Communication

Bifunctional Unnatural Sialic Acids for Dual Metabolic Labeling of Cell-Surface Sialylated Glycans

Lianshun Feng, Senlian Hong, Jie Rong, Qiancheng You, Peng Dai, Rongbing Huang, Yanhong Tan, Weiyao Hong, Can Xie, Jing Zhao, and Xing Chen

J. Am. Chem. Soc., **Just Accepted Manuscript** • DOI: 10.1021/ja402326z • Publication Date (Web): 31 May 2013

Bifunctional Unnatural Sialic Acids for Dual Metabolic Labeling of Cell-Surface Sialylated Glycans

Lianshun Feng,[⊥,†,‡] Senlian Hong, [¶,†,‡] Jie Rong,[⊥,†,‡] Qiancheng You,[†] Peng Dai,[†] Rongbing Huang,[†] Yanhong Tan,[†] Weiyao Hong,[†] Can Xie,[*,¶] Jing Zhao,[*,⊥] and Xing Chen[*,†,§,∥]

[⊥]School of Chemical Biology and Biotechnology, Shenzhen Graduate School of Peking University, Shenzhen 518055, China. [†]Beijing National Laboratory for Molecular Sciences, Department of Chemical Biology, College of Chemistry and Molecular Engineering, [¶]School of Life Sciences, [§]Synthetic and Functional Biomolecules Center, and [∥]Peking-Tsinghua Center for Life Sciences, Peking University, Beijing 100871, China.

Supporting Information Placeholder

ABSTRACT: Sialic acid analogues containing a unique chemical functionality or chemical reporter have been metabolically incorporated into sialylated glycans. This process is termed metabolic glycan labeling and has been emerged as a powerful tool for studying sialylation as well as other types of glycosylation. Currently, this technique can only install a single functionality. Here we describe a strategy for dual labeling of sialylated glycans using a new class of bifunctional sialic acid analogues. We designed and synthesized sialic acid analogues containing two distinct chemical reporters at both N-acyl and C-9 positions. We demonstrated that these bifunctional unnatural sialic acids were metabolically incorporated into cellular glycans and the two chemical reporters exerted their distinct functions. This approach expands the capability of metabolic glycan labeling on probing sialylation and glycan-protein interactions.

Sialic acids are often the outermost monosaccharides of cell-surface glycans in vertebrates.[1] Sialylated glycans, linked on glycoproteins or glycolipids, play important roles in diverse biological and pathological processes. For example, sialyl Lew^X, a sialic acid-containing tetrasaccharide, serves as a ligand for selectins, which plays a crucial role in leukocyte homing.[2,3] Sialylated glycolipids such as gangliosides regulate cell-cell and cell-pathogen interactions.[4] In addition, a high level of sialic acid expression is usually found in tumor cells, which has been shown to correlate with metastatic potential in several cancer types.[5] Despite the fundamental significance of sialylated glycans, it remains challenging to fully understand the functions of sialylation using conventional biochemical and genetic methods.[6]

In recent years, metabolic glycan labeling has emerged as a powerful approach to chemically probe sialylation as well as several other types of glycosylation in live cells and within living animals.[7,8] In this strategy, the cell's sialic acid biosynthetic pathway is harnessed to incorporate unnatural sialic acids that are modified with a bioorthogonal functional group. The bioorthogonal functionality can be conveyed at either *N*-acyl side chain or C-9 position of sialic acid analogues (Figure 1A). The metabolic labeling of sialic acids can also been achieved by feeding cells with the analogues of *N*-acetylmannosamine (ManNAc), the sialic acid biosynthetic precursor. For unnatural ManNAc analogues, the bioorthogonal functional group can be installed on *N*-acyl side chain, but not on the C-6 position, which corresponds to the C-9 position of sialic acid. The C-6 hydroxyl group of ManNAc is enzymatically modified by ManNAc 6-kinase during its biosynthetic conversion to sialic acid, so modification at C-6 position is not tolerable. In a second step, the cell-surface bioorthogonal group is reacted with a fluorescent probe bearing a complementary functional group for imaging sialic acids or reacted with an affinity tag for enrichment and identification of sialylated glycoproteins, using a bioorthogonal reaction. Azide and alkyne are the two most popular bioorthogonal groups, which have been exploited for metabolic labeling of sialic acids. The Bertozzi group demonstrated that azide could be incorporated into cell-surface glycans using *N*-azidoacetylmannosame (ManNAz)[9] or azido sialic acid (SiaNAz).[10] Our group recently used 9-azido sialic acid (9AzSia) to metabolically label sialylated glycans.[11] Metabolic incorporation of alkyne was reported by the Wong group using *N*-(4-pentynoyl)mannosamine (ManNAl), which was converted to cell-surface alkynyl sialic acid (SiaNAl).[12]

