Electronic Supplementary Material (ESI) for Analyst.

Supporting Information

for

Enzymatic glycoengineering-based spin labelling of cell surface sialoglycans to enable their analysis by electron paramagnetic resonance (EPR) spectroscopy

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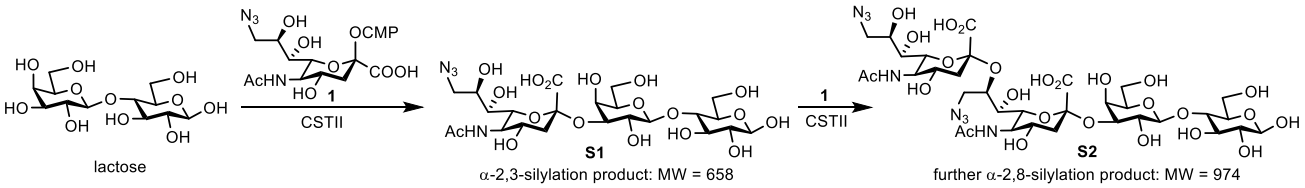
I. Materials and Methods

Materials. HeLa cell line was purchased from American Type Culture Collection (ATCC, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin (10000 U/mL), and phosphate buffered saline (PBS) were purchased from Thermo Fisher Scientific (USA). Dibenzocyclooctyne-fluorescein (DBCO-FAM) was purchased from Lumiprobe (USA). CMP-Neu5Ac9Az was synthesized by the reported method, and its NMR and MS spectra matched with that in the literature (S. Han, B. E. Collins, P. Bengtson, and J. C. Paulson, *Nat. Chem. Biol.*, 2005, 1, 93; B. Cheng, L. Dong, Y. Zhu, R. Huang, Y. Sun, Q. You, Q. Song, J. C. Paton, A. W. Paton, and X. Chen, *ACS Chem. Biol.*, 2019, 14, 2141). The CSTII enzyme was prepared according to the reported method using a pET22b vector (J. Cheng, H. Yu, K. Lau, S. Huang, H. A. Chokhawala, Y. Li, V. K. Tiwari, and X. Chen, *Glycobiology*, 2008, 18, 686; M. Sun, Y. Li, H. A. Chokhawala, R. Henning, and X. Chen, *Biotechnol. Lett*., 2008, 30, 671) with the gene custom-synthesized by GeneArt® from Thermo Fisher Scientific (USA). All other reagents used were purchased from Thermo Fisher Scientific (USA).

Investigation of CSTII-mediated sialyation. Lactose (1.5 mg, 4.0 μmol) and Neu5Ac9Az-CMP (0.30 mg, 0.47 μmol) were dissolved in 50 mM Tris-HCl buffer (50 μL) containing 20 mM $MgCl_2$. The pH of this solution was adjusted using sodium hydroxide solution to be set between 8 and 9. To the solution was added CSTII (150 μg), which was followed by adding Tris-HCl buffer to reach a volume of 250 μL. Therefore, the final concentrations of lactose, Neu5Ac9Az-CMP, and CSTII for the reaction were 16.0 mM, 1.88 mM, and 0.6 mg/mL, respectively. The solution was incubated at 37 °C. During the process, 50 μL of aliquots were taken from the reaction mixture at different time points (5, 15, 30 and 60 min) and quenched with an equal volume of ice-cooled methanol. These samples were centrifuged at 14,000 rpm for 15 min, and the supernatant was isolated and analyzed with TLC (development solvent: isopropanol:H_2O:NH_4OH 8:3:2) and LC-MS (electron spray ionization ESI, negative ionization mode)

to detect α2,3- and α2,8;α2,3-disialylated products. Both TLC and MS results showed that only α2,3-sialylated lactose (*i.e.,* the GM3 glycan **S1**, MW = 658) was formed at the time points of 5, 15 and 30 min, whilst at 60 min, di-sialylated lactose (*i.e.,* the GD3 glycan **S2**, MW = 974) was also formed in a trace amount, which was observable by MS but not by TLC.



