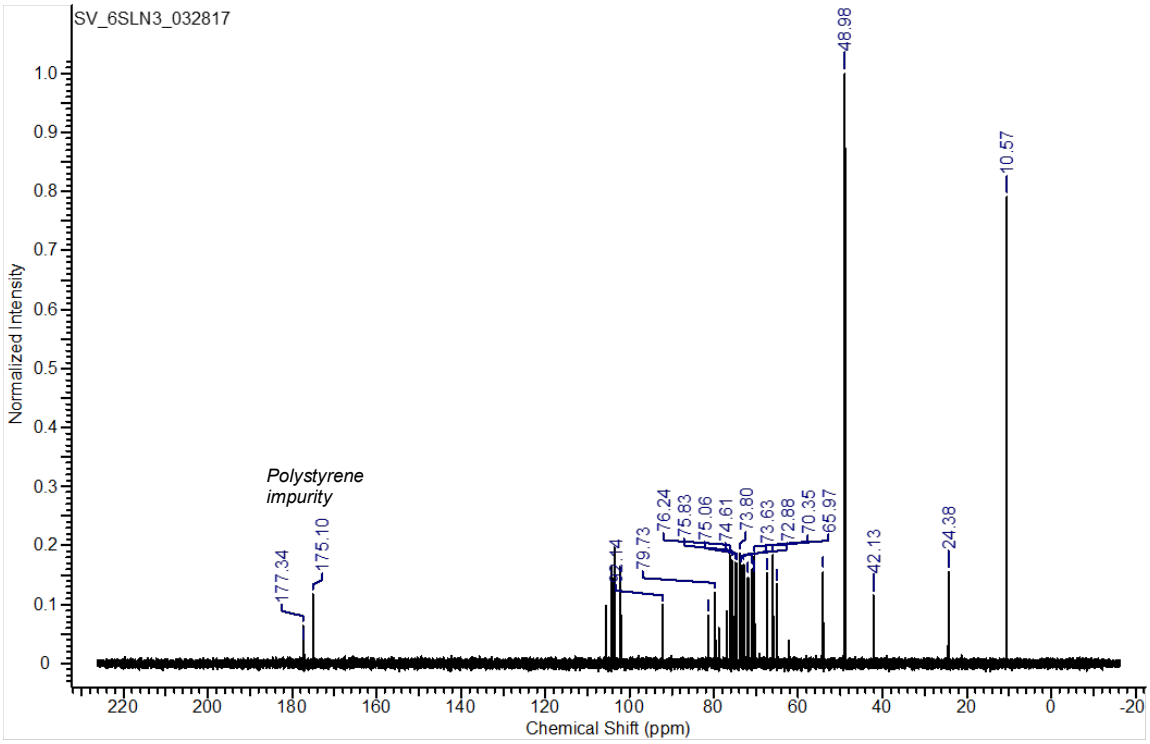
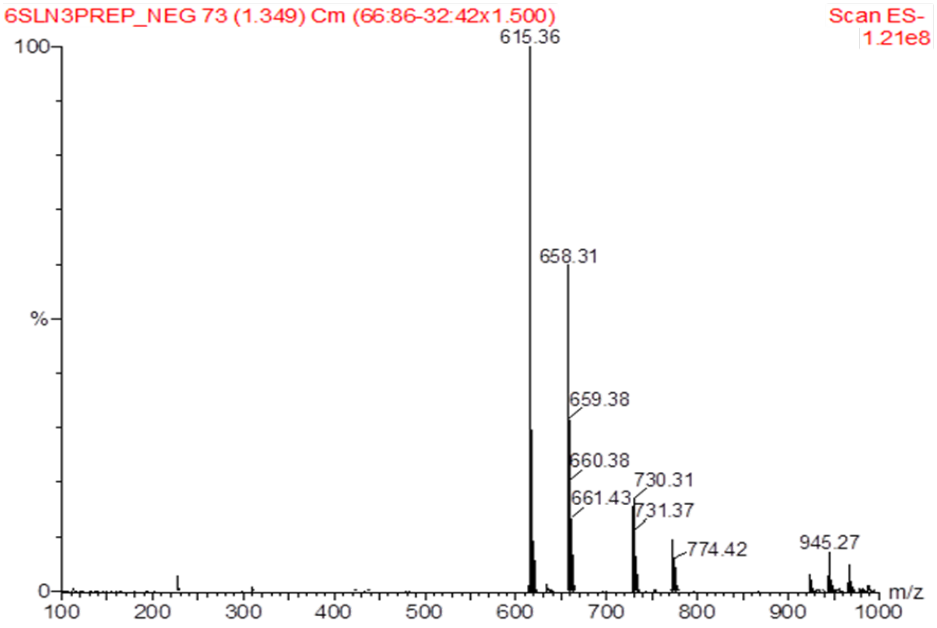
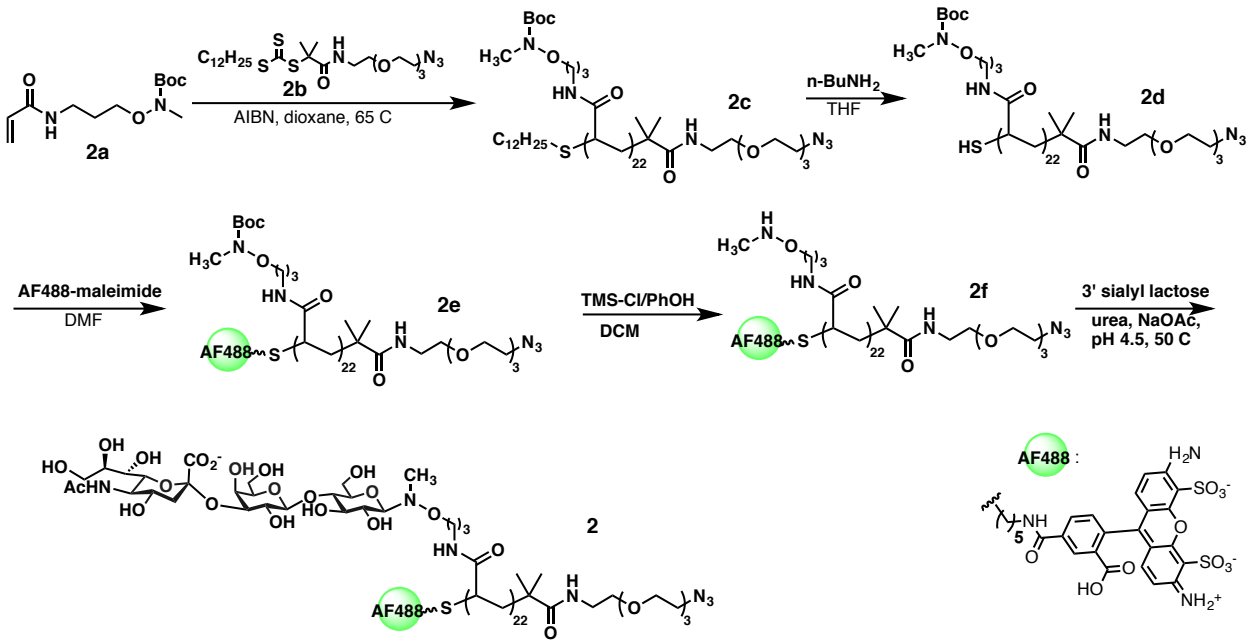