Another class of interesting functional group installed on unnatural sugars is photocrosslinker. In the same study by the Bertozzi group,[10] aryl azide was shown to be efficiently incorporated into sialylglycoconjugates using the sialic acid analogue bearing N-acyl aryl azide (i.e., SiaNAAz). The Paulson group later developed the sialic acid analogue containing aryl azide at C-9 position (9AAzSia) to capture the glycan ligand of CD22, a key regulator of B cell signaling.[13] More recently, the Kohler group showed that diazirine installed at N-acyl side chain of sialic acid (i.e., SiaDAz) and ManNAc (i.e., ManDAz) could be similarly used for studying sialic acid-protein interactions.[14]

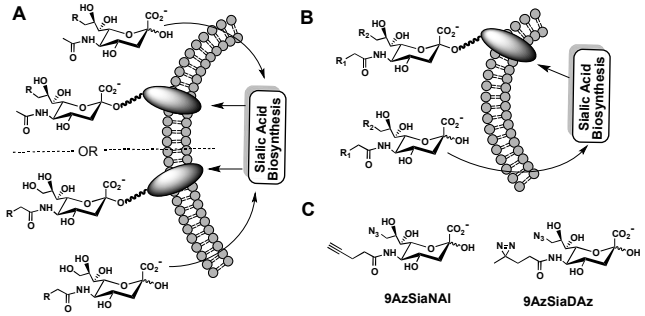
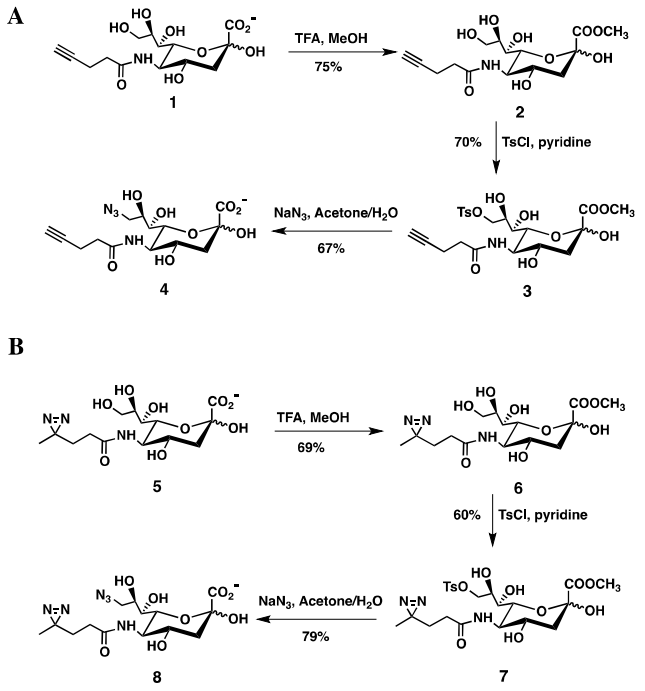


Figure 1. (A) Metabolic labeling of sialylated glycans using sialic acid analogues containing an unnatural functional group (e.g., bioorthogonal group or photocrosslinker) at N-acyl side chain or C-9 position. (B) Dual labeling strategy for simultaneously installing two unnatural functionalities at both N-acyl side chain and C-9 position using bi-functional unnatural sialic acids. (C) Two bifunctional sialic acid analogues used in this study.

