Facile Click-Mediated Cell Imaging Strategy of Liposomal Azido Mannosamine Lipids via Metabolic or Nonmetabolic Glycoengineering

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ABSTRACT: Two Ac4ManNAz (AAM) derivatives with octadecanoic ester (C18 ester) and octadecyl ether (C18 ether) attached to the anomeric hydroxyl groups were synthesized and used in preparation of liposomes. Both liposomes show strong cell-labeling efficiencies on MDA-MB-231 cancer cells. The cell surface-anchored azide group can react with DBCO-Cy5 via Cu-free click chemistry. The two liposomes exhibit different azide placement mechanisms; C18-ether-AAM-treated cells have azido placement through direct insertion, while C18-ester-AAM-treated cells express azido more through metabolic glycoengineering.

■ RESULTS AND DISCUSSION

Octadecanoic ester of Ac4ManNAzOH was synthesized (Scheme S1 and Figure 1A) by removing the acetyl group at the anomeric position of AAM by Staudinger ligation or Huisgen reaction under physiological conditions, such as dibenzocyclooctyne (DBCO), that can undergo Cu-free click chemistry with azide. Such cell-labeling strategies have been developed for cell indemnification, cancer cell targeting, and imaging. Besides mannose derivatives, other N-azido-modified monocarbohydrate moieties have also been reported to bear cell-labeling capabilities, such as Ac3GlcNAz,10−12 Ac3GalNAz,10,12,13 Ac4ManNAz,10,14−16 Ac3FucAz12 and Ac3ArabAz.12 Recently, caged azido-unnatural sugars have been reported for targeted cell labeling.17,18

To facilitate metabolic saccharide engineering cell labeling, nanostructures have a clear advantage over small-molecule reagents because of high cargo loading, modulated cell internalization capability, and potentially substantially elongated circulation half-life. As a typical cell-labeling azido carbohydrate, AAM has been attempted for improved cell labeling through its incorporation of various nanomedicine platforms, in particular liposome,19−21 the most widely used nanomedicine platform. As the anomeric position of AAM has been demonstrated to be critical for the metabolic activities of AAM in the cell,16 we have modulated the activities of the sugar derivatives via the anomeric conjugation of controlling chemical structures. To enable easy incorporation of AAM to liposomes, we have previously explored the cell labeling of C6 and C12 anomeric-modified AAM. In this study, we designed and synthesized C18-containing AAM and explored its capabilities of cell labeling when it is incorporated in liposomes. The C18 hydrophobic moiety was introduced to Ac3ManNAz by replacing the anomeric acetyl group with an octadecanoic ester (C18 ester) or octadecyl ether (C18 ether). The cell-labeling efficiencies and mechanisms of these two C18-AAMs were evaluated via Cu-free click chemistry established on DBCO-Cy5 (Scheme 1).

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Scheme 1. Cell Labeling by Liposomal Octadecanoic Ester (C18-Ester-AAM) and Octadecyl Ether (C18-Ether-AAM) of AAM via Metabolic or Nonmetabolic Membrane Incorporation

**Figure 1.** (A) Introducing octadecanoic ester (C18 ester) and octadecyl ether (C18 ether) at the anomeric of AAM. (B) Formation of AAM derivatives containing liposome. (C) Particle size analysis of AAM derivative-containing liposomes. (D) Analysis of azido carbohydrate release from the lipid-loaded liposomes through the reverse-titration method of DBCO-Cy3.

octadecanoyl chloride. Octadecyl ether of Ac₅ManNAzOH was synthesized directly from Ac₅ManNAz by 1-octadecanol in the presence of the Lewis acid catalyst of BF₃·Et₂O. The single β-isomer (Figure S16) instead of the α/β complex of AAM...
octadecanoic ester was obtained via neighboring group participation (Figure 1A). After introduction of the hydrophobic C18 group to AAM, both AAM derivatives have significantly reduced water solubilities compared to AAM (Figure S1).