Figure S3. ESI-MS of 6'-sialyllactose azide (**1**).



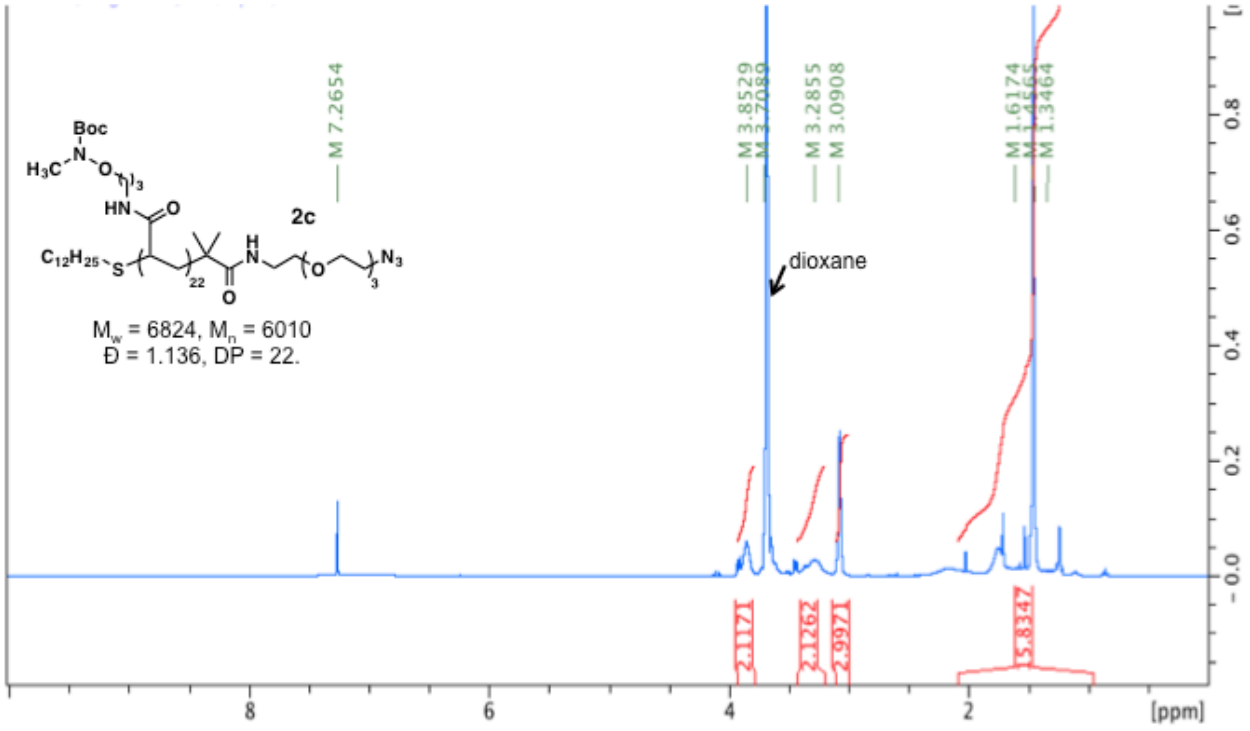
Scheme 1. Overview of 3'sialyllactose glycopolymer (**2**) synthesis.



Synthesis of 3'-sialyllactose glycopolymer (2) from monomer (2a).

RAFT polymerization. The RAFT polymerization of this Boc-protected *N*-methylaminooxypropylacrylamide (**2a**) monomer has previously been described.[4] A Schlenk flask equipped with a magnetic stir bar was charged with an azide-terminated chain transfer agent (**2a**, 38.58 mg, 80.9 μmol, 0.07 mol% with respect to **2a**), followed by the radical initiator AIBN (11.25 mg. 68.5 μmol, 0.06 mol% with respect to **2a**), and the *tert*-butyl (3-acrylamidopropoxy)methyl carbamate monomer (**2a**; 296 mg, 1.15 mmol, delivered as 420 μL of a 360 mM solution in anhydrous dioxane), and anhydrous dioxane (172 mg). After degassing with six free-pump-thaw cycles, the reaction was allowed to proceed at 65 °C for seven hours. The reaction was quenched by submerging the flask in a dry ice-acetone bath. The mixture was then diluted in ether, and precipitated three times in hexanes. The resulting residue was concentrated in $CHCl_3$ and dried under high-vacuum to yield polymer (**2c**; 184 mg, 62.2%). ^{1}H NMR ($CDCl_3$, 300 MHz): δ (ppm): 3.90-3.65 (bs, 2H), 3.35-2.80 (bm, 5H), 1.80-1.05 (bm, 16H). GPC (DMF, 0.2% LiBr): Mw = 6824, Mn = 6010, Đ = 1.136, DP (n) = 22.