All of the currently available sialic acid analogues can only endow sialylated glycans with mono-functionality. We were interested in expanding the metabolic labeling strategy to simultaneously incorporate two functionalities into sialic acids. We envisioned that the bifunctional sialic acids, once incorporated, would enable biological applications that are not possible with the monofunctional analogues. For example, two bioorthogonal functional groups could be used to conjugate two probes for dual-color imaging. A bioorthogonal group could be used to enrich the sialic acid-binding proteins when combined with a photocrosslinker. We reasoned that the biosynthetic machinery would tolerate sialic acid analogues with two distinct chemical reporters simultaneously installed at N-acyl side chain and C-9 position, respectively, based on its promiscuity for modification at either position (Figure 1B).

Scheme 1. Synthesis of 9AzSiaNAl and 9AzSiaDAz.



To test our hypothesis, we designed and synthesized two classes of bifunctional sialic acid analogues: 9AzSiaNAl contains two bioorthogonal functional groups, azide and alkyne, at C-9 position and N-acyl side chain, respectively; 9AzSiaDAz contains an azide at C-9 and a photocrosslinker, diazirine, at N-acyl side chain (Figure 1C and Scheme 1). To synthesize 9AzSiaNAl, we first generated SiaNAl (**1**) as previously reported.[15] The azido group was then installed on the C-9 position of SiaNAl to give 9AzSiaNAl (**4**), with overall yield of 35% (Scheme 1A). Similarly, 9AzSiaDAz (**8**,) was synthesized from SiaDAz (**5**)[14] with overall yield of 33% (Scheme 1B).

First, we determined whether 9AzSiaNAl and 9AzSiaDAz can be metabolically incorporated into cellular sialylglycoconjugates in various cell lines including CHO, HeLa, Daudi cells, and BJA-B K20 cells. The cells were treated with 9AzSiaNAl, 9AzSiaDAz, or 9AzSia as a positive control for 24 h, and detected for cell-surface azides using the copper-free click chemistry reaction[16] with aza-dibenzocyclooctyne-biotin conjugate (DBCO-biotin).[17] In this study, we used a DBCO-biotin conjugate containing a sulfo group (*Sulfo*-DBCO-biotin) for better water solubility. The cells were then stained with streptavidin-Alexa Fluor 647, and analyzed by flow cytometry (Figure 2A, and Supporting Information (SI) Figure S1, S2, S3, and S4). 9AzSiaNAl and 9AzSiaDAz-treated CHO cells both exhibited robust fluorescence labeling in a dose-dependent manner, indicating the metabolic incorporation of the bifunctional sialic acid analogues (Figure 2A, and SI Figure S1). The incorporation efficiencies of the bifunctional sialic acid analogues are at the comparable level of 9AzSia, indicating simultaneously incorporation of two chemical reporters at both the *N*-acyl group and C-9 is well tolerated by the sialic acid biosynthetic machinery. Similar results were obtained in HeLa cells (SI Figure S2), Daudi cells (SI Figure S3), and K20 cells (SI Figure S4). The relative incorporation efficiencies of three sialic acid analogues vary slightly in different cell lines and at different concentrations, but they are all in the same order of magnitude. These results demonstrate that the bifunctional sialic acid analogues can be generally used in various cell lines.

To further confirm the bifunctional sialic acids are incorporated into glycoproteins, CHO cells treated with 9AzSiaNAl, 9AzSiaDAz, and 9AzSia were reacted with *Sulfo*-DBCO-biotin, and then lysed. The cell lysates were characterized by anti-biotin Western blot. Similar to 9AzSia, 9AzSiaNAl and 9AzSiaDAz were both metabolically incorporated into a wide repertoire of glycoproteins (Figure 2B).

Interestingly, when we detected the cell-surface azides using the copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) assisted by the ligand BTTAA,[18] a biocompatible variant of click chemistry with improved reaction kinetics developed by the Wu group, similar levels of fluorescence were observed in cells treated with 9AzSiaNAl and 9AzSia (SI Figure S5). This implies that the cross-reaction between cell-surface 9AzSiaNAl is not significant, probably due to steric hindrance between sialylated glycans.

