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Non-natural sialic acid derivatives with *o*-nitrobenzyl alcohol substituents for light-mediated protein conjugation and cell imaging†

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We have synthesized two sialic acid derivatives substituted with an *ortho*-nitrobenzyl alcohol (***o*-NBA**) group that can undergo light-mediated conjugation with primary amines at their 5- or 9-carbon position. The ***o*-NBA** derivatives were shown to react with multiple lysine residues of human serum albumin (HSA) when exposed to 365 nm light irradiation within 10 min. The resulting sugar conjugates were characterized by mass spectroscopy and used for fluorescence-based cell imaging.

Glycosylation is an important post-translational modification (PTM) of proteins.^{1,2} Protein glycosylation plays an essential role in cell physiology mediating immune responses, cell adhesion, cell–cell interactions and signal transduction.³ Abnormal glycosylation levels is a hallmark for health related conditions including inflammation, autoimmune disorders, neurodegenerative diseases and cancer.^{4–7} Sialic acids are a common terminal sugar of N- and O-linked glycans. Sialylation is abnormally upregulated in a diverse range of cancers including breast,⁸ pancreatic,⁹ ovarian,¹⁰ brain,¹¹ lung,¹² prostate,¹³ colorectal,¹⁴ renal,¹⁵ skin¹⁶ and liver cancer.¹⁷

In order to detect glycosylation in live cells, non-natural sugar probes have been developed. A general strategy is to install biorthogonal handles (such as an azide) to the side chain of a monosaccharide without perturbing its natural metabolic activity.^{18–20} Then after incubation with live cells, a biorthogonal unit (such as an alkyne) linked with a fluorophore is added to react with the metabolically processed sugar probe, thus enabling the spatiotemporal monitoring of biomolecular glycosylation.^{21,22} This elegant strategy has not only significantly advanced glycobiology, but provided insight into carbohydrate-based drug discovery.^{23,24} However, while sugar probes for metabolic labelling of glycans have been extensively reported, those capable of capturing sugar-binding proteins remain much less explored.

To achieve the *in situ* capture of proteins, a reactive handle needs to be introduced into the biomolecules for biorthogonal conjugation. The prototypical reactive units commonly used include benzophenone, diazirine, aryl azide, tetrazole, and thienyl-substituted α -ketoamide.²⁵ Recently developed photocatalytic biorthogonal reactions are particularly advantageous for live cell imaging because of their biocompatibility and the ability to achieve biomolecular labelling in an on-demand fashion.²⁶ Recently, Chen *et al.* developed a strategy using *ortho*-nitrobenzyl alcohol (***o*-NBA**) as a light-activatable reactive handle to modify nucleic acids, and the resulting non-natural biomolecular probes were shown to sensitively capture nucleic acid-binding proteins.²⁷ This is because the nitroso benzaldehyde intermediate generated by UV light irradiation can undergo selective cyclization with primary amines of proteins.^{28–31} This prompted us to explore the use of ***o*-NBA** as a reactive handle to modify sugars for light-mediated protein conjugation.

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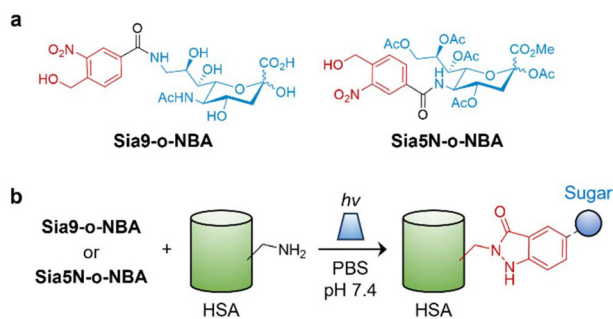


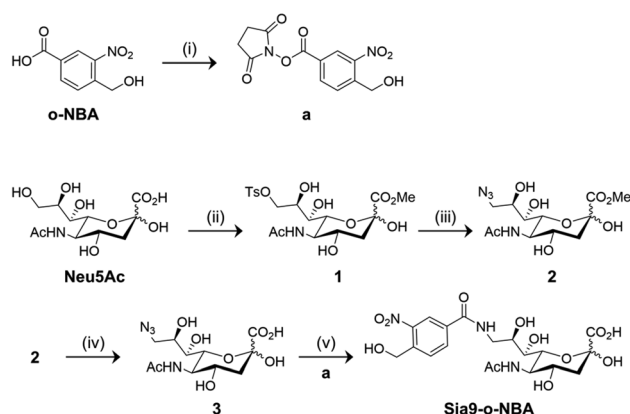
Fig. 1 (a) Structure of the *o*-NBA-modified sialic acid derivatives for (b) light-mediated covalent conjugation with human serum albumin (HSA).