Figure. S4 ^{1}H NMR analysis of polymer backbone 2c.



End-deprotection and fluorophore labeling. Polymer **2c** (9.38 mg) end-deprotection was achieved by reaction with n-butylamine in THF (20 mM, 0.5 mL) for 2 hours at 0 °C. Following dilution in ether and precipitation with excess hexanes (3x), the end-deprotected polymer (**2d**) was isolated as a white solid (7.99 mg, 81.3%). ^{1}H NMR ($CDCl_3$, 300mHz) δ (ppm): 3.90-3.65 (bs, 2H), 3.35-2.80 (bm, 5H), 1.80-1.05 (bm, 16H). GPC (DMF, 0.2% LiBr): Mw = 6826, Mn = 6030, Đ = 1.132, DP ≈ 22. UV–Vis 310 nm (CH_2Cl_2, 100 μM) = 0.075. The isolated material (7.99 mg) was then reacted with an AlexaFluor 488 C_5-maleimide/DMF solution (1.1 eq; 2 mM, 694 μL) overnight at room temperature. The resulting material was diluted in ether, precipitated in excess hexanes (3x), dand ried under high-vacuum to yield the AlexaFluor488-labeled polymer (**2e**; 6.63 mg, 82.9%).

Fig. S5. GPC analysis of polymer backbone (**2c**; solid) and end-deprotected polymer (**2d**; dotted). A higher molecular weight species is observed in **2d** (dotted) due to spontaneous oxidation of the free thiols of the polymer to form disulfide bridges.

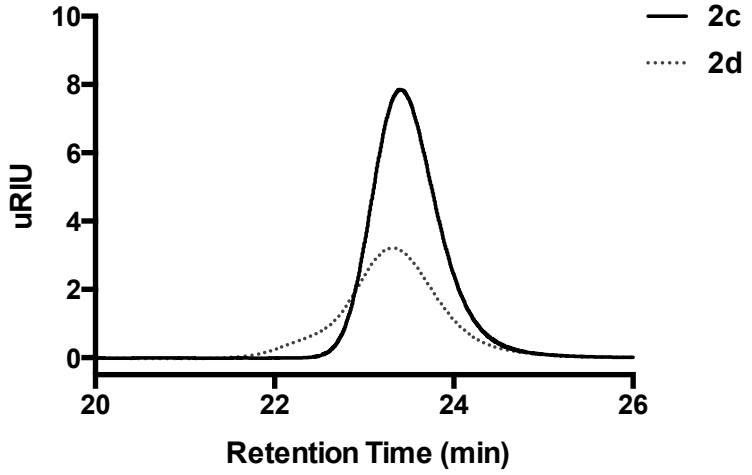


Fig. S6. UV-Vis analysis of polymer backbone (**2c**; solid) **and end-deprotected polymer** (**2d**; dotted)**.** The loss of the peak at 310 nm for **2d** indicates removal of the trithiocarbonate protecting group.

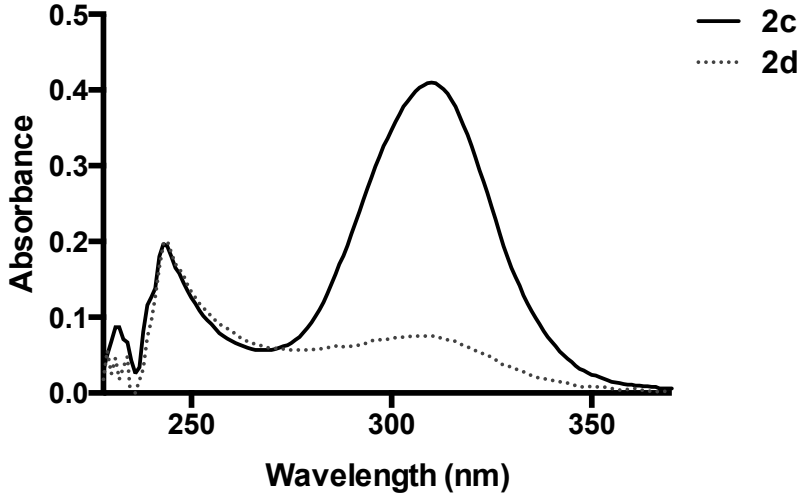
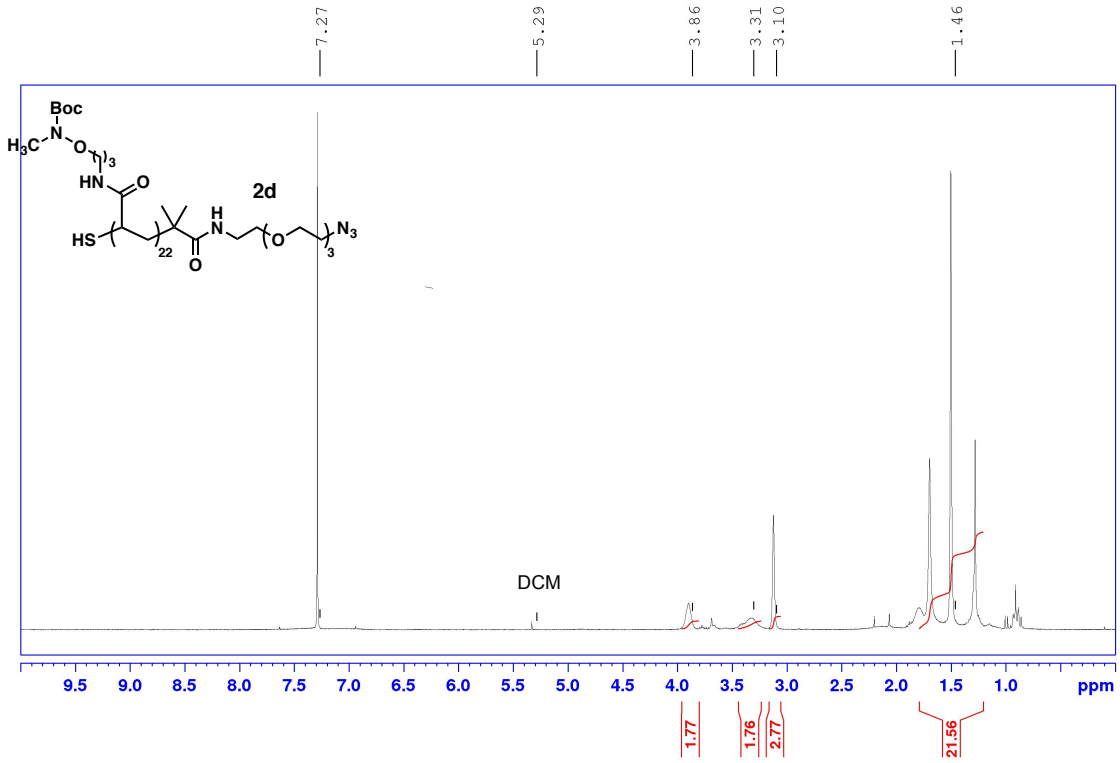


