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Cell-Selective Metabolic Glycan Labeling Based on Ligand-Targeted Liposomes

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Supporting Information

ABSTRACT: A cell-specific metabolic glycan labeling strategy has been developed using azidosugars encapsulated in ligand-targeted liposomes. The ligands are designed to bind specific cell-surface receptors that are only expressed or up-regulated in target cells, which mediates the intracellular delivery of azidosugars. The delivered azidosugars are metabolically incorporated into cell-surface glycans, which are then imaged via a bioorthogonal reaction.

Cell-surface glycans play key roles in mediating various molecular recognition events. For example, sialic acid-containing glycoconjuates are involved in bacterial infection, viral invasion, and leukocyte homing. Aberrant glycosylation is implicated in disease (e.g., cancer) progression.[1] Probing the dynamic changes of glycan biosynthesis and structures is, therefore, of great importance for augmenting our understanding of glycobiology and improving disease diagnosis and therapeutics. The metabolic glycan labeling technique has recently emerged as an appealing approach for detecting and imaging glycans on live cells and within living animals.[2−7] This approach exploits the underlying biosynthetic machinery to metabolically incorporate a sugar analogue into cellular glycans. It was first shown by Reutter and coworkers that the propanoyl group can be incorporated into sialylglycoconjugates.[8] Subsequently, Bertozzi's group extended this approach to install bioorthogonal functional groups (e.g., azide) into cell-surface glycans, followed by a bioorthogonal reaction to covalently attach a biophysical (e.g., fluorescent) probe bearing a complementary functional group (e.g., alkyne). By intercepting the sialic acid biosynthesis, *N*-acetylmannosamine analogue bearing azide (i.e., *N*-azidoacetylmannosamine, ManNAz)[2] or sialic acid analogues, both *N*-azidoacetyl sialic acid (SiaNAz)[9] and 9-azido sialic acid (9AzSia),[10] have been used to metabolically incorporate azides into cell-surface sialoglycoconjugates. Probing mucin type O-linked glycosylation, O-GlcNAcylation, and fucosylation has also been realized using *N*-azidoacetylgalactosamine (GalNAz),[3] *N*-azidoglucosamine (GlcNAz),[4] and 6-azido fucose (6AzFuc),[5] respectively. However, one limitation of the metabolic labeling approach has been the lacking of cell type selectivity, that is, the azidosugars can be readily incorporated into numerous mammalian cell lines that have been studied, and distribute in various tissues when injected into living mice.[6]

We envisioned that the extension of the metabolic glycan labeling to possess cell-type selectivity would tremendously enhance the utility of this technology. For example, it would allow one to probe the glycosylation dynamics in a designated organ, or to image the glycans on a specific population of cells. Bertozzi's group has recently demonstrated this concept using a ManNAz derivative caged with a peptide substrate for the prostate-specific antigen (PSA) protease that can be selectively uncaged outside the PSA-secreting cells for subsequent cellular uptake.[11] However, this approach is limited to target extracellular proteases, and cell surface receptors including many important biomarkers cannot be targeted. Furthermore, each protease that is intended to target demands a new design and synthesis of the caged unnatural sugars. To overcome these limitations, we sought to develop a general strategy for cell-specific glycan labeling that combines the unnatural sugar methodology with the means of targeted liposomal delivery. For decades, liposomes have been extensively explored for targeted drug delivery, and a number of liposome-based drugs are currently on the market.[12,13] Herein, we present the cell-specific metabolic glycan labeling using ligand-targeted liposomes to deliver unnatural sugars to target cells in a cell-surface receptor dependent manner (Figure 1). In this strategy, unnatural sugars, for example,

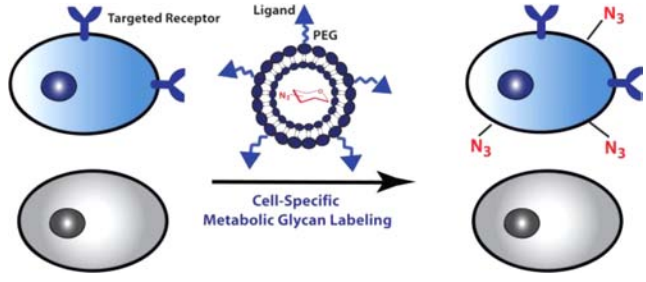

Figure 1. Schematic of the cell-specific metabolic glycan labeling using ligand-targeted liposomal delivery. Unnatural monosaccharide derivatives (e.g., azidosugars) encapsulated in ligand-targeted liposomes are selectively delivered into the target cells in a receptor-mediated manner. The delivered azidosugars are then metabolically incorporated into cell-surface glycans.

azidosugars, are encapsulated in ligand-targeted liposomes. The ligands bind to specific cell-surface receptors that are only expressed or up-regulated in target cells, which mediates the intracellular delivery of azidosugars via endocytosis. The delivered azidosugars are metabolically incorporated into cell-surface glycans, which can then be detected or imaged using a bioorthogonal reaction.

