Expanding the diversity of unnatural cell surface sialic acids

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Main Text

Novel chemical reactivity can be introduced onto cell surfaces through metabolic oligosaccharide engineering.[1,2] This technique exploits the substrate promiscuity of cellular biosynthetic enzymes to deliver unnatural monosaccharides bearing bioorthogonal functional groups into cellular glycans. For example, derivatives of *N*-acetylmannosamine (ManNAc) are converted by the cellular biosynthetic machinery into the corresponding sialic acids and subsequently delivered to the cell surface in the form of sialoglycoconjugates (Figure 1A). Analogs of *N*-acetylglucosamine (GlcNAc) and *N*-acetylgalactosamine (GalNAc) are also metabolized and incorporated into cell surface glycans, likely through the sialic acid and GalNAc salvage pathways, respectively.[3-6] Furthermore, GlcNAc analogs can be incorporated into nucleocytoplasmic proteins in place of β-*O*-GlcNAc residues.[7] These pathways have been exploited to integrate unique electrophiles such as ketones and azides into the target glycoconjugate class. These functional groups can be further elaborated in a chemoselective fashion by condensation with hydrazides[8] and by Staudinger ligation[9], respectively, thereby introducing detectable probes onto the cell (shown schematically in Figure 1B).

We have previously demonstrated that *N*-levulinoylmannosamine (ManLev, **1a**, Scheme 1) is metabolized by cells to *N*-levulinoyl sialic acid (SiaLev) (**2a**), which is then appended to glycoconjugates that are ultimately expressed on the cell surface.[3,8] Increasing the length or steric bulk of the *N*-acyl side chain of **1a** abrogates cell

surface expression of the corresponding sialic acids.[10] A rate-determining step in the *de novo* biosynthesis of unnatural sialic acids appears to be the phosphorylation of ManNAc at the 6-OH by ManNAc 6-kinase (Figure 1A).[10] Accordingly, Reutter and coworkers were able to introduce a broader spectrum of sialic acid analogs into cellular glycans by feeding cells the sialic acid derivative directly, thereby bypassing the bottleneck enzyme (Figure 1A).[11] These observations suggest that sialic acid analogs may be more efficient delivery vehicles for bioorthogonal functional groups than their mannosamine precursors. To this end, we synthesized a series of sialic acid derivatives and evaluated the efficiency of their metabolism to cell surface sialosides. In most cases, the sialic acids were superior to the corresponding mannosamines. These analogs also permitted cell surface expression of novel groups that would not be tolerated by the *de novo* pathway, including an aryl azide with potential photocrosslinking capability.

We synthesized a panel of sialic acids bearing ketone side chains of various length at the *N*-acyl position, a site known to be tolerant of unnatural modifications in the context of the mannosamine precursor (i.e. ManLev, **1a**). Sialic acid analogs **2a-c** (Scheme 1) were synthesized by the neuraminic acid (NeuAc) aldolase-catalyzed condensation [12] of pyruvate and the corresponding mannosamine derivatives[10](**1a-c,** Scheme 1). Compounds **1a-c** and **2a-c** were incubated with the human T cell lymphoma Jurkat for three days to evaluate their metabolic incorporation into cell surface glycans. Subsequent treatment of the cells with biotin hydrazide followed by

FITC-avidin allowed analysis of cell surface ketone expression by flow cytometry.[3] As depicted in Figure 2, keto sialic acids produced a higher level of cell surface ketones than the corresponding keto mannosamine analogs. The most significant difference was observed with the levulinoyl analogs **2a** and **1a**; the sialic acid analog produced 3-fold more cell surface ketones than the mannosamine analog. The metabolic efficiency of longer side chain analogs was reduced in both the mannosamine and sialic acid series, but 5-oxo-hexanoyl analog **2b** was integrated into cell surface glycans at significant levels, unlike its mannosamine congener. Even the further extended homolog **2c** was expressed on the cell surface at levels significantly above background.