Scheme S1: CSTII-catalyzed sialylation reactions of lactose

EGE-based fluorescence labeling of cells and flow cytometry analysis: HeLa cells were cultured in DMEM media containing 10% FBS and 100 U mL^{-1} of Penicillin–Streptomycin until the 80~90% confluency was achieved. The cells were treated with trypsin and harvested as pellets by spinning at 600 g for 7 min. The cell pellets were washed twice with PBS buffer (pH = 7.4) containing 2% BSA. About 1 million cells were counted and resuspended in 50 µL of DMEM (without FBS) in a fresh tube. In the meantime, Neu5Ac9Az-CMP (0.1 mmol) was dissolved in 50 µL of 20 mM Tris-HCl buffer (containing 20 mM $MgCl_2$). After the pH of this solution was adjusted to be 8, CSTII (150 µg) was added, and the volume was adjusted with Tris-HCl buffer to 250 µl. For the controls, only Tris-HCl buffer (no CSTII). This solution was briefly vortexed and then transferred into the cell suspension. The mixture was incubated at 37 ºC for 30 min with occasional agitation through pipetting air to prevent cells from settling down. The cells were washed three times with FACS buffer (PBS containing 2% BSA, pH = 7.4). Then, a DBCO-FAM solution (50 µM, 100 µL) in FACS buffer was added and the solution was kept at room temperature (rt) in the dark for 60 min. Finally, the cells were washed again three times with FACS buffer before being analyzed on an Attune™ NxT flow cytometer using a blue excitation laser (488 nm wavelength).

Sialidase pre-treatment: After about 1 million HeLa cells were resuspended in 50 μL of DMEM without FBS as described above, Sialidase A (NEB, 100 U) was added per manufacturer's protocol and DMEM medium without FBS was added to reach a final volume of 200 μL. The cell suspension was incubated at 37 °C for 1 h with continuous shaking at 40 rpm. The reaction was quenched by adding chilled FACS buffer, followed by washing with FACS buffer for three times. Then the cells were subjected to CSTII-mediated EGE as described above and fluorescence or spin labeling.

Microscopic analysis of cells: About 10,000 cells were seeded in a culture dish. In the following day, the cells were washed with ice-cooled PBS buffer three times. After washing, to each dish was added Neu5Ac9Az-CMP (0.1 mM, 100 μL) in Tris-Cl buffer with pH adjusted to 8, which was followed by the addition of CSTII (50 μg), and the final volume was made up to 300 μL by adding DMEM. The culture dish was incubated at 37 °C for 30 min in a shaker at 40 rpm. The reaction was quenched with addition of ice-cooled FACS buffer (1 mL), followed by washing with FACS buffer three times. The cells were resuspended in FACS buffer solution of DBCO-FAM (50 μM, 200 μL) and incubated at rt for 1 h. The cells were washed three times with FACS buffer and then incubated in a PBS solution of 4′,6-diamidino-2-phenylindole (DAPI) (300 nM, 1 mL) at rt for 5 min. Thereafter, the cells were washed three times and then fixed with 4% paraformaldehyde (PFA). Finally, the cells were visualized under a fluorescence microscope.

EGE-based spin-labeling of cells: About 5 million cells were counted, resuspended in DMEM (50 μL) without FBS, incubated with Sialidase A (100 U), and washed as described above. Thereafter, these pre-treated cells were resuspended in DMEM (without FBS) and incubated with Neu5Ac9Az-CMP and CSTII for EGE as described above. In the control groups, cells were treated by the exactly same protocol but without CSTII. After the cells were washed three times with PBS buffer containing 2% BSA (pH = 7.4), a DBCO-SL solution (50 μM, 500 μl) was added and the suspension was incubated at rt in the dark

for 1 h. The cells were washed with PBS buffer containing 2% BSA three times, pelleted, and finally subjected to EPR analysis.

CW-EPR analysis of spin-labeled cells. The pellet of above spin-labeled cells was resuspended in PBS buffer (40 μL) containing 0.8% agarose. This cell suspension was immediately loaded into a 50 μL-microcapillary pipette tube (Hirschmann), and the sample was allowed to solidify before CW-EPR data collection. X-Band (9.5 GHz) CW-EPR absorption spectra were collected at 30 °C by a protocol reported in the literature (M. Jaiswal, T. Tran, Q. Li, X. Yan, M. Zhou, K. Kundu, G. Fanucci, and Z. Guo, *Chem. Sci*., 2020, 11, 12522) using a Magnettech MiniScope MS-5000 benchtop spectrometer equipped with a dielectric resonator. The spectra were reported as an average of 16 scans with 120 mT sweep width, 0.2 mT modulation amplitude, 100 kHz modulation frequency, and 1 mW incident microwave power (2 mW incident microwave power on the Bruker E500). All of the EPR spectra were area normalized to the cell number, and all spectra were baseline-corrected and processed using the LabVIEW software provided by C. Altenbach and W. Hubbell (https://sites.google.com/site/altenbach/labview-programs).