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To demonstrate our strategy, folate-targeted liposomes (f-LPs) were employed to target 9AzSia to folate receptor (FR)-expressing cells. FR is overexpressed in a number of epithelial-derived tumors including ovarian, breast, lung, and colorectal cancers, and f-LPs have been widely exploited for targeted drug delivery to cancers.[14] HeLa cells, a FR expressing cell line, were chosen as the initial target cells. HeLa cells cultured in a folic acid-depleted medium have up-regulated expression of FR,[15,16] which is herein referred to as FR^+ HeLa. It is known that different cell lines vary in the efficiency of metabolic incorporation of unnatural sugars.[17,18] Therefore, we chose to first test our strategy in FR^+ HeLa and HeLa cells, two cell populations that differ only in the FR expression level, to demonstrate the efficiency of the receptor-mediated targeted glycan labeling. Similarly, KB, another FR expression cell line, and FR^+ KB were used to test the generic nature of the approach.

We first verified the metabolic incorporation of 9AzSia in both HeLa and FR^+ HeLa cells, using the copper(I)-catalyzed azide–alkyne cycloaddition (CuAAC) assisted by the ligand BTTAA[19] to conjugate the fluorescent probe to the cell surface azides. HeLa and FR^+ HeLa cells exhibited similar levels of cell surface labeling in a dose dependent manner upon treatment with 9AzSia, as shown by the flow cytometry and confocal fluorescence microscopy analysis (Supporting Information (SI) Figure S1). Similar metabolic incorporation of 9AzSia was observed in KB and FR^+ KB cells in a dose-dependent manner (SI Figure S2).

To prepare the f-LPs encapsulating 9AzSia (f-LP-9AzSia), we chose nanometer-scale (~ 200 nm), PEGylated liposomes that are composed of DOPC, cholesterol, and DSPE-PEG2000-FA (molar ratio, 50:50:0.5; see Supporting Information for details). The liposomal formulation was adopted from that in current clinical use. Encapsulation of 9AzSia resulted in a final 9AzSia to lipid molar ratio of approximately 1.2:1, while maintaining the nanodimensions of the liposomes (about 10% increase in diameter) (SI Table S1). The f-LP-9AzSia was stable for more than one month, exhibiting unchanged diameter and 9AzSia to lipid ratio (SI Figure S3A,B). The stability is rendered by the PEGylation on liposome surface, as the liposomes without PEG coating aggregated after 2 weeks (SI Figure S3A).

Typically, millimolar concentrations of unprotected sialic acid analogues are needed in order to achieve a significant level of metabolic labeling (SI Figure S1). We therefore asked whether the ligand-targeted liposomes could deliver sufficient unnatural sugars into the cytosol. We treated FR^+ HeLa cells with f-LP-9AzSia at various concentrations (0–500 μM, calculated based on 9AzSia concentration) for 24 h at 37 °C. The cells were then washed, labeled with biotin–alkyne using BTTAA-assisted CuAAC, stained with Alexa Flour 488–streptavidin, and analyzed using flow cytometry. Robust fluorescence was observed in a dose-dependent manner (Figure 2A). Significant fluorescence labeling was achieved at concentration as low as 10 μM. Azide expression increased sharply as varying f-LP-9AzSia concentration from 5 to 100 μM, approaching saturation at 100 μM. Remarkably, f-LP-9AzSia achieved the same level of cell surface incorporation at concentrations about 40-fold lower than free 9AzSia (e.g., the labeling with 25 μM of f-LP-9AzSia was higher than 1 mM of 9AzSia). By comparison, protection of the polar carboxyl group as its methyl ester and hydroxyl groups by peracetylation was shown to improve the metabolic efficiency of sialic acid analogues by about 20-fold by permitting passive diffusion through membranes.[9] Similar results were obtained with FR^+ KB cells (SI Figure S4). The dependence of 9AzSia incorporation on the incubation time of f-LP-9AzSia was studied by changing the culture medium to remove excess liposomes at various time points. The azide expression level gradually increased over 24-h period (SI Figure S5).