We next synthesized azide-containing sialic acid derivative **2d** for direct comparison with its previously studied mannosamine counterpart **1d**[9] (Scheme 1). Compounds **2d** and **1d** were incubated with Jurkat cells and subsequently labeled with a phosphine reagent conjugated to the FLAG peptide (phosphine-FLAG).[4] Treatment of cells with a FITC-labeled anti-FLAG antibody enabled analysis of cell surface azide expression by flow cytometry. In contrast to the trend observed with the ketone derivatives, compounds **1d** and **2d** showed similar metabolic efficiency (Figure 3A). In this case, phosphorylation by ManNAc 6-kinase may not impede conversion of **1d** to **2d**, and a more downstream step may be rate-limiting. Notably, previous work has shown that compound **1d** is processed by sialoside biosynthetic enzymes more efficiently than ManLev (**1a**)[4], a result that might reflect a more efficient phosphorylation step for **1d**.

Having demonstrated that larger acyl substituents can be escorted to the cell surface by sialic acid precursors, we sought to extend metabolic oligosaccharide engineering to novel functionalities such as photocrosslinkers. Thus, aryl azide analogs **1e** and **2e** were synthesized (Scheme 1) and subsequently incubated with Jurkat cells for three days. The cells were reacted with phosphine-FLAG, treated with the FITC-labeled anti-FLAG antibody and analyzed by flow cytometry (Figure 3A). As expected, treatment of cells with mannosamine analog **1e** produced minimal cell surface azides. This compound is likely to be a poor substrate for the enzymes in the *de novo* sialic acid biosynthetic pathway due to its large *N*-acyl side chain.[10] Remarkably, however, sialic acid analog **2e** yielded a robust cell surface azide signal, indicating that the aryl azide functionality was tolerated by the downstream enzymes. To our knowledge, this is the first example of the metabolic introduction of a photoactivatable cross-linking agent to mammalian cell surface glycans.

Previous work in several labs has shown that protection of polar functional groups on sugars can increase their cellular uptake by permitting passive diffusion through membranes.[13-15] Esters have been particularly useful as they can be readily cleaved by cytosolic esterases, permitting further metabolic conversion. Indeed, peracetylated mannosamine derivatives can be fed to cells at concentrations 200-fold lower than the free sugars while achieving the same number of cell surface products. Reutter and coworkers previously demonstrated that the methyl ester of sialic acid is

metabolized similarly to the free form, suggesting that the ester is cleaved inside cells.[11]

To further improve the metabolic efficiency of our analogs, we investigated protected derivatives. Compound **2d** was protected as its methyl ester (**4d**) and this derivative was tested for incorporation into cell surface glycans. As shown in Figure 3A, compound **4d** was converted to the cell surface sialoside with the same efficiency as compound **2d**. We further protected the hydroxyl groups to provide peracetylated sialic acid analogs **5d** and **5e**. Analysis of their cell surface products showed that the compounds are efficiently metabolized at extracellular concentrations in the micromolar range (Figure 3B). Overall, we found the fully protected sialic acids to be utilized by cells between 8 and 20-fold more efficiently than their unprotected counterparts.

In conclusion, sialic acid derivatives are efficient vehicles for delivery of bulky functional groups to cell surfaces and masking of their hydroxyl groups improves their cellular uptake and utilization. Furthermore, the successful introduction of photoactivatable aryl azides into cell surface glycans opens up new avenues for studying sialic acid-binding proteins and elucidating the role of sialic acid in essential processes such as signaling and cell adhesion.

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Figure Legends

Figure 1. Unnatural monosaccharides can deliver novel functional groups to cell surface glycans. A. Conversion of ManNAc to cell surface sialic acid and points of interception with unnatural substrates. B. Schematic of metabolic oligosaccharide engineering and cell surface modification.

Figure 2. Metabolism of keto sugars by Jurkat cells. Cells were incubated with the indicated compounds at various concentrations for three days, reacted with biotin hydrazide and treated with FITC-avidin according to reference 3. The cells were then analyzed by flow cytometry. The data are reported as fluorescence intensity (%FI) relative to cells treated with **1a** (2.5 mM, 100%) and error bars represent the standard deviation of at least two replicate experiments.