We then formulated liposomes containing C18-ester-AAM or C18-ether-AAM. C18-ester-AAM or C18-ether-AAM could be easily incorporated into liposomes with high efficiency, and the liposome formulation was further improved through extrusion of the lipid mixture with 1,2-distearyl-sn-glycero-3-phosphocholine (DSPC), cholesterol, and 1,2-distearyl-sn-glycero-3-phosphoethanolamine-N-[amino(poly(ethylene glycol))]-2000 (DSPE-PEG2000) (Figure 1B). The liposome is the most widely used nanomedicine delivery platform, and therefore, such a study can potentially be representative to understand cell labeling by nanolabeling agents. AAM-loaded liposomes were prepared as the control. The sizes of the prepared liposomes were 199.5 nm (AAM), 196 nm (C18 ester), and 192.2 nm (C18 ether), respectively, as analyzed by dynamic light scattering (DLS) with the corresponding sugar loading efficiency (Figure 1C) of 72.3% (AAM, Figure S2), 52.4% (C18 ester, Figure S3), and 53.8% (C18 ether, Figure S4). The AAM got the highest loading efficiency, which is presumably due to its higher water solubility, and thus, the incorporation of AAM to the liposome likely follows a different process compared to C18-AAM that mainly incorporates into the hydrophobic segments of the liposomes. Sugar release of the three types of liposomes was analyzed by the reverse-titration method of Click conjugation of DBCO-Cy5. Both C18-ester and C18-ether-AAM-containing liposomes show a much lower tendency of premature release of azido sugar than that of the AAM liposome control at 37 °C in PBS, substantiating that they might be more viable platforms for the controlled intracellular delivery of cell-labeling agents (Figure 1D). The labeling efficiencies of both C18 ester and C18 ether were evaluated with MDA-MB-231 breast cancer cells. The cells were incubated with liposomes for 2 or 12 h at AAM-associated lipid concentration of approximately 10 μM, followed by treatment with DBCO-Cy5 for 1 h to enable complete Click reaction. The cells incubated with PBS were used as the negative control, and the cells incubated with free AAM were used as the positive control.

The cell-labeling efficiencies of C18-ether-AAM- and C18-ester-AAM-containing liposomes were studied by flow cytometry. Both C18-ester and C18-ether-AAM could undergo rapid cell labeling, evidenced by the flow cytometry analysis of the treated cells as compared to controls (Figure 2A). Because metabolic processing of the liposome containing C18-ether-AAM and C18-ester-AAM would take much longer than 2 h in order for azido to be placed on the cell surface, it is reasonable to believe that the surface azide groups were largely via fusion of the C18-ether-AAM and C18-ester-AAM into the cellular membrane lipid structures. Western blot analysis of the C18-ether-AAM and C18-ester-AAM liposome-treated cells supported such analysis given the low azido contents of the cells post 2 h incubation of C18-ether-AAM or C18-ester-AAM liposomes followed by reaction with DBCO-Cy5 (Figure 2B). When the cells were treated with C18-ether-AAM and C18-ester-AAM liposomes for 12 h followed by reaction with DBCO-Cy5, C18-ester-AAM liposome/DBCO-Cy5-treated cells showed the highest fluorescence intensity and therefore the highest contents of the azido group on the cell surface, presumably due to both the metabolic processes of the internalized C18-ester-AAM to incorporate azido group on the surface sialic acids and the fusion of C18-ester-AAM into the cell membrane. The C18-ether-AAM liposome showed no activity for the metabolic labeling of azide on the cell surface, as evidenced in the Western blot analysis of cells treated with C18-ether-AAM liposome. The fluorescence intensities of C18-ether-AAM liposome/DBCO-Cy5-treated cells for 12 h, as shown in Figure 2A, were comparable to AAM- or AAM liposome-treated cells for 12 h, suggesting that the incorporated azide groups on the cell membrane with fused C18-ether-AAM are stable for at least 12 h.