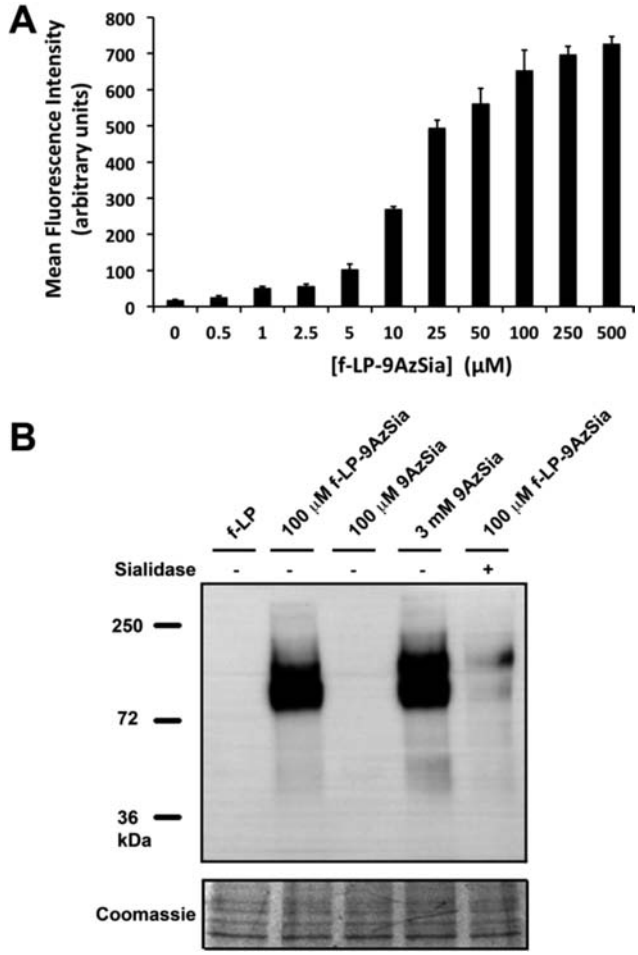

Figure 2. Efficient metabolic glycan labeling by f-LP-9AzSia. (A) Flow cytometry analysis of FR^+ HeLa cells treated with f-LP-9AzSia at various concentrations. The treated cells were labeled with biotin–alkyne and Alexa Flour 488–streptavidin, and analyzed by flow cytometry. Error bars represent the standard deviation from three replicate experiments. (B) Immunoblot analysis of species from FR^+ HeLa cells metabolically labeled with f-LP-9AzSia and 9AzSia. The cells were incubated with f-LP, 100 μM f-LP-9AzSia, 100 μM 9AzSia, or 3 mM 9AzSia, lysed, and reacted with biotin–alkyne. For the sialidase cleavage experiment (the right-most lane), the cells treated with 100 μM f-LP-9AzSia were incubated with the enzyme for 30 min before cell lysis. An immunoblot was performed using standard procedures, detecting with HRP-conjugated anti-biotin. Equal protein loading was confirmed using Coomassie blue staining.

To confirm that f-LP-9AzSia indeed incorporates 9AzSia into sialylglycoproteins, we characterized the f-LP-9AzSia treated cell lysates in anti-biotin Western blot, and the observed protein bands were consistent with the labeled glycoproteins in 9AzSia treated cell lysates (Figure 2B). Three millimolar 9AzSia was needed to achieve the comparable level of band intensity to that observed in 100 μM f-LP-9AzSia treated cells, which is in agreement with the flow cytometry results. We further incubated the f-LP-9AzSia treated cells with sialidase, an enzyme that cleaves sialic acid residues, and evaluated the cell-surface azide levels. As expected, sialidase treatment significantly reduced the cell surface labeling (Figure 2B). We concluded that 9AzSia encapsulated in

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targeted liposomes was metabolically incorporated into cell-surface glycans with high efficiency, in a dose- and time-dependent manner.

We next evaluated the selectivity of f-LP-9AzSia for targeted glycan labeling between HeLa and FR$^+$ HeLa cells, two cell populations that differ only in the FR expression level (Figure 3).