Figure 3. Metabolism of azido sugars in Jurkat cells. Cells were incubated with the indicated compounds for three days, reacted with phosphine-FLAG, treated with a FITC-labeled anti-FLAG antibody, and analyzed by flow cytometry. A. The data are reported as fluorescence intensity (%FI) relative to cells treated with 5 mM **1d** (100%). B. The data are reported as fluorescence intensity (%FI) relative to cells treated with 20 μM **3d** (100%). In both panels, error bars represent the standard deviation of at least three replicate experiments.

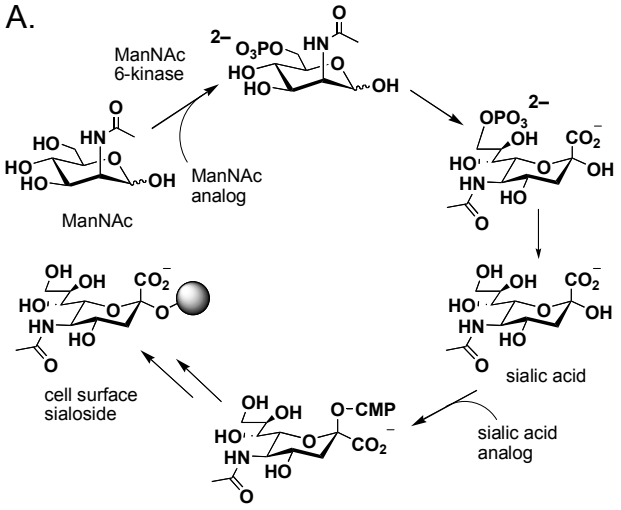
Scheme 1

i. Acylation with unnatural side chain. ii. Sodium pyruvate, 10% NaN_3, NeuAc Aldolase, potassium phosphate (pH

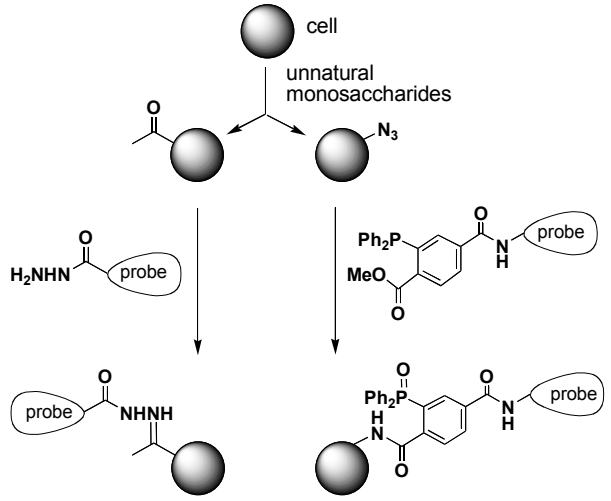
7.2), 37 °C, overnight. iii. Acetic anhydride, pyridine, rt, overnight. iv. cat. Acetyl chloride, MeOH, rt, overnight.

Figure 1

A.


ManNAc
6-kinase
ManNAc
analog
ManNAc
sialic acid
cell surface
sialoside
sialic acid
analog

B.


