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Fluorination of mammalian cell surfaces via the sialic acid biosynthetic pathway

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ABSTRACT

Metabolic oligosaccharide engineering has been employed to introduce fluorine-containing groups onto mammalian cell surfaces. Incubation of HeLa, Jurkat, and HL60 cells in culture with fluorinated sialic acid and mannosamine analogues resulted in cell-surface presentation of fluorinated glycans. Metabolic conversion of fluorinated precursors was detected and quantified by DMB-derivatization and HPLC ESI-MS analysis. Between 7% and 72% of total membrane-associated sialosides were fluorinated, depending on the precursor used and the cell type. Fluorination of mammalian cell surfaces provides a means for introducing a bioorthogonal surface for modulating noncovalent interactions such as those involved in cell adhesion.

The ability to decorate the exterior of living cells with covalently attached chemical entities not normally found on cell surfaces provides a means for low background detection and highly specific chemical modification of the modified cells. Metabolic glycoengineering has proven to be a very successful tactic for attaching unnatural functionalities to cells.[1–3] In this approach, a synthetic monosaccharide similar in structure to a natural precursor in a biosynthesis pathway for a cell-surface glycan, but bearing an unnatural functional group, is incubated with cells. If the modified monosaccharide enters the cell and is processed by the biosynthetic enzymes similarly to the natural precursor, then the resulting cell-surface glycan bears the unnatural functional group. The sialic acid pathway has been extensively used for metabolic glycoengineering of cell surfaces because of its tolerance of precursors with unnatural *N*-acyl groups. Both unnaturally acylated neuraminic acids[4–6] and mannosamines[7–14] are processed by the pathway and have been used to present unnatural functional groups on the surfaces of cells, both in culture and in live rodents.[15–17] A wide range of unnatural groups have been successfully installed onto cell-surface sialoconjugates via glycoengineering, including chain-extended *N*-acyl groups such as *N*-propanoyl-,[4] *N*-butanoyl-,[12,13] and *N*-pentanoyl-,[18] as well as N-acyl groups comprising fluoromethyl-,[6] trifluoromethyl-,[6,19] keto-,[20] azido-,[21] thio-,[11] succinato-,[6] and aryl[5,12] moieties at the 5-position of sialic acid, and amino-, acetamido-, succinatamido-, iodo-, thio-, methylthio-, and methylsulfonyl- in place of the 9-position hydroxyl group of sialic acid.[6] Since none of these functional groups is normally found on cell surfaces, their installation imbues the engineered cells with covalent or noncovalent chemical properties distinct from those normally found on cells. This technique has been effective in modulating several biological phenomena including cell adhesion,[10,19,22] differentiation,[8,9] viral infection,[18,23] and immunogenicity.[12] Recently our laboratory has extended this strategy to permit presentation of fluoroalkyl groups on the glycocalyx of cultured mammalian cells.[19] Fluorination of cell surfaces in vivo imparts several new attributes to the cells because: (1) fluorocarbons provide a means of generating interaction interfaces that are simultaneously hydrophilic and lipophobic, a feature that has been valuable in modulating interaction in other engineered biomolecules;[24,25] and (2) fluorine is virtually absent in soft tissue. Accordingly, fluorination of cells results in reduced adhesion to normal (unfluorinated) extracellular matrix biomolecules,[19] and may provide a means of patterning cells on surfaces via altered noncovalent interactions. Also, since tumor cells typically express elevated levels of sialic acids, especially as sialyl Lewis^x and sialyl Lewis^a epitopes, on their glycocalyx compared with non-transformed cells,[26,27] treatment with fluorinated precursors in the sialic acid pathway may allow low-background detection and imaging of tumors by fluorine MRI.

The present study examines the scope and limitations for fluorination of cells using the sialic acid biosynthetic pathway, and reports the adhesion of labeled cells to the matrix biomolecule fibronectin. A panel of unnatural mannosamine and sialic acid analogues with differing fluoroalkyl groups (Scheme 1) was synthesized and incubated with cells. Biosynthetic conversion and