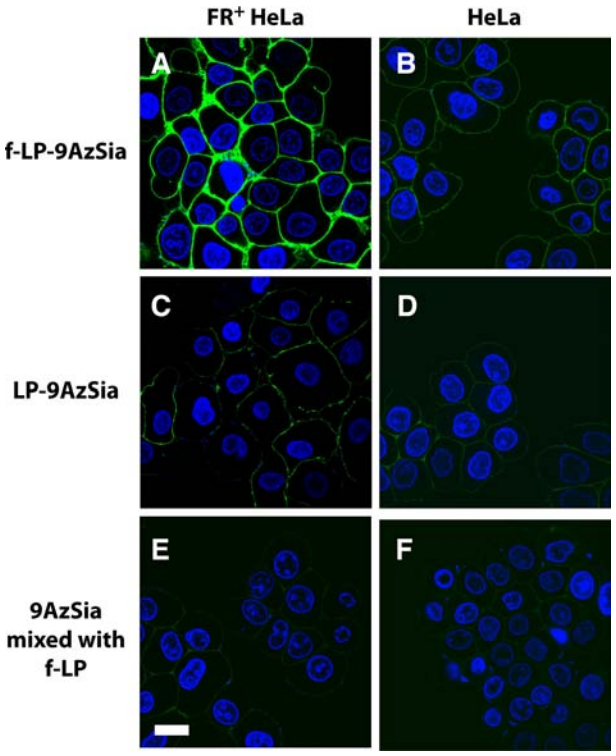

Figure 3. Cell-selective imaging of glycans targeted by f-LP-9AzSia. Confocal fluorescence microscopy images of FR$^+$ HeLa cells (A, C, E) and HeLa cells (B, D, F) treated with 100 μM f-LP-9AzSia, 100 μM LP-9AzSia, or the mixture of 100 μM 9AzSia and f-LP. The treated cells were fluorescently labeled by biotin−alkyne and Alexa Flour 488−streptavidin. The nuclei were visualized by staining with Hoechst 33342 (blue signal). Scale bar: 20 μm.

As shown by confocal fluorescence microcopy, FR$^+$ HeLa cells treated with f-LP-9AzSia showed substantial cell surface fluorescence (Figure 3A), whereas much weaker fluorescence was observed in HeLa cells (Figure 3B), consistent with lower FR expression. Similarly, treating cells with 9AzSia encapsulated in liposomes without folic acid ligands (LP-9AzSia), or the mixture of 9AzSia and f-LP resulted in weak or minimal fluorescence labeling (Figure 3C−F).

We further quantified the targeting efficiency of f-LP-9AzSia using flow cytometry. The metabolic incorporation of 9AzSia in FR$^+$ HeLa and HeLa cells treated with f-LP-9AzSia at concentrations ranging from 5 to 250 μM was compared (Figure 4A). FR$^+$ HeLa cells displayed 2.5- to 5.5-fold higher labeling than HeLa cells, with the highest selectivity at 10−50 μM. Similar targeting selectivity was observed between KB and FR$^+$ KB cells (SI Figure S6). We also quantified the incorporation of LP-9AzSia and observed slightly higher labeling in FR$^+$ HeLa cells (SI Figure S7), which is presumably because of the increased endocytosis caused by folic acid starvation. Nevertheless, the incorporation of LP-9AzSia is at a background level comparing to the receptor-mediated uptake of f-LP-9AzSia. Furthermore, the targeted labeling by f-LP-9AzSia can be blocked by 1 mM free folic acid (SI Figure S8). Therefore, the observed selectivity of