Here, we designed and synthesized two sialic acid derivatives modified with an *o*-NBA group attached to the 5- or 9-position capable of light-mediated coupling with primary amines (Fig. 1a). As a proof-of-concept, the resulting sugar probes were used for light-catalyzed conjugation with human serum albumin (HSA) in which lysine residues (with amino side chain) are abundant (Fig. 1b). The resulting sugar-HSA conjugates were characterized by mass spectroscopy and were used for cell imaging using an encapsulated fluorescent imaging agent.

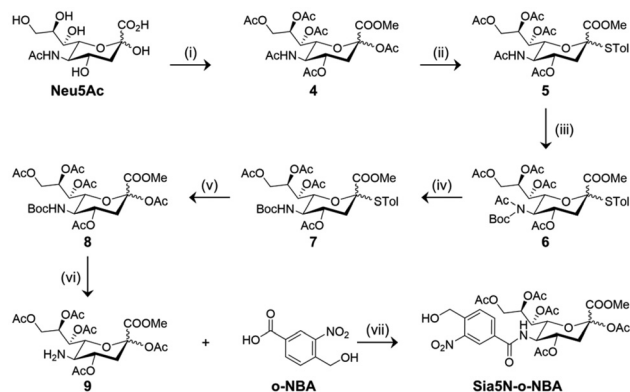
C-2 substituted peracetylated *N*-acetyl mannosamine (ManNAc) has been used as a non-natural sugar probe to detect cell sialylation after undergoing a series of biochemical transformations.²³ However, introducing bulky functional groups to the C-2 position of ManNAc may compromise substrate recognition by endogenous enzymes that mediate sialylation.³² To address this issue, non-natural sialic acid derivatives are being developed to minimize perturbation of the subsequent sialylation biochemical pathways.^{33,34} According to previous literature reports, we synthesized the key sialic acid intermediates **3** and **9**,^{35–37} and the subsequent introduction of an *o*-NBA group by an amidation reaction allowed for the 9- (Sia9-*o*-NBA) and 5-substituted (Sia5N-*o*-NBA) non-natural sialic acid derivatives to be successfully prepared, respectively (Schemes 1 and 2).

Sialic acid intermediate **3** was obtained according to previous literature methods.^{35,36} Briefly, the commercially available *N*-acetylneuramic acid (**Neu5Ac**) was transformed into its methyl ester in the presence of Dowex 50WX2 H⁺ resin to protect the carboxyl acid group. The hydroxyl group at the C9 position of this intermediate was then treated with tosyl chloride in pyridine to obtain the tosylated derivative **1**. Azide **3** was obtained through the reaction of sodium azide with **1** in methanol to afford intermediate **2**, followed by saponification with LiOH. Finally, **3** was reduced with Pd/C in water to yield an amine intermediate, followed by amidation with *N*-hydroxysuccinimide-activated *o*-NBA (**a**) to give Sia9-*o*-NBA in 15% yield (Scheme 1).

Likewise, intermediate **9** was obtained according to a previous literature report.³⁷ Starting from **Neu5Ac**, esterification and then per-acetylation afforded a fully protected sugar product **4**. Treatment of **4** with *p*-thiocresol (TolSH) in the pres-



Scheme 1 Synthesis of Sia9-*o*-NBA. Conditions and reagents: (i) *N*-hydroxy succinimide (NHS), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride-HCl (EDC-HCl) at room temperature (rt) for 2 h. (ii) Dowex 50WX2 H⁺ resin in MeOH at 60 °C for 2 h, and then tosyl chloride in pyridine from 0 °C to rt overnight. (iii) NaN₃ in MeOH at reflux for 24 h. (iv) LiOH in MeOH/H₂O at rt for 2 h. (v) H₂, Pd/C at rt overnight, and then **a** in triethylamine at rt overnight.