In addition, we evaluated the metabolic incorporation of the peracetylated 9AzSiaDAz (i.e., Ac_4-9AzSiaDAz, **9**, see the Supporting Information for the synthesis). Peracetylation has been used to significantly improve the metabolic efficiency of ManNAc analogues, up to hundreds of folds, by

facilitating the passive diffusion across the plasma membrane.[19] By contrast, peracetylation of sialic acid analogues has shown much less improvements.[10] In agreement with the previous reports, Ac_4-9AzSiaDAz exhibited only 3- to 5- fold increase of the incorporation efficiency at different concentrations, comparing to the free 9AzSiaDAz (SI Figure S6). We therefore used the free sialic acid analogues for the following studies.

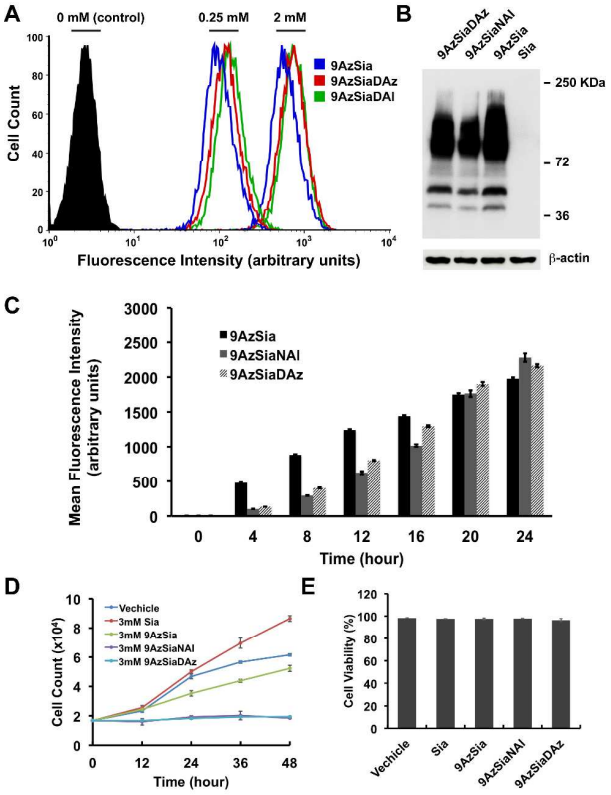


Figure 2. Evaluation of metabolic incorporation and biological effects of bifunctional sialic acid analogues. (A) CHO cells were treated with 9AzSia, 9AzSiaNAl, and 9AzSiaDAz at various concentrations for 24 h. The treated cells were labeled with *Sulfo*-DBCO-biotin and streptavidin-Alexa Fluor 647, and analyzed by flow cytometry. (B) Immunoblot analysis of glycoproteins from CHO cells metabolically labeled with unnatural sialic acids. The cells treated with 9AzSia, 9AzSiaNAl, 9AzSiaDAz, and sialic acid (Sia) were reacted with *Sulfo*-DBCO-biotin, and then lysed. The cell lysates were probed with HRP conjugated anti-biotin (top panel). Equal protein loading was confirmed using an anti-β-actin antibody (bottom panel). (C) The time-dependence of the incorporation of 9AzSia, 9AzSiaNAl, and 9AzSiaDAz at 3 mM concentration in CHO cells analyzed by flow cytometry. (D) Effects of bifunctional sialic acid analogues on the growth of CHO cells. The cells were treated with 3 mM 9AzSia, 9AzSiaNAl, 9AzSiaDAz, Sia, and vehicle, the number of cells was counted at various time points. (E) Effects of bifunctional sialic acid analogues on cell viability. CHO cells were incubated with 3 mM 9AzSia, 9AzSiaNAl, 9AzSiaDAz, and Sia for 24 h, the percentage of viable cells was counted using 7-AAD viability assay. Error bars represent the standard deviation from three replicate experiments.