In the further confocal microscopy analysis, free AAM in DMSO and liposomal AAM as the positive control indicated the procedure of the cell-labeling test effective, and 2 h incubation was not sufficient for the metabolic pathway of sialic biosynthesis. All of the liposomes of AAM, C18-ester-AAM, and C18-ether-AAM could label MDA-MB-231 cells after 12 h incubation, followed by reaction with DBCO-Cy5 for visualization (Figure 3). The C18-ester-AAM liposome shown the ability of cell surface labeling with even stronger efficiency than AAM after the treatment of DBCO-Cy5. The C18-ester-AAM liposome could also label the cells with strong fluorescence intensity after treatment of DBCO-Cy5, but its location was different with C18-ester-AAM in 12 h incubation, while the results of 2 h were similar. The C18-ether-AAM liposome maintained the potent ability of intracellular labeling via the fusion pathway, which has shown similar results after 2 and 12 h. However, the C18-ester-AAM liposome exhibited a similar cell-labeling result as C18-ether-AAM liposome in 2 h incubation but shown a significantly different result in 12 h incubation, which was sufficient for the metabolic pathway of sialic biosynthesis. Compared with the 2 h incubation group, both C18-ester-AAM and C18-ether-AAM can label the cells in 2 h due to their fusion pathway from the similar hydrophilic tails, while the liposomal AAM or free AAM in DMSO could not. The differences between the 2 and 12 h on labeling positions of C18-ester-AAM also indicated that C18-ester-AAM could be continuously metabolized onto the cell membrane in 12 h.
which labeled the cell membrane clearly. Instead, C18-ether-AAM just acculturated on fusion in 12 h.

**SUMMARY AND CONCLUSIONS**

In summary, we demonstrated different cell-labeling mechanisms of the C18-ester-AAM liposome and the C18-ether-AAM liposome via facile Click chemistry on DBCO-Cy5 treatment of MDA-MB-231 cells. The results of flow cytometry, SDS-PAGE, and confocal microscopy indicated that the two liposomes exhibit different azide placement mechanisms; C18-ether-AAM-treated cells have azido placement through direct insertion, while C18-ester-AAM-treated cells express azido more through metabolic glycoengineering. Additionally, liposomal formation of hydrophilic AAM derivative lipids could keep strong cell-labeling ability directly through the nonmetabolic glycoengineering way.

**EXPERIMENTAL SECTION**

**General Experimental Details.** All reagents were obtained from commercial sources. Room temperature refers to ambient temperature, and 0 °C was maintained in an ice-water bath. Thin-layer chromatography (TLC) was performed using silica gel 60 with F254 indicator on glass plates (Merck), and flash chromatography was performed using a Merck 40–63 μm silica gel. Solvent ratios for the purification of compounds by flash chromatography are reported as percent volume (v/v). Reactions were performed using oven-dried glassware apparatus under an inert atmosphere with anhydrous solvents. Dichloromethane (DCM), hexane, and ethyl acetate (EA) were purified by passing them through alumina columns and kept anhydrous in molecular sieves. NMR spectroscopy was performed in deuterated solvents at 20 °C. Chemical shifts (δ) are given in parts per million (ppm) relative to TMS, and the solvent residual signal is used as a reference. Abbreviations for peak multiplicities are s (singlet), d (doublet), t (triplet), dt (doublet of triplet), m (multiplet), and br (broad). Mass spectra were measured using an electrospray ionization (ESI) mass spectrometer. Details for the spectrometers and other information are given in the Supporting Information.