Scheme 2 Synthesis of Sia5N-*o*-NBA. Conditions and reagents: (i) Dowex 50WX2 H⁺ resin in MeOH at 60 °C for 2 h, and then Ac₂O and DMAP in pyridine at rt for 2 h. (ii) *p*-Thiocresol and BF₃·Et₂O in CH₂Cl₂ at rt overnight. (iii) Boc₂O and DMAP in THF at 60 °C for 8 h. (iv) MeONa/MeOH at rt for 2 h, and then Ac₂O and DMAP in pyridine at rt for 2 h. (v) *N*-Bromosuccinimide in acetone/H₂O at rt for 2 h, and then Ac₂O and DMAP in pyridine at rt for 2 h. (vi) Trifluoroacetic acid in CH₂Cl₂ at rt for 2 h. (vii) 2-(7-Azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate and diisopropylethylamine in CH₂Cl₂ overnight.

ence of BF₃·Et₂O as the Lewis acid catalyst for 24 h afforded thioglycoside **5**. In order to selectively remove the C5 *N*-acetyl group, an additional *t*-butoxycarbonyl (Boc) group was introduced to protect the secondary amine, affording intermediate **6**. Subsequently, deacetylation in a mixture of MeONa/MeOH followed by acetylation with Ac₂O and 4-dimethylaminopyridine (DMAP) gave the C5 Boc-protected sugar **7**. A short (2 h) treatment of **7** with *N*-bromosuccinimide (NBS) in a mixture of acetone/water set free the C1 alcohol, which was immediately acetylated with Ac₂O/DMAP to give **8**. The Boc group of **8** was selectively removed with trifluoroacetic acid to obtain amine **9**, which underwent amidation with *o*-NBA in



the presence of 2-(7-azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HATU) and diisopropylethylamine (DIPEA) to produce **Sia5N-o-NBA** in 18% yield (Scheme 2).

With the non-natural sialic acid derivatives in hand, we examined their capacity for light-mediated protein conjugation using HSA as a model. The protecting group-free **Sia9-o-NBA** was used for the study. To a phosphate buffered saline (PBS, 0.01 M, pH 7.4) solution of HSA, an excess of **Sia9-o-NBA** was added. The resulting mixture was exposed to 365 nm light irradiation for 10 min, and then incubated at 37 °C for another 30 min. Subsequently, the unreacted sialic acid derivatives were removed through dialysis to afford the **Sia9-o-NBA-HSA** conjugates, which were then digested with trypsin for LC-MS/MS analysis. The results showed that 20 lysine sites of HSA were modified with **Sia9-o-NBA** after light

irradiation (Fig. 2). These results indicate the effectiveness of the sialic acid derivative for light-mediated protein conjugation.

Given that sialic acid-binding proteins are commonly detected in a variety of mammalian cells, we attempted to use the sialic acid-HSA conjugates for fluorescence-based cell imaging. Chlorin e6 (**Ce6**), which is known to bind HSA in its IB domain, was used as the fluorescent imaging agent.³⁸ HSA without and with conjugation with **Sia9-o-NBA** were incubated with **Ce6** for 6 h, followed by dialysis to obtain **Ce6@HSA** and **Ce6@Sia9-o-NBA-HSA**. Fluorescence spectroscopy indicated the presence of the typical **Ce6** emission band for all protein conjugates when dissolved in PBS solution (Fig. S1†).

RAW264.7 (mouse macrophage) and HeLa (human cervical cancer) cells were used for the imaging experiments. We observed that the HSA-based imaging agents were internalized by both cell lines after incubation for 1 hour at a **Ce6** concentration of 5 μ M (Fig. 3a). The fluorescence intensity of the acquired images was then quantified using a Columbus™ Image Data Storage and Analysis System (Fig. 3b). Quantitative analysis revealed a higher cellular uptake of **Ce6@Sia9-o-NBA-HSA** by RAW264.7 than HeLa cells. This difference is probably caused by a higher Siglec (a family of sialic acid-binding proteins) expression level in immune cells.³⁹ Indeed, a subsequent analysis by quantitative real-time polymerase chain reaction corroborated the significantly higher expression level of several Siglec subtypes in RAW264.7 than HeLa cells (Fig. 3c). As a result, our sialic acid-conjugated HSA could be used to target macrophages for a diverse range of biomedical applications.

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1  MKWVTFISLL  FLESSAYSRG  VFRRDAHKSE  VAHRFKDLGE  ENFKALVLIA
51  FAQYLQQCPF  EDHVKLNVNE  TEFAKTCVAD  ESAENCCKSL  HTLFGDKLCT
101 VATLRETYGE  MADCCAKQEP  ERNECFLQHK  DDNPNLPRLV  RPEVDVMCTA
151 FHDNEETFLK  KYLYEIARRH  PYFYAPLELLF  FAKRYKAAFT  ECCQAADKAA
201 CLLPKLDELR  DEGKASSAKQ  RLKCASLQKF  GERAFKAWAV  ARLSQRFPKA
251 EFAEVSKLVT  DLTKVHTECC  HGDLLECADD  RADLAKYICE  NQDSISSKLK
301 ECCEKPLLEK  SHCIAEVEND  EMPADLPSLA  ADFVESKDVC  KNYAEAKDVF
351 LGMFLYEYAR  RHPDYSVVLL  LRLAKTYETT  LEKCCAAADP  HECYAKVFDE
401 FKPLVEEPQN  LIKQNCELFE  QLGEYKFQNA  LLVRYTKKVP  QVSTPTLVEV
451 SRNLGKVGSK  CCKHPEAKRM  PCAEDYLSVV  LNQLCVLHEK  TPVSDRVTKC
501 CTESLVNRRP  CFSALEVDET  YVPKEFNAET  FTFHADICTL  SEKERQIKKQ
551 TALVELVKHK  PKATKEQLKA  VMDDFAAFVE  KCCKADDKET  CFAEEGKKLV
601 AASQAALGL