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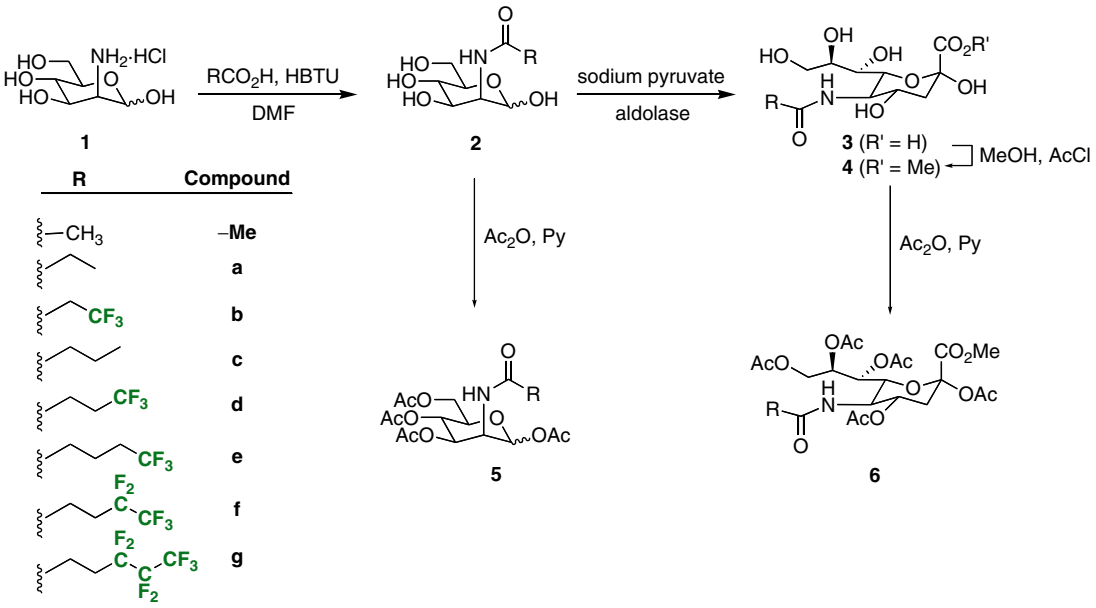

Scheme 1. Synthesis of modified mannosamines and sialic acids.

expression on cell surface was measured by hydrolysis of the oligosaccharides and HPLC analysis of a fluorescent derivative,[28] and cell adhesion was measured to fibronectin-coated culture plates.

Mannosamine analogues **5a–g** (Scheme 1) were prepared by HBTU-mediated acylation of mannosamine with the corresponding alkanoic acid, followed by peracetylation. Sialic acids analogues **6a–g** were prepared from the corresponding acylated mannosamines **2a–g** by sialic acid aldolase (EC 4.1.3.3) catalyzed condensation with pyruvate, followed by formation of the methyl esters with methanolic HCl and peracetylation. The compounds were prepared as the peracetylated derivatives to facilitate diffusion across the cell membrane.[5]

To determine if cells would process the fluorinated mannosamines and display them on their cell-surface glycans, cultured HL60,[29] Jurkat, and HeLa cells were incubated with **5a–g** and the membrane-bound sialic acids were released by acid hydrolysis, derivatized with the fluorogenic reagent 1,2-diamino-4,5-methylene-dioxybenzene (DMB), and analyzed by HPLC, according to Hara et al.[28] Synthetic sialic acid standards **3a–g** were also derivatized to establish retention times. The results were similar for all three cell types, with HL60 typically showing the greatest extent of cellular membrane presentation of the unnatural sialic acids (Table 1). The incorporation was confirmed by HPLC analysis and the identities of the peaks were confirmed by co-injection of the synthetic standards and LC ESI MS. Incubation with fluorinated mannosamines **5b** and **5d** resulted in cells displaying fluorinated sialic acids comprising 22% and 63% of their total cellular membrane sialic acids. However, incubation with mannosamines **5e–g**, possessing longer fluoroalkyl chains, resulted in no detectable cellular membrane fluorination.

Incubation of cells with fluorinated sialic acids **6b**, **d**, **e**, and **f** resulted in higher levels of cellular membrane presentation of the modified glycans than with the corresponding mannosamine, as expected since sialic acid is later in the biosynthetic pathway than mannosamine. This circumvents the most stringent enzyme in the pathway with respect to substrate tolerance.[30] For example, incubation of HL60 cells with **6d** resulted in cells displaying the fluorinated sialic acid comprising 72% of their total cellular membrane sialic acids (Table 1). Incubation of HL60 cells with **6f** lead to modification of 7% of the cellular membrane sialic acids with the pentafluoropentanoyl group, a modification not possible by incubation with mannosamine **5f**. Incubation with **6g** led to no detectable cellular membrane modification, suggesting that the size limit for the fluoroacylated sialic acid to be processed by the biosynthetic pathway had been exceeded.

To rule out the possibility that the modified sialic acids detected were due to contamination by the precursor with which the cells were incubated rather than cellular membrane-derived, we performed a pulse-chase experiment with two different analogues. HL60 cells were incubated with compound **6d** for 72 h, then **6b** was added at an equimolar concentration for 4 h prior to harvesting the cells. Analysis of the membranes as described above showed only **6d** on the cellular membrane, with no detectable **6b**. This result confirms that the detected modified sialic acids are not the result of precursor contamination, and establishes that flux through the biosynthetic pathway from fluorinated sialic acids to cellular membrane glycans does not occur to any significant extent in 4 h.