**Ac4ManNaAz (2, AAM) and Ac3ManNaAzOH (3).** These two compounds were prepared from D-mannosamine hydrochloride in multisteps according to the literature.3

**Synthesis of C18 Ester of Ac3ManNaAzOH (4, C18-Ester-AAM).** Ac3ManNaAzOH (20 mg, 51.5 μmol) and TEA (10 μL, 70 μmol) were dissolved in anhydrous DCM (3 mL). Dodecanoic acid chloride (12.8 μL, 55.8 μmol) or octadecanoic acid chloride (23.7 mg, 78 μmol) was added at 0 °C. The mixture was warmed to room temperature and stirred overnight. The reaction was worked up by pouring the mixture into saturated NaHCO3 aq and washed by saturated NaCl aq. The organic phase was dried over Na2SO4, warmed to room temperature and stirred overnight. The crude material was then purified by silica gel chromatography, eluting with hexane–ethyl acetate (1:1, v/v) to obtain white solid (23.5 mg, 70%, 2/5 α/β isomers).3H NMR (500 MHz, CDCl3) δ 6.63 (d, J = 9.0 Hz, 1H), 5.90 (s, 1H), 5.15 (d, J = 9.7 Hz, 1H), 5.06 (dd, J = 9.8, 3.9 Hz, 1H), 4.72 (dd, J = 7.9, 2.7 Hz, 1H), 4.23 (dd, J = 11.5, 4.4 Hz, 1H), 4.11 (d, J = 7.1 Hz, 1H), 4.07 (s, 2H), 3.82 (dd, J = 9.6, 2.0 Hz, 1H), 2.36–2.33 (m, 2H, 1.20 (s, 4H), 2.06 (s, 3H), 2.00 (s, 3H), 1.62–1.57 (m, 2H), 1.25 (s, 28H), 0.87 (s, J = 7.0 Hz, 5H).13C NMR (125 MHz, CDCl3) δ 171.16, 170.47, 170.12, 169.54, 167.22, 90.15, 77.28, 77.03, 76.78, 73.34, 71.43, 65.02, 61.71, 60.39, 52.62, 49.73, 33.86, 31.93, 29.70, 24.14, 22.69, 20.63, 14.12. ESI-HRMS (m/z) C33H45N10O2: calculated: 654.3840; found: 677.3730 [M + Na]+.

**Synthesis of C18 Ether of Ac3ManNaAzOH (5, C18-Ether-AAM).** Ac3ManNaAz (65 mg, 0.17 mmol) and 1-octadecanol (49 mg, 0.18 mmol) were dissolved in anhydrous DCM (5 mL). The mixture was stirred overnight at room temperature and then washed with saturated sodium NaHCO3 aq or saturated NaCl aq. The organic phase was collected and...
concentrated to yield a yellow oil. The crude product was purified by silicone gel column chromatography using ethyl acetate/hexane (1/1, v/v) as the eluent to obtain white solid (65 mg, 58%, β-isomer). 1H NMR (500 MHz, CDCl3) δ 6.49 (d, J = 9.3 Hz, 1H), 5.13 (t, J = 10.1 Hz, 1H), 4.59 (dd, J = 9.3, 4.3, 1.7 Hz, 1H), 4.13 (dd, J = 12.3, 2.5 Hz, 1H), 4.08 (d, J = 16.6 Hz, 1H), 4.02 (d, J = 16.6 Hz, 1H), 3.99–3.96 (m, 1H), 2.12 (s, 3H), 2.05 (s, 3H), 1.98 (s, 3H), 1.60 (p, J = 6.8 Hz, 3H), 1.25 (s, 28H), 0.87 (t, J = 6.9 Hz, 4H). 13C NMR (125 MHz, CDCl3) δ 170.55, 170.00, 169.78, 166.66, 98.66, 77.28, 77.03, 76.78, 69.34, 68.75, 68.09, 65.85, 62.21, 50.38, 51.94, 29.71, 29.67, 29.64, 29.57, 29.39, 29.24, 26.09, 22.70, 20.81, 20.70, 20.64, 14.13. ESI-MS (m/z) C32H56N4O9: calculated: 640.4047; found: 663.3945 [M + Na]+.

**REFERENCES**