Line shape analyses and simulations of EPR spectra. EPR spectra were simulated using the chili and esfit functions of EasySpin. The A- and g-tensors utilized were previously determined: gxx = 2.0070, gyy = 2.0062, gzz = 2.0033, Axx = 6.7 G, Ayy = 6.7 G, and Azz = 35 G (M. Jaiswal, T. Tran, Q. Li, X. Yan, M. Zhou, K. Kundu, G. Fanucci, and Z. Guo, *Chem. Sci*., 2020, 11, 12522). The other parameters used in the EPR line shape simulation include linewidth parameter (that reflects relaxation effects and spin–spin interaction from nearest neighbor nitroxide moieties at 18-20 Ang distance), correlation time of motion (τ_c), and the ordering potential C20, which was allowed to vary to assess the motional order parameter S. Each EPR spectrum was simulated using 2-component simulation, which contained a fast motion component to describe the sharp peaks and a slower motion component to capture the broadened

areas of the spectrum. Simulations with only single component fast motions could not sufficiently recapitulate the broad features in the spectra.

II. Additional Results and Data

Table S1: FACS results of fluorescence-labeled cells with CSTII-mediated EGE for different time

	Mean Fluorescence Intensity (MFI)				
	Control	EGE 5 min	EGE 15 min	EGE 30 min	EGE 60 min
Trial #1	1131	2560	3686	6312	8570
Trial #2	1344	2676	3623	6560	8937
Trial #3	1288	2616	3652	5386	8763
Mean	1254.33	2617.33	3653.66	6086.00	8756.66
SD*	63.82	33.532	18.22	357.67	106.116

* SD: standard deviation

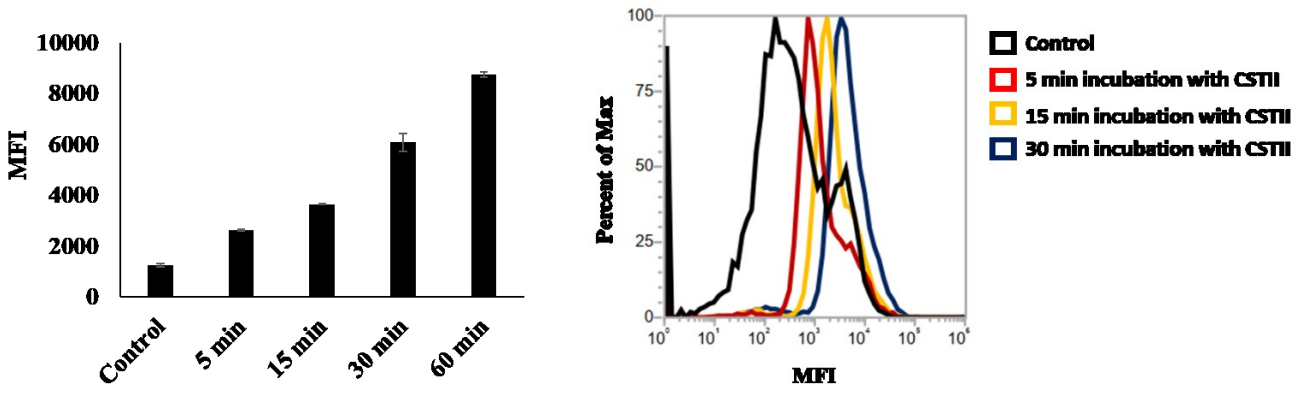


Figure S1: FACS results of HeLa cells treated first with a sialidase, and then with Neu5Ac9Az-CMP (control) or Neu5Ac9Az-CMP plus CSTII for 5, 15, 30 and 60 min, and finally with DBCO-FAM.