We next performed detailed investigations on the biological effects of bifunctional sialic acid analogues. We first assessed the time-dependent incorporation of the bifunctional sialic acid analogues, in comparison with the monofunctional counterpart. CHO cells were incubated with 3 mM 9AzSia, 9AzSiaNAl, and 9AzSiaDAz for various periods of time, and the incorporation of the unnatural sialic acids was analyzed by flow cytometry. The metabolic incorporation of all three analogues increased over time and reached a similar maximal level after approximately 20 h (Figure 2C). The incorporation of 9AzSia ascended more steeply than the two bifunctional analogues in the first 12 h, after which it became more slowly until reaching the saturation. We next evaluated the effects of the sialic acid analogues on cell growth. At the highest concentration used, 3 mM, 9AzSiaNAl and 9AzSiaDAz exhibited significant inhibitory effects on cell growth of CHO cells (Figure. 2D) and HeLa cells (SI Figure S7A). Reduced cell growth was also previously observed when feeding cells with high concentration (above 100 μM) of peracetylated ManNAc analogues bearing diazirine.[20] Furthermore, we evaluated the cytotoxicity of the sialic acid analogues using 7-aminoactinomycin D (7-AAD) cell viability assay. No apparent toxic effects were observed at all concentrations for either 9AzSiaNAl or 9AzSiaDAz (Figure 2 E and SI Figure S7B). These results indicate that the bifunctional sialic acid analogues impair cell growth, probably by imposing cellular stress, but do not affect cell viability.

Introduction of dual or multiple bioorthogonal functional groups into biomolecules including DNA,[21] proteins,[22,23] glycans,[24,25] and lipids[26] have recently drawn great attention. We further demonstrated the bi-functionalities of cell-surface 9AzSiaNAl by using two-color fluorescence imaging (Figure 3). 9AzSiaNAl-treated HeLa cells were first reacted with DBCO-Fluor 488 to label cell-surface azides using copper-free click chemistry. The cell-surface alkynes were then reacted with azide-Alexa Fluor 647 using BTTAA-assisted CuAAC. As expected, the cells showed two-color labeling and two fluorescent dyes exhibited perfect co-localization, showing exclusively the merged yellow color (Figure 3A). Cells treated with 9AzSia or SiaNAl were only labeled with single color. To further confirm that the dual labeling of azide and alkyne occur simultaneously on the same 9AzSiaNAl molecules, we compared the cells treated with a mixture of 3 mM of 9AzSia and 3 mM SiaNAl by two-color co-localization and Förster resonance energy transfer (FRET) imaging. As expected, merged yellow color, though seemingly to a less extend, was also observed in cells treated with the mixture of two monofunctional sialic acid analogues, since some sialic acids may reside closely on cell surfaces (Figure 3B). The FRET is extremely sensitive to the donor-acceptor distance and the range of effective FRET is approximately 1-10 nm. We reasoned that dual labeling of the azide and alkyne of 9AzSiaNAl results in intramolecular FRET, which should be more effective than intermolecular FRET occurred in cells treated with the mixture of 9AzSia and SiaNAl. We observed significant FRET-induced fluorescence in HeLa cells treated with 9AzSiaNAl, while only very week signal was observed in cells treated with 9AzSia/SiaNAl mixture (Figure 3C, left column). Quantification of the FRET efficiency by donor dequenching after acceptor photobleaching showed FRET efficiencies in 9AzSiaNAl- and 9AzSia/SiaNAl-treated cells to be ~48% and ~6%, respectively (Figure 3C, right column).

These results indicate that the cell-surface 9AzSiaNAl was effectively dually labeled.