cell
unnatural
monosaccharides
probe
probe
probe
probe

Scheme 1

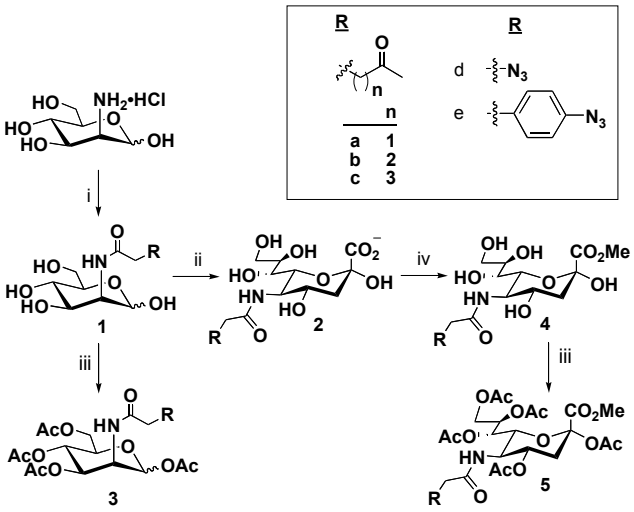


Figure 2

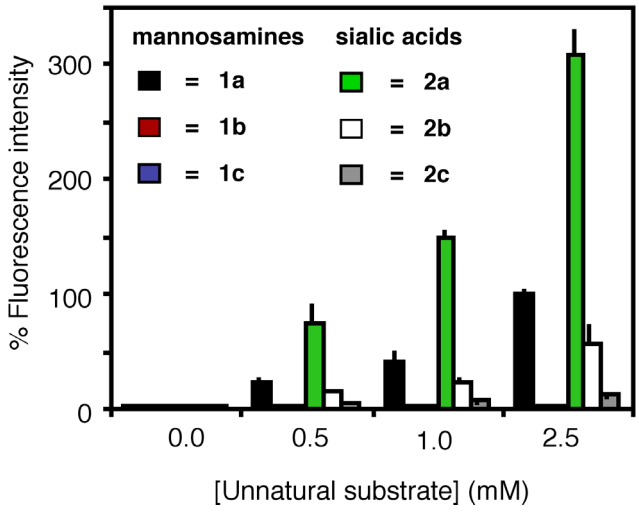
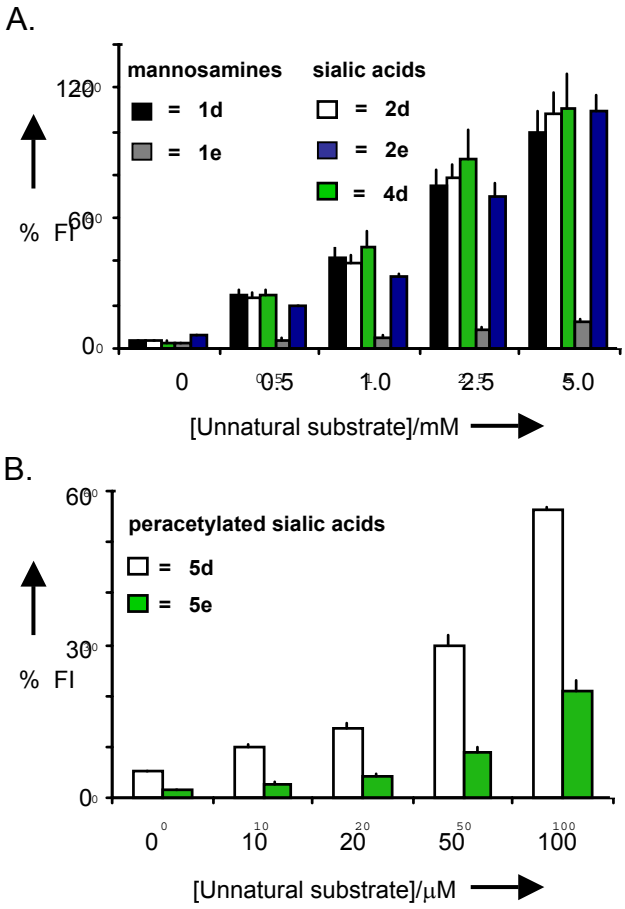
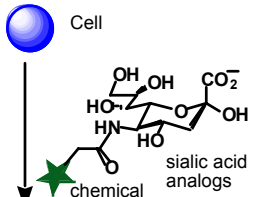

mannosamines
= 1a
= 1b
= 1c
sialic acids
= 2a
= 2b
= 2c
% Fluorescence intensity
300
200
100
0
0.0
0.5
1.0
2.5
[Unnatural substrate] (mM)

Figure 3



Graphical Abstract



Introduction of sialic acid analogs to cells, rather than their upstream *N*-acetylmannosamine (ManNAc) precursors, improves the delivery of ketones to the cell surface and enables the incorporation