Table S2: Flow cytometry results of fluorophore-labeled cells after treatment with PNGase F

	Mean Fluorescence Intensity (MFI)		
	Control	Labeled Cell	Labeled Cell + PNGase F
Trial #1	486	2854	2001
Trial #2	496	2835	1977
Mean	491	2844	1989
SD	7.07	13.44	17.00
Students t test (P =)		2.88612E-10***	6.82E-08***

*** $P << 0.01$ compared to the control: statistically very significant.

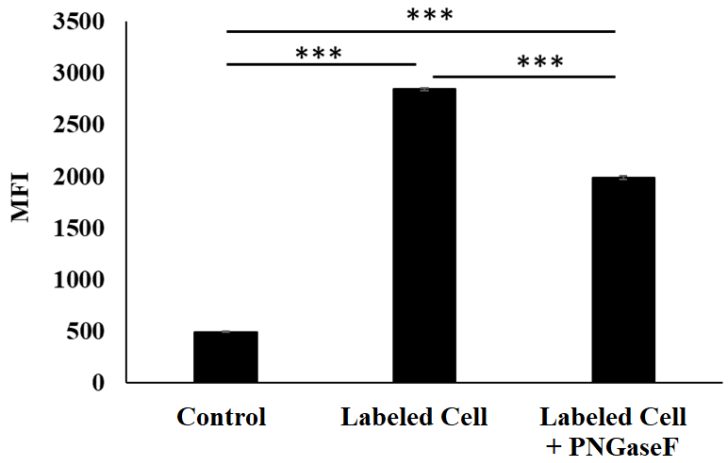


Figure S2: FACS results of fluorescence-labeled HeLa cells with or without PNGase F treatment. HeLa cells were sequentially treated with a sialidase, Neu5Ac9Az-CMP, and then DBCO-FAM (control); with a sialidase, CMP-Neu5Ac9Az + CSTII, and then DBCO-FAM (labelled cell); with a sialidase, CMP-Neu5Ac9Az + CSTII, DBCO-FAM, and then PNGAse F (labelled cell + PNGase F). *** denotes that the difference between paired groups is statistically very significant.

Table S3. Simulation results from both 1-component and 2-component fit of HeLa cells treated with CSTII, Neu5Ac9Az-CMP, and then DBCO-SL

Parameters	1-comp fit	2-comp fit	
		Comp 1	Comp 2
FWHM (G)	0.62	1.46	0.27
t_c (ns)	0.49	0.40	7.5
Percentage (%)	100	42	58
C20	0	0	0
S	0	0	0
RMSD	0.0623	0.0305	

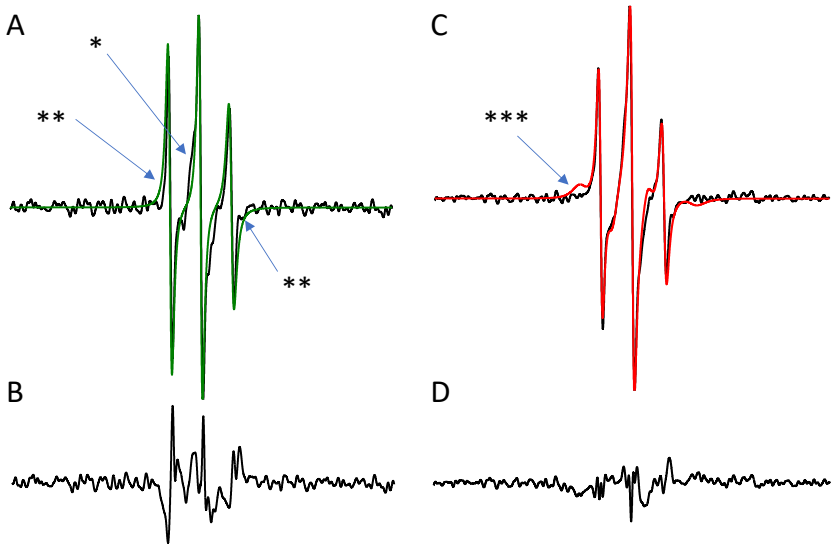


Figure S3: Comparison of EPR spectral simulations of control subtracted HeLa cells after incubation with Neu5Ac9Az-CMP with CSTII and DBCO-SL using (A) single component versus (C) two-component simulations. Residuals are shown for (B) single component versus (D) two-component simulations. Single asterix shows where the linewidth of the central line cannot be adequately recapitulated by a single component. Double asterix indicate where the high-field and low-field transition breads are overestimated by a single component fit. Triple Asterix indicates where the two-component slow-motion component overestimates the baseline.