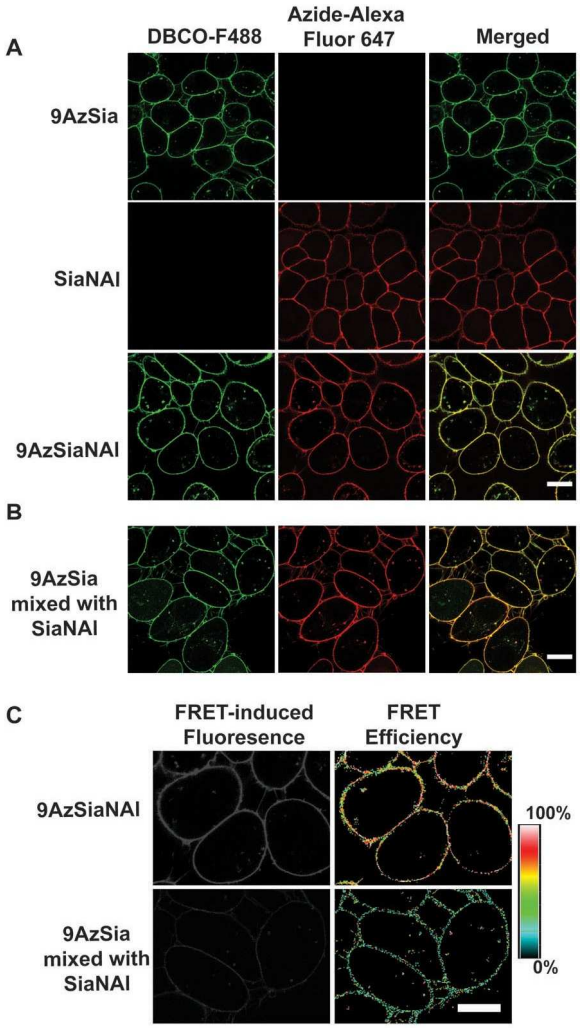


Figure 3. Two-color fluorescence imaging and FRET analysis of 9AzSiaNAl incorporated glycans on cell surfaces. (A) HeLa cells were treated with 3mM 9AzSia, SiaNAl or 9AzSiaNAl for 24 h. The treated cells were reacted with DBCO-Fluor 488 and Alexa Fluor 647-azide. (B) HeLa cells were treated with a mixture of 3 mM 9AzSia and 3 mM SiaNAl, followed by conjugation with DBCO-Fluor 488 and Alexa Fluor 647-azide. (C) FRET-induced fluorescence imaging (left column) and FRET efficiency imaging (right column) of HeLa cells treated with 9AzSiaNAl or with the mixture of two monofunctional sialic acid analogues. FRET-induced fluorescence was excited using the 488-nm laser and collected with the 640-nm long-pass filter. FRET efficiency for each pixel was measured by donor dequenching after acceptor photobleaching. The color-coded scale bar is for FRET efficiencies. Scale bar: (A)-(C), 20 μm.

9AzSiaDAz is designed to simultaneously endow sialic acid-containing glycans with a bioorthogonal functional group and a photocrosslinker. Once metabolically incorporated, the diazirine upon UV light activation can covalently crosslink sialic acid-binding proteins while the azide simultaneously enables the enrichment of the target proteins by conjugation with an affinity tag functionalized with an alkyne. To demonstrate the feasibility of using 9AzSiaDAz to identify sialic acid-binding proteins, we chose to capture the dimerization of the sialic acid binding IgG-like lectin (Siglec) CD22, which has been showed to be mediated by sialic acid-CD22 interactions via photocrosslinking,[13,14] as a proof-of-principle experiment. Daudi cells, a B cell line expressing CD22, were cultured with 2 mM 9AzSiaDAz for 24 h, followed by UV irradiation for 20 min to crosslink sialic acid with its binding partners. The cells were also treated with SiaDAz and natural sialic acid for comparison. The cells were lysed, and reacted with alkyne-biotin. Then, the tagged, photocrosslinked complexes were purified using streptavidin beads and analyzed by Western blot using an anti-CD22 antibody. We observed the photocrosslinked CD22 in 9AzSiaDAz-treated cells (SI Figure S8). The absence of UV irradiation resulted in only monomeric CD22, indicating the UV induced photocrosslinking via diazirine. For cells treated with SiaDAz, no CD22 was observed due to the lacking of the affinity tag. These results collectively demonstrate that once incorporated into cell-surface sialylated glycans, diazirine and azide on 9AzSiaDAz simultaneously exert their distinct functions, photocrosslinking and affinity enrichment, respectively.

In Summary, bifunctional sialic acid analogues, 9AzSiaNAl and 9AzSiaDAz have been developed for simultaneously incorporating two distinct chemical reporters into cellular sialylated glycans. We demonstrated that 9AzSiaNAl could be used for two-color and FRET imaging. Further applications include probing the biosynthesis and turnover dynamics of sialylated glycans as a function of time by conjugating distinct probes to azide and alkyne at different time points. This can potentially be used to categorize glycoproteins by their turnover rates.