By comparing the HPLC peak fluorescence intensities of the DMB derivatives of the cellular membrane-derived fluorinated sialic acids with a standard curve generated from the synthetic standards, an estimate of total sialic acid and of the modified sialic acid

Table 1
Presentation of modified sialic acid derivatives on cellular membranes of HL60 cells treated with compounds **5** and **6**

Incubation compound	*N*-Ac-Neu acid[a]	Modified sialic acid[a]	Total sialic acid[a]	No. of CF_3 groups/cell[b]
None	1.00	—	1.00	—
5a	1.10	0.60	1.70	—
5b	0.90	0.20	1.10	2.0×10^7
5c	1.00	0.20	1.20	—
5d	0.30	0.50	0.80	4.4×10^7
6b	0.29	0.62	0.91	4.5×10^7
6d	0.35	0.89	1.24	6.8×10^7
6e	0.84	0.30	1.14	3.9×10^7
6f	1.49	0.12	1.61	1.5×10^7
6g	1.20	—	1.20	—

The relative amounts were determined by DMB-labeling, integrated areas in HPLC, and the cell count.

[a] Values are normalized to *N*-acetylneuraminic acid (**3**–Me) in untreated cells.

[b] Calculated number of CF_3 groups based on total sialic acid as determined by the DMB labeling standard curve.

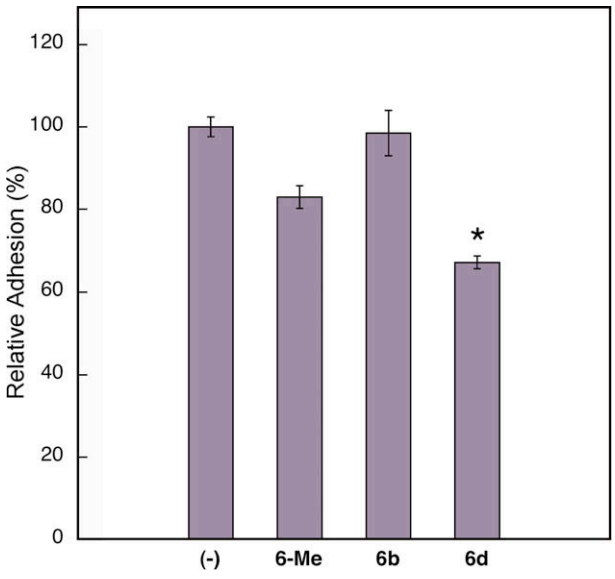

Figure 1. Adhesion of HL60 cells bearing unnatural sialic acids on their surface to fibronectin. Cells incubated with **6**–Me, **6b**, **6d**, or no additive (–) were fluorescently labeled with Calcein-AM and their adhesion to fibronectin-coated wells was measured by fluorescence emanating from adhered cells (see Supplementary data). Only **6d** showed a significant ($*P \leqslant 0.05$) reduction in adhesion relative to untreated cells. Bars represent the average of at least five replicates.

per cell was obtained. In every case where incorporation of a fluorinated derivative occurred, there were 10^7–10^8 CF_3 groups present on the membranes of each cell (Table 1).

The extent to which fluorinated sialic acids on the cell surface alter cellular adhesion to fibronectin was assessed by first incubating cells with **5a–d**, **6a–d**, or **6**–Me at 200 μM concentrations for 72 h to present the various unnatural sialic acid glycans on the cell surface, then labeling the cells with live-cell specific fluorescent dye Calcein-AM. The fluorescent cells were allowed to adhere to wells in microtiter plates coated with fibronectin collagen. Cells were allowed to adhere to the plates for 2 h, then the wells were washed with PBS buffer. The fluorescence in each well before and after washing revealed the extent of cell adhesion to protein adsorbed on plates. Cells treated with fluorinated sialic acid **6d** exhibited significantly reduced adhesion compared with untreated cells (Fig. 1).

In conclusion, we have probed the tolerance of the sialic acid glycan biosynthetic pathway in cultured mammalian cells toward unnatural fluorinated sialic acid and mannosamine derivatives. Our studies indicated that mannosamines containing *N*-acyl groups of up to four carbon atoms with a terminal trifluoromethyl group and neuraminic acids containing *N*-acyl groups of up to five carbon and five fluorine atoms are processed and expressed on the cell membranes. Interestingly, two substrates with the same length chain but with different degree of fluorination have different metabolic efficiencies. $Ac_5SiaC_5F_3$ **6e** bearing five carbon atoms with a trifluoromethyl group was metabolized and incorporated with higher efficiency than $Ac_5SiaC_5F_5$ **6f** bearing the same number of carbon atoms with an additional two fluorine atoms. Of the three cell types tested, HL60 cells showed higher levels of incorporation than HeLa or Jurkat cells, but all three cell types behaved qualitatively similarly. Cells with surfaces modified by trifluorobutanoyl groups showed reduced adhesion to fibronectin. Studies are underway to elucidate the physical basis for this phenomenon.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.09.010.

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