Figure S7. ^{1}H NMR analysis of end-deprotected polymer backbone **2d.**



Boc deprotection and glycan ligation. The AlexaFluor488-labeled polymer backbone **2e** (6.63 mg) was reacted with a freshly prepared mixture of chlorotrimethylsilane (3 M) and phenol (1 M) in DCM (0.5 mL) for 2 hours, dark, at RT. The mixture was then precipitated in ether following dilution in methanol. After drying the precipitate under a nitrogen stream, the product was dissolved in water, isolated by a PD-10 column, frozen, and lyophilized to yield the Boc-deprotected polymer **2f** (2.69 mg, 61%). ^{1}H NMR (D_2O, 300 MHz) δ (ppm): 3.90-3.65 (bs, 2H), 3.35-2.80 (bm, 5H), 1.80-1.05 (bm, 16H). UV–Vis spectrophotometry was used to determine AlexaFluor488 labeling efficiency of this water-soluble polymer: **2f**, (AlexaFluor488, λ_{max}: 495 nm in H_2O) = 58%. The resulting polymer **2f** (2.69 mg) was then dissolved in a sodium acetate buffer (1 M NaOAc, 1 M urea, pH 4.5) such that the solution contains 200 mM of the polymer side chains. Then, a PCR tube (0.5 mL) containing 3'-sialyllactose (9.12 mg, 1.1 eq.) was charged with the solution of **2f (**63.3 μL**)**, and the viscous solution was heated to 50 °C in a thermocycler for 72 hours. The resulting glycopolymer was purified via QuickSpin DNA columns using deuterated phosphate buffer (150 mM NaCl, 100 mM phosphate, pD 7.4), to yield **2** (0.5 mL). The final concentration was determined by UV-Vis analysis and the AlexaFluor488 labeling efficiency ([pol] = 908 μM). ^{1}H NMR analysis was used to determine glycan ligation efficiencies (45%).

Figure S8. ^{1}H NMR analysis of Boc-deprotected polymer (**2f**).