9AzSiaDAz has been metabolic incorporated into cell-surface glycans and the installed diazirine and azide can be used to photocrosslink binding proteins and affinity enrichment, respectively. When SiaDAz is used to capture glycoprotein interactions, it is not straightforward to detect the cell-surface incorporation of SiaDAz. Lectins recognizing sialic acids or recognizing core oligosaccharide structures that are masked by the addition of sialic acids were used to detect the incorporation of SiaDAz on live cells.[14,27] This method is only applicable in cell lines with deficiencies in sialic acid biosynthesis such as K20 cells[28], a subclone of the BJA-B B-cell lymphoma cell line that is deficient in UDP-GlcNAc 2-epimerae activity. However, the quantification of incorporation efficiency can be complicated by the fact that the diazirine modification might influence lectin binding. Notably, our bifunctional 9AzSiaDAz enables the direct detection of the cell surface display of photocrosslinking diazirine-containing sialic acids, even in cells with intact sialic acid biosynthetic machinery, as demonstrated in CHO, HeLa, and Daudi cells. More importantly, this photocrosslinking bifunctional sugar will find invaluable applications in identifying sialic acid-binding proteins. For mono-functional photo sugars such as SiaDAz and 9AAzSia, one would have to tag a sialylated protein of interest, so that only binding partners of a specific protein could be identified at a time. In our strategy, the sialic acid is directly tagged, which renders the possibility of globally identifying all sialic acid-binding proteins. In addition, more bi-functional sialic acid analogues with

desired properties can be developed, given the generic nature of the synthetic process.

Notably, bifunctional ManNAc analogues that contains two functionalities at N-acyl chain and C-6 position can not be metabolically converted into the corresponding sialic acid, due to the participation of C-6 hydroxyl group in the enzymatic transformation of ManNAc to sialic acid. Recently, the Hakenberger group reported a ManNAc analogue bearing an azide at C-4 position could be metabolically converted to the corresponding C-7 substituted sialic acid and incorporated into cell-surface sialylated glycans.[29] It will be interesting to test whether the C-4 and N-acyl bifunctionalized ManNAc analogues can be explored for metabolic glycan labeling, which is currently under investigation in our lab.

ASSOCIATED CONTENT

Supporting Information

Chemical synthesis and experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

xingchen@pku.edu.cn
jingzhao@pkusz.edu.cn
canxie@pku.edu.cn

Author Contributions

‡These authors contributed equally.

Notes

The authors declare no competing financial interests.

ACKNOWLEDGMENT

This work was supported by the National Basic Research Program of China (973 Program) (No. 2012CB917303) and the National Natural Science Foundation of China (No. 21172013, and No. 91127034). J. Z. thanks the Shenzhen Government (JC201104210113A) and Guangdong Government (S20120011226). We thank Prof. Michael Pawlita and Prof. James Paulson for sharing BJAB K20 cells.

REFERENCES

(1) Varki, A.; Cummings, R. D.; Esko, J. D.; Freeze, H. H.; Hart, G. W.; Etzler, M. E. *Essentials of Glycobiology*; 2nd ed.; Cold Spring Harbor Laboratory Press: Cold Springer Harbor, NY, 2008.

(2) Somers, W. S.; Tang, J.; Shaw, G. D.; Camphausen, R. T. *Cell* **2000**, *103*, 467.

(3) Rosen, S. D. *Annu. Rev. Immunol.* **2004**, *22*, 129.

(4) Lopez, P. H. H.; Schnaar, R. L. *Curr Opin Struct Biol* **2009**, *19*, 549.

(5) Dube, D. H.; Bertozzi, C. R. *Nat. Rev. Drug Discov.* **2005**, *4*, 477.

(6) Chen, X.; Varki, A. *ACS Chem. Biol.* **2010**, *5*, 163.