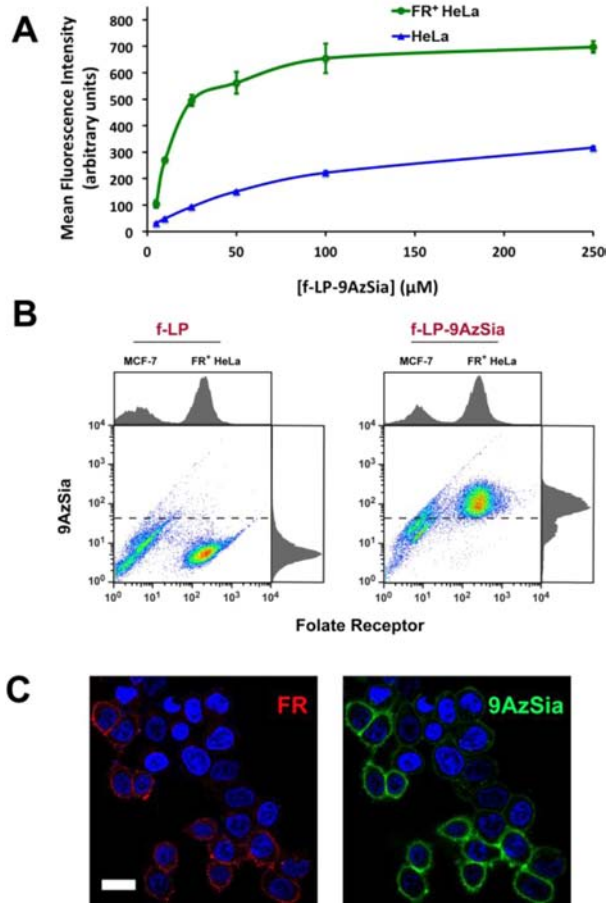

Figure 4. (A) Quantitative analysis of the targeting selectivity of f-LP-9AzSia between FR$^+$ HeLa and HeLa cells by flow cytometry. After being treated with f-LP-9AzSia at concentrations ranging from 5 to 250 μM, the cells were labeled with biotin−alkyne and Alexa Flour 488−streptavidin conjugate. Error bars represent the standard deviation from three replicate experiments. (B) Flow cytometry analysis of selective glycan labeling in co-cultured MCF-7 and FR$^+$ HeLa cells. The mixed cells were treated with 100 μM f-LP-9AzSia or f-LP, followed by labeling cell-surface folate receptor with anti-LK26 antibody, and labeling azide with biotin−alkyne and Alexa Flour 488−streptavidin conjugate. (C) Confocal fluorescence microscopy images of the co-cultured MCF-7 and FR$^+$ HeLa cells targeted by f-LP-9AzSia. FR$^+$ HeLa cells with up-regulated folate receptor expression (red signal) are correlated with the targeted 9AzSia labeling (green signal) on cell surface. The nuclei were visualized by staining with Hoechst 33342 (blue signal). Scale bar: 20 μm.

targeted glycan labeling primarily reflects the difference of FR level between two cell populations.[15,16]

Finally, we explored the selective glycan labeling in a mixed population of two cell types. To do so, MCF-7, an FR-negative human breast tumor cell line, was co-cultured with FR$^+$ HeLa in the folic acid-depleted medium. f-LP-9AzSia discriminated between the two cell types, only resulting in significant incorporation of 9AzSia in FR$^+$ HeLa cells (Figure 4B,C and SI Figure S9). Quantification analysis showed that FR$^+$ HeLa was labeled about 4-fold higher than MCF-7 cells by f-LP-9AzSia at 100 μM (SI Figure S10). Furthermore, LP-9AzSia did not result in significant labeling in both cell populations. These results, collectively, demonstrate that the ligand-targeted liposomes selectively deliver 9AzSia into the target cells in a receptor dependent manner.

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In summary, we have developed a ligand-targeted liposome-based strategy for cell-selective metabolic glycan labeling. The platform of liposomal delivery permits the facile introduction of myriad alternative ligands, in addition to folic acid, that target numerous receptor types. The ligands include antibodies, aptamers, carbohydrates, and peptides. Notably, there is a wealth of knowledge on the targeted liposomal delivery of drugs using these ligands, which can be readily adopted for targeted glycan metabolic labeling.

One of the promising applications of this strategy is for cell-specific or tissue-specific imaging and detection of glycosylation in vivo, which is currently being pursued in our laboratory. It should be noted that the targeted metabolic glycan labeling approach will be especially advantageous for applications using the imaging modalities such as magnetic resonance imaging (MRI) and positron emission tomography (PET), where co-staining and co-localization studies are not allowed. Another direction we are pursuing is to extend this technique to cell-selective glycoproteomic analysis. Our proof-of-principle studies showed that sialylated proteins could be selectively captured and enriched in FR^+ HeLa cells and a known sialylated protein, N-cadherin, was selectively detected in the enriched silylated proteins from FR^+ HeLa cells (SI Figure S11). Further proteomic fingerprinting of the enriched glycoproteins in different cell types is under investigation.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures, supporting tables, and supporting figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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