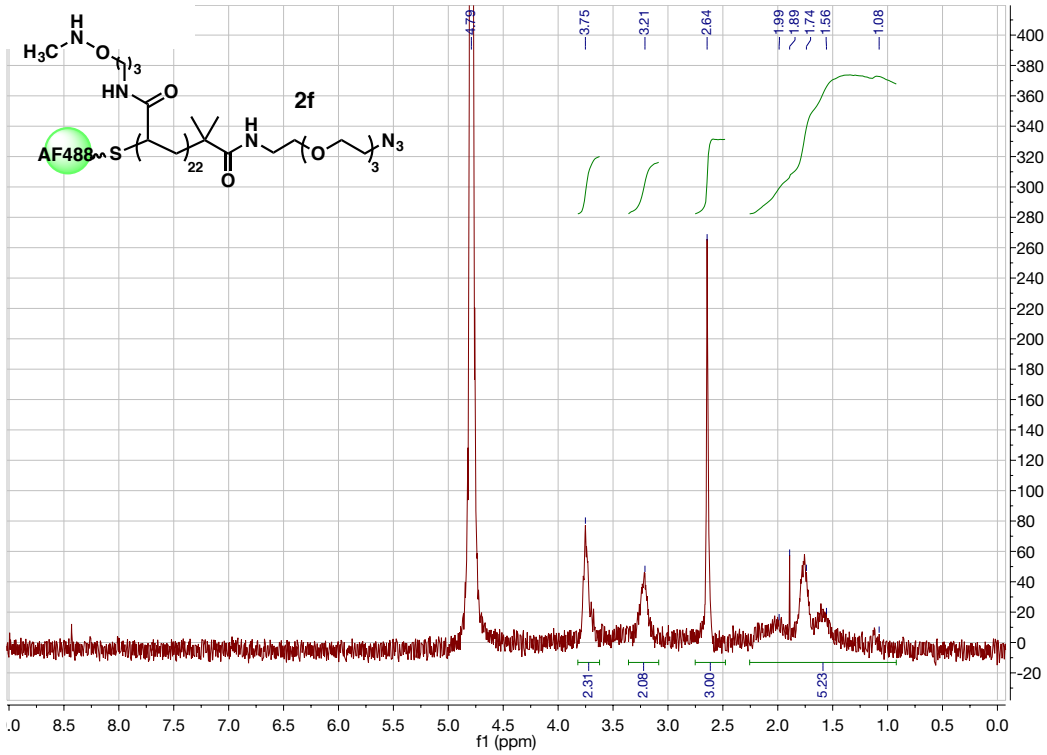
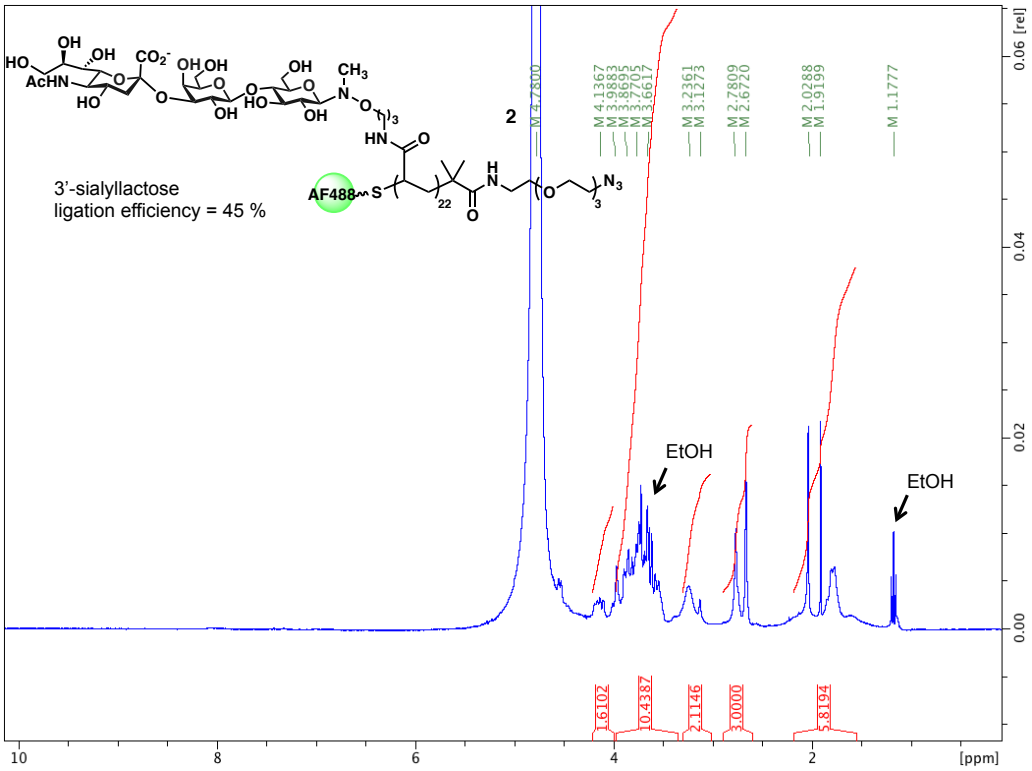


Figure S9. ^{1}H NMR analysis of 3'-sialyllactose azide glycopolymer (**2**).



Synthesis and characterization of lipid conjugates.

6'-sialyllactose-azide (**1**) or 3'-sialyllactose glycopolymer-azide (**2**) were reacted with 1.1 eq. of DSPE-PEG(2000)-DBCO (**3**, 10 mM) in a 10% d6-DMSO/D_2O solution overnight at RT. The resulting crude mixture was used directly for experiments.

Scheme 2. Synthesis of glycolipid and glycopolymer conjugates **(4** and **5**).

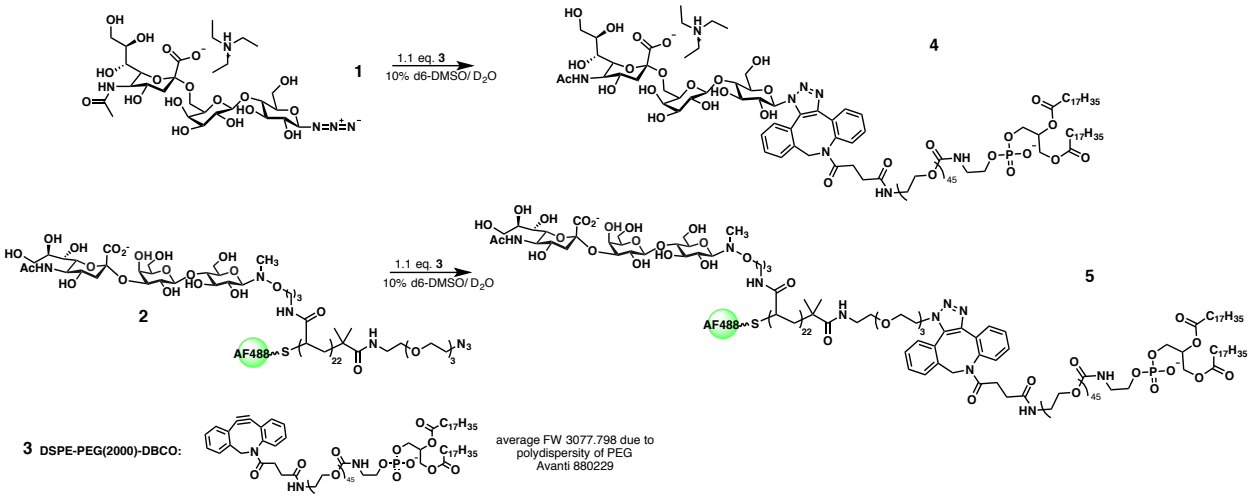


Fig. S10. ^{1}H NMR of 6'-sialyllactose glycolipid conjugate (**4**).