(7) Keppler, O. T.; Horstkorte, R.; Pawlita, M.; Schmidt, C.; Reutter, W. *Glycobiology* **2001**, *11*, 11R.

(8) Laughlin, S. T.; Bertozzi, C. R. *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 12.

(9) Saxon, E.; Bertozzi, C. R. *Science* **2000**, *287*, 2007.

(10) Luchansky, S. J.; Goon, S.; Bertozzi, C. R. *ChemBioChem* **2004**, *5*, 371.

(11) Xie, R.; Hong, S.; Feng, L.; Rong, J.; Chen, X. *J. Am. Chem. Soc.* **2012**, *134*, 9914.

(12) Hsu, T.-L.; Hanson, S. R.; Kishikawa, K.; Wang, S.-K.; Sawa, M.; Wong, C.-H. *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 2614.

(13) Han, S.; Collins, B. E.; Bengtson, P.; Paulson, J. C. *Nat. Chem. Biol.* **2005**, *1*, 93.

(14) Tanaka, Y.; Kohler, J. J. *J. Am. Chem. Soc.* **2008**, *130*, 3278.

(15) Chang, P. V.; Chen, X.; Smyrniotis, C.; Xenakis, A.; Hu, T.; Bertozzi, C. R.; Wu, P. *Angew. Chem. Int. Ed.* **2009**, *48*, 4030.

(16) Agard, N. J.; Prescher, J. A.; Bertozzi, C. R. *J. Am. Chem. Soc.* **2004**, *126*, 15046.

(17) Debets, M. F.; van Berkel, S. S.; Schoffelen, S.; Rutjes, F. P. J. T.; van Hest, J. C. M.; van Delft, F. L. *Chem. Comm.* **2010**, *46*, 97.

(18) Besanceney-Webler, C.; Jiang, H.; Zheng, T.; Feng, L.; Soriano Del Amo, D.; Wang, W.; Klivansky, L. M.; Marlow, F. L.; Liu, Y.; Wu, P. *Angew. Chem. Int. Ed.* **2011**, *50*, 8051.

(19) Du, J.; Meledeo, M. A.; Wang, Z.; Khanna, H. S.; Paruchuri, V. D. P.; Yarema, K. J. *Glycobiology* **2009**, *19*, 1382.

(20) Bond, M. R.; Zhang, H.; Kim, J.; Yu, S.-H.; Yang, F.; Patrie, S. M.; Kohler, J. J. *Bioconj. Chem.* **2011**, *22*, 1811.

(21) Gramlich, P. M. E.; Warncke, S.; Gierlich, J.; Carell, T. *Angew. Chem. Int. Ed.* **2008**, *47*, 3442.

(22) Kele, P.; Mezö, G.; Achatz, D.; Wolfbeis, O. S. *Angew. Chem. Int. Ed.* **2009**, *48*, 344.

(23) Neumann, H.; Wang, K.; Davis, L.; Garcia-Alai, M.; Chin, J. W. *Nature* **2010**, *464*, 441.

(24) Chang, P. V.; Prescher, J. A.; Hangauer, M. J.; Bertozzi, C. R. *J. Am. Chem. Soc.* **2007**, *129*, 8400.

(25) Patterson, D. M.; Nazarova, L. A.; Xie, B. J.; Kamber, D. N.; Prescher, J. A. *J. Am. Chem. Soc.* **2012,** 134, 18638.

(26) Hulce, J. J.; Cognetta, A. B.; Niphakis, M. J.; Tully, S. E.; Cravatt, B. F. *Nat. Methods* **2013**, *10*, 259.

(27) Bond, M. R.; Zhang, H.; Vu, P. D.; Kohler, J. J. *Nat. Protoc.* **2009**, *4*, 1044.

(28) Keppler, O. T.; Hinderlich, S.; Langner, J.; Schwartz-Albiez, R.; Reutter, W.; Pawlita, M. *Science* **1999**, *284*, 1372.

(29) Möller, H.; Böhrsch, V.; Bentrop, J.; Bender, J.; Hinderlich, S.; Hackenberger, C. P. R. *Angew. Chem. Int. Ed.* **2012**, *51*, 5986.

Insert Table of Contents artwork here