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Fig. 2 Analysis of HSA modified with **Sia9-o-NBA** by LC-MS/MS; the modified lysine residues are underlined and in bold red as follows: K36, K75, K97, K117, K161, K186, K198, K214, K229, K249, K286, K300, K341, K375, K383, K499, K549, K584, K588 and K594.

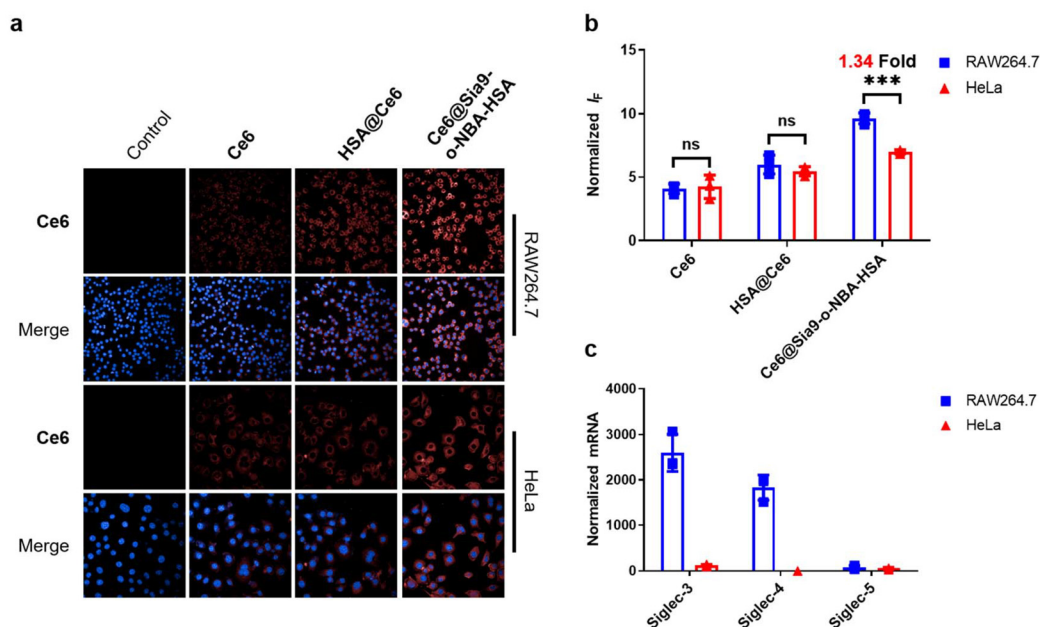


Fig. 3 (a) Fluorescence imaging and (b) quantification of different cells incubated with **Ce6**-encapsulated HSA derivatives (5 μ M) for 1 h. (c) Relative mRNA expression level of three Siglec subtypes in RAW264.7 and HeLa determined by polymerase chain reaction. The excitation/emission channels used for **Ce6** and Hoechst 33342 (to stain nucleic acids) are 405/650–760 nm and 405/435–480 nm, respectively.



Conclusions

We synthesized two sialic acid derivatives bearing the **o**-NBA group at their 5- or 9-positions. The use of 365 nm light catalyzed the conjugation between both non-natural sugar derivatives with HSA. LC-MS/MS analysis indicated that the sugar-protein conjugates were successfully produced after light activation for 10 min, and a subsequent cell imaging assay was suggestive of different uptake efficiency by macrophages that highly express sialic acid-binding proteins. We anticipate that these derivatives will be useful for the light-mediated capture of sialic acid binding proteins,^{40,41} as well as for imaging applications in biological samples and live cells.^{42–45} Relevant chemical biological studies are currently underway in our laboratory.

Data availability

All data associated with this study have been included in either the manuscript or the supplementary file associated with the manuscript.

Conflicts of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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