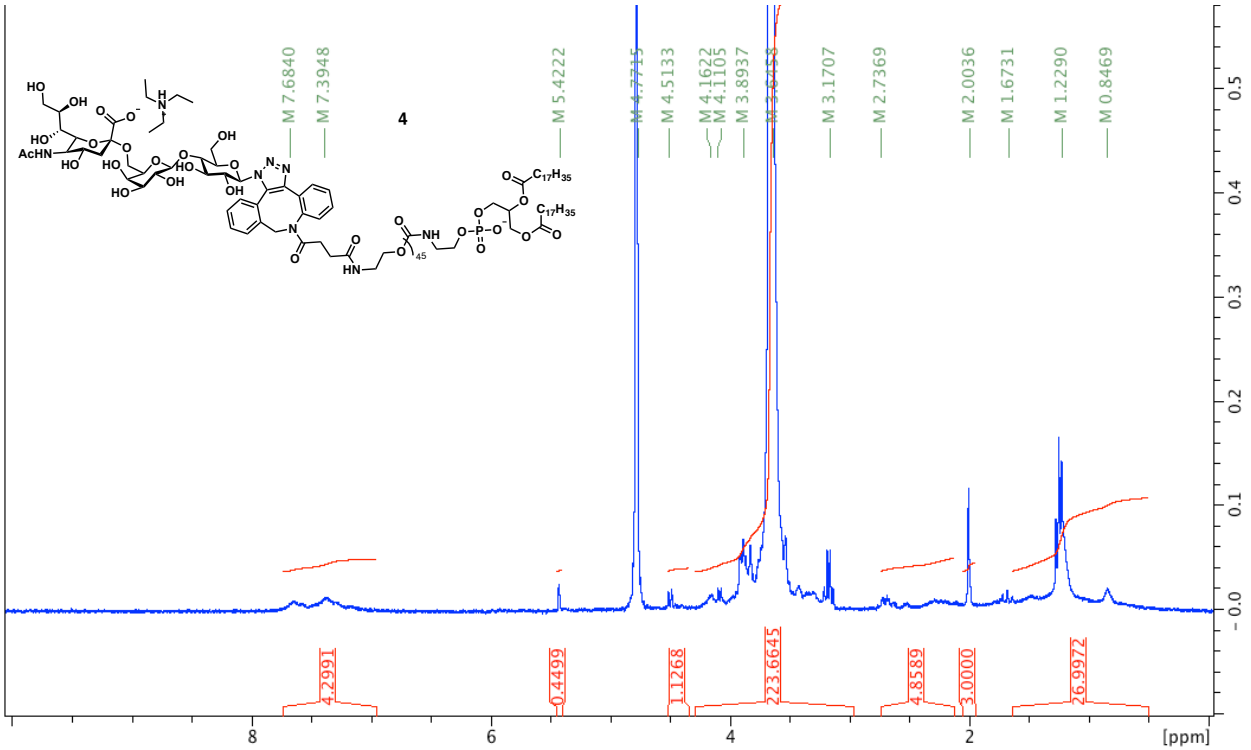
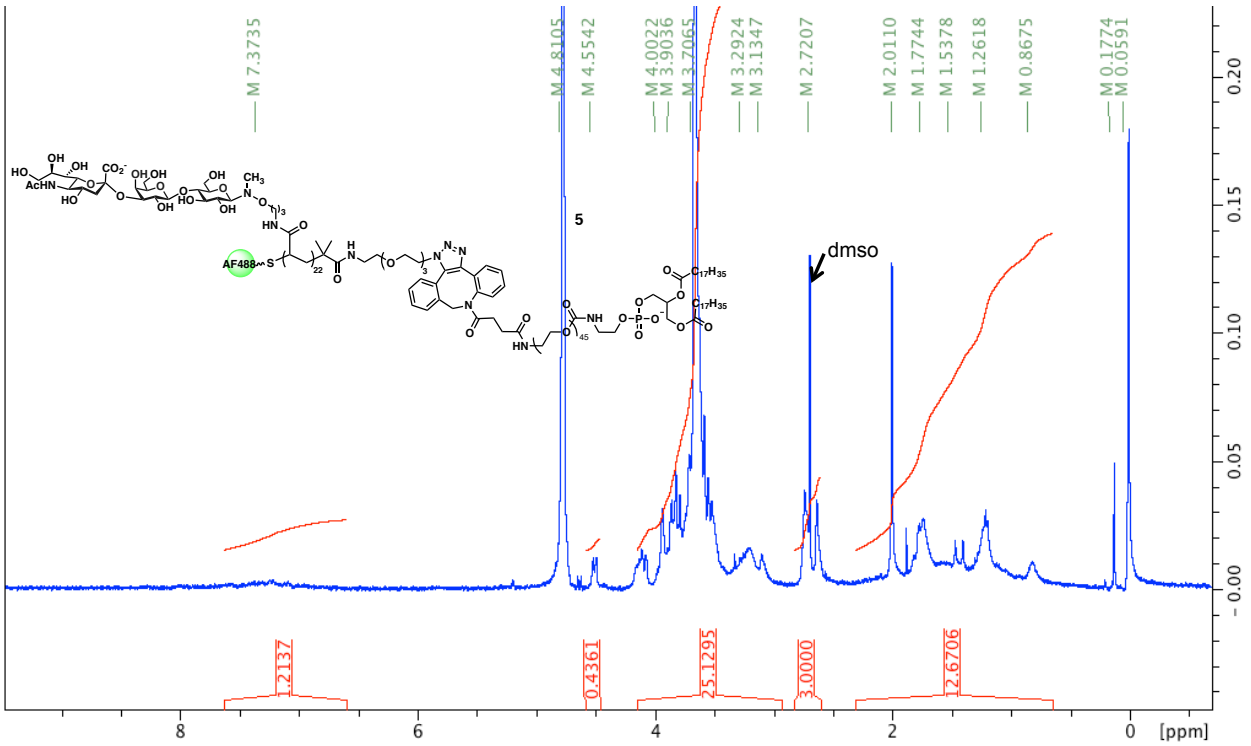


Figure S11. ^{1}H NMR of glycopolymer lipid conjugate (**5**).



Biological materials and methods. CHO-Lec2 cells (originally named Pro-5WgaRII6A) were obtained from ATCC (Cat # CRL-1736). CHO-Lec2s were cultured in MEM α (no nucleosides; Gibco Cat# 12561056) + 10% FBS (Origin: Australia; Life Technologies 1009133, lot 1647565). CHO-Lec2s were cultured as adherent cells on tissue-culture treated plastic dishes, and passaged every two days 1:10, by trypsinization with 0.05% Trypsin-EDTA (Gibco Cat # 25300120). The following conjugates were obtained commercially: biotinylated *Maackia amurensis lectin* II (bMALII, Cat # B-1265, 1 mg/mL) from Vector Labs (Burlingame, CA); Dylight649 conjugated *Sambucus nigra lectin* (Dy649-SNA, Cat # Dy649-6802-1) and biotinylated *Sambucus nigra lectin* (bSNA, Cat # B-1305, 1 mg/mL) were purchased from EY Labs (San Mateo, CA); Cy5 conjugated streptavidin (Cy5-Strep, Cat # SA1011) from Molecular Probes (Grand Island, NY).

Cellular remodeling with 6'-SL glycolipid or 3'-SL glycopolymer. CHO-Lec2 cells were seeded to confluency 24 hours prior to incubation on tissue-culture treated 24-well plates. Next day, cells were washed once with DPBS (-Ca, -Mg), and incubated with desired concentrations of the material in MEMα for 1 hour at 37 °C, 5% CO_2. Cells were visually checked for general health after incubation with the polymers by microscopy. No significant effects on cellular morphology were observed up to 1 mM final concentrations of either material. The cells were then washed twice with DPBS, trypsinized for 3-5 minutes to detach adherent cells, neutralized with an equal volume of MEMα + 10%FBS, and harvested into 1.5 mL eppendorf tubes. Cell suspensions were then pelleted (300 xg, 4 min, RT), washed once with DPBS, and fixed (1% PFAPBS) for 30-60 minutes at 4°C. After two sequential pelleting and washing steps, the cell suspensions were then stained for sialic acids with bMALII (1:100; 10 μg/mL) or Dy649 (1:100; 5 μg/mL) in 1% BSA/DPBS (+Ca, + Mg) overnight at 4°C, with gentle agitation. bMAL samples were washed and pelleted twice prior to labeling with Cy5-Streptavidin (1:500) in 1% BSA/DPBS (+Ca, + Mg) for an additional hour at 4 °C. Samples were further washed and pelleted twice prior to flow cytometry analysis. Graphs were generated using GraphPad Prism (v 6.0). Bar graphs and XY-graphs are depicted as means ± SD of duplicate wells.

Flow cytometry. CHO-Lec2 or CHO-K1 (WT) cells were dissociated as before, and analyzed on a BD Accuri C6 flow cytometer. During collection, cells were gated using a FSC-H vs. SSC-H scatter plot, and 10, 000 cells in the relevant gate were collected per sample. FlowJo (v. 10) was then used to analyze the results.

Fluorescence microscopy. All imaging and processing was performed with a Zeiss AxioVert.A1 epifluorescence microscope and ZEN software, respectively. For visualizing polymer incorporation via the AlexaFluor488 fluorophore, cells in 24-well plates were fixed in 4% PFA/PBS for 10 mins at RT, washed twice with DPBS, and imaged directly in DPBS. To visualize SNA staining, cells were similarly fixed, and were then incubated with bSNA (1:25; 40 μg/mL) in 1% BSA/DPBS (+Ca, +Mg) overnight at 4°C, with gentle agitation. After washing twice, cells were further incubated in Cy5-Streptavidin (1:400) in 1% BSA/DPBS (+Ca, + Mg) for an additional hour at RT. Cells were imaged directly in DPBS after two additional washes.

Figure. S12. Comparison of CHO-K1 (WT) and CHO-Lec2 sialic acid content. CHO-Lec2 mutants display significantly less α(2-6) and α(2-3) sialic acids, as indicated by reduced SNA and MALII lectin staining, respectively.

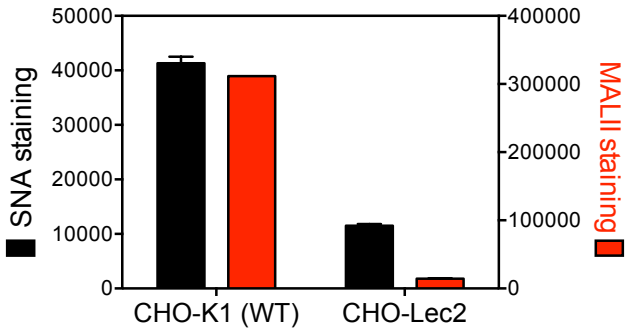


Figure S13. SNA staining (red) of CHO-Lec2 cells remodeled with or without increasing concentrations of (**4**), compared to CHO-K1 (wild-type) cells. Scale bar: 50 μm.

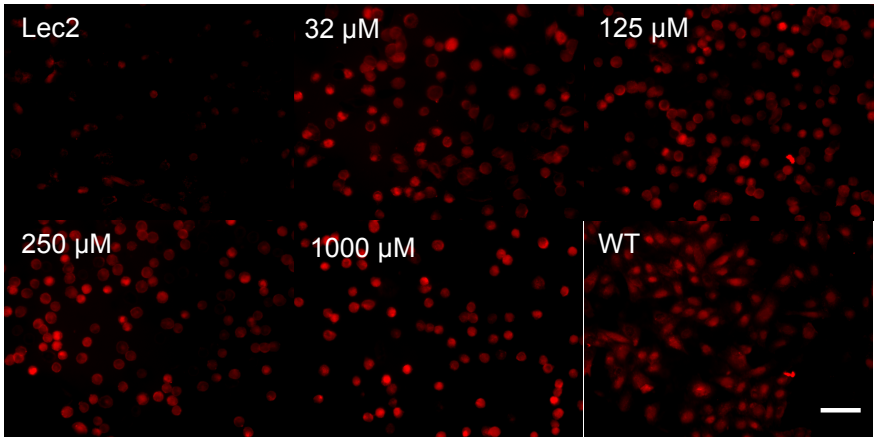


Figure S14. AlexaFluor488 polymer fluorescence of CHO-Lec2 cells remodeled with increasing concentrations of (**5**). Scale bar: 50 μm.

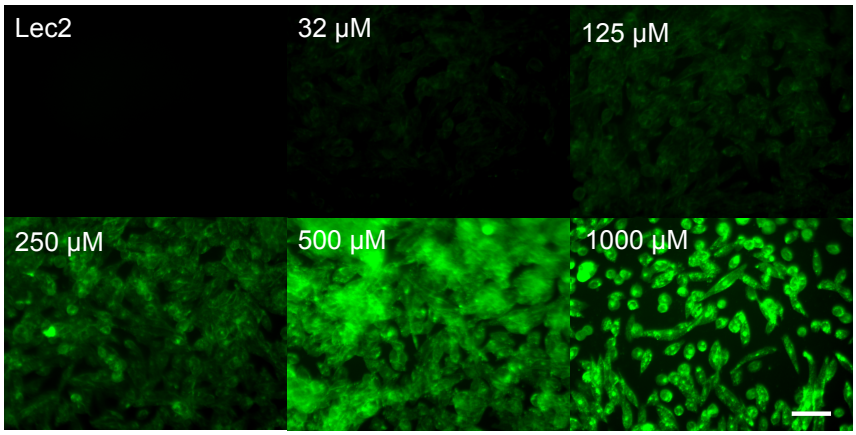


Figure S15. AlexaFluor488 polymer fluorescence to evaluate nonspecific binding of azido-polymer **2** versus incorporation of DSPE-terminated polymer **5**. Left: fluorescence microscopy, scale bar: 50 μm. Right: Flow cytometry analysis.

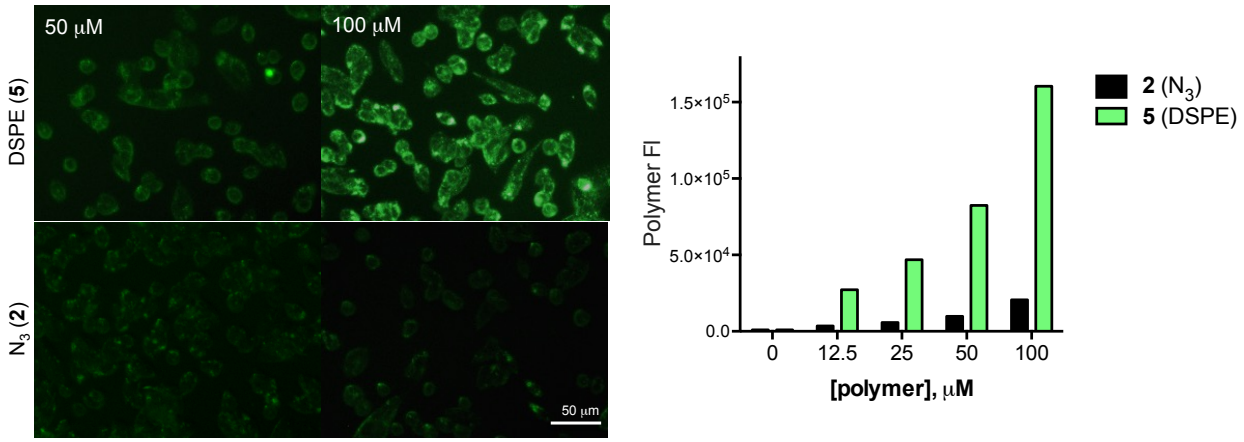
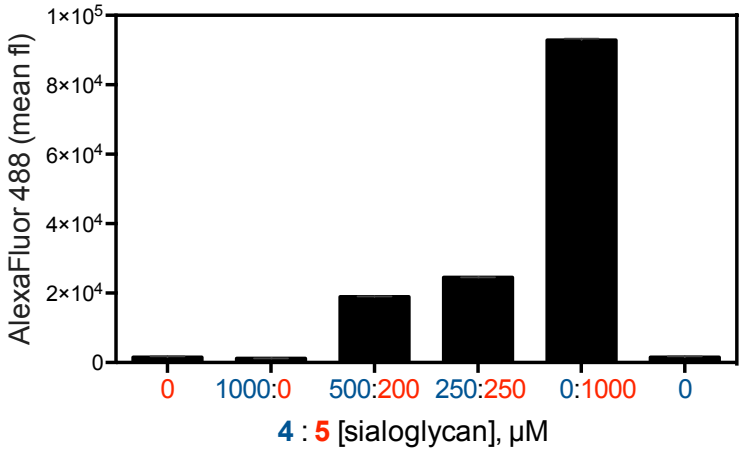


Figure S16. AlexaFluor488 polymer fluorescence of CHO-Lec2 cells resulting from the incubation of AlexaFluor488-labeled 3'-sialyllactose polymer **5** mixed with 6'-sialyllactose glycolipid **4**. Polymer fluorescence **5** still responds in a dose-dependent manner, even when mixed with **4.**



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