Supporting Information

Novel Liposomal Azido Mannosamine Lipids on Metabolic Cell Labeling and Imaging via Cu-Free Click Chemistry

Li Shen,*, † Kaimin Cai, † Jin Yu, † and Jianjun Cheng * †

† Ocean College, Zhejiang University, Zhoushan 316021, China
‡ Department of Materials Science and Engineering, University of Illinois at Urbana-Champaign Urbana, Illinois 61801, United States

*E-mail: shenli@zju.edu.cn
*E-mail: jianjunc@illinois.edu

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General methods

Materials: D-Mannosamine hydrochloride and other materials were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. Reactions were performed using oven-dried glassware apparatus under an atmosphere of nitrogen or argon with anhydrous solvents. Anhydrous dichloromethane (DCM), hexane, and ethyl acetate were purified by passing them through alumina columns and kept anhydrous in molecular sieves. All other reagents were used as obtained from commercial sources. Room temperature refers to ambient temperature. Temperatures of 0°C were maintained using an ice-water bath. Thin-layer chromatography was performed using silica gel 60 with F254 indicator on glass plates (Merck). Flash chromatography was performed using Merck 40-63 μm silica gel. Solvent ratios for the purification of compounds by flash chromatography are reported as percent volume (v/v).

Instrumentation: Nuclear magnetic resonance (NMR) analyses were conducted on a Varian U500 or a Carver-Bruker 500 (500 MHz) spectrometer. All MS analyses were performed on Waters quadrupole and time-of-flight (Q-TOF) Ultima ESI mass spectrometer. High-performance liquid chromatography (HPLC) analyses were performed on a Shimadzu CBM-20A system (Shimadzu, Kyoto, Japan) equipped with a SPD20A Photodiode Array (PDA) detector (190 nm-800 nm) and an RF10Axl fluorescence detector, and an analytical Phenyl-Hexyl column (Phenomenex, 5 μm, 100*4.6 mm, Torrance, California, USA). Extrusion of liposomes was conducted with an Avanti mini-extruder (Avanti, Alabaster, AL, USA). The Diameter and diameter distribution of liposomes and microbubbles were determined by using a ZetaPlus dynamic light scattering (DLS) detector (15 mW laser, incident beam = 676 nm, Brookhaven Instruments, Holtsville, NY, USA). Confocal laser scanning microscopy images were taken on a Zeiss LSM 700 Confocal Microscope (Carl Zeiss, Thornwood, NY, USA). Fluorescence intensity of cells was measured on an IN-Cell Analyzer 2200 system (GE Healthcare Life Sciences, Pittsburgh, PA, USA). Protein bands were visualized with Image Quant LAS 4010 (GE Healthcare, Little Chalfont, UK).

Cell culture: The MDA-MB-231 triple negative breast cancer cell line was purchased from American Type Culture Collection (Manassas, VA, USA). Cells were cultured in DMEM medium containing 10% FBS at 37°C in 5% CO₂ humidified air.
**Water solubility of Ac₄ManNAz (AAM) derivative lipids**

Ac₄ManNAz derivative lipid was dissolved in acetonitrile to 10 mM as stock solution, 10 μL of which was dispersed in 990 μL DI water. The mixture was centrifuged for 1 min at 5000 rpm. The clear solution above was measured by HPLC based on the standard curve.

![Figure S1. Water solubility of Ac₄ManNAz (AAM) derivative lipids](image)

**Chemical stability of Ac₄ManNAz (AAM) derivative lipid**

Ac₄ManNAz derivative lipid-loaded liposomes (200 μM) were incubated in 800 μL of PBS containing 10% FBS at 37°C. 100 μL of incubated sample was taken out at selected time points (0 h, 1 h, 2 h, 4 h, 8 h, 12 h and 24 h), and added with 300 μL of acetonitrile for protein-precipitation, after centrifuging (12000 rpm, 5 min). Then the above clear solution was detected via HPLC and the concentration of Ac₄ManNAzOH lipid was calculated from standard curve (Figure S2).

![Figure S2. Chemical stabilities of Ac₄ManNAz (AAM) derivative lipids in FBS](image)
Preparation of Ac₄ManNAz derivative lipid-loaded liposomes.

1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC, 21 mg), cholesterol (7 mg) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG2000, 7 mg) were dissolved in anhydrous chloroform (300 μL), followed by the addition of Ac4ManNAz derivative lipid (2.5 μmol) in chloroform (250 μL). The solvent was evaporated in vacuum and left to dry for 12 h to form a lipid film. Add 2.5 mL of phosphate buffer saline (PBS) into the glass tube of lipid film. Sonicate the solution in plus (3 s on and 2 s off) for 4 min with holding the glass tube. After vortex the tube, the liposomal suspension was then fine sized by extruding successively through a mini extruder equipped with a membrane filter (400 nm and 220 nm pore size) and two syringes for at least 15 times. Ac4ManNAz derivative lipid-loaded liposomes were then purified by a Sephadex G25 column using PBS as the eluent to remove small molecules. Collected liposomal solution was characterized by DLS and stored at 4°C for use. To determine the loading of encapsulated Ac4ManAz derivative lipid, prepared liposome solution (5.0 mg/mL) was diluted by acetonitrile-water solution (1:1, v/v) with a final concentration of 10% (v/v). HPLC measurements were conducted to determine the amount of encapsulated Ac4ManAz derivative lipid in prepared liposomes based on the standard curve.

**Figure S3.** Determination of sugar loading efficiency of C2 ester-loaded liposomes via HPLC. The red line was the fitted standard curve of C2 ester, plotted as the integrated absorbance signal of C2 ester at 220 nm versus the concentration of C2 ester. The solution of disrupted liposomes (1000 μM) showed a C2 ester concentration of 723 μM, as shown by the arrow. The sugar loading efficiency was calculated as: 100%*(723 μM)/(1000 μM) = 72% (mol /mol).
Figure S4. Determination of sugar loading efficiency of C6 ester-loaded liposomes via HPLC. The red line was the fitted standard curve of C6 ester, plotted as the integrated absorbance signal of C6 ester at 220 nm versus the concentration of C6 ester. The solution of disrupted liposomes (1000 μM) showed a C6 ester concentration of 407 μM, as shown by the arrow. The sugar loading efficiency was calculated as: 100%*(407 μM)/(1000 μM) = 41% (mol/mol).

Figure S5. Determination of sugar loading efficiency of C12 ester-loaded liposomes via HPLC. The red line was the fitted standard curve of C12 ester, plotted as the integrated absorbance signal of C12 ester at 220 nm versus the concentration of C12 ester. The solution of disrupted liposomes (1000 μM) showed a C12 ester concentration of 498 μM, as shown by the arrow. The sugar loading efficiency was calculated as: 100%*(498 μM)/(1000 μM) = 50% (mol/mol).
Sugar release of Ac₄ManNAz derivative lipid-loaded liposomes.

Ac₄ManNAz derivative lipid-loaded liposomes were dispersed in PBS (pH = 7.4, 1 mL) to 50 μM as final concentration, and then 800 μL of the solution was transferred into a dialysis bag. The dialysis bag was immersed in PBS (19.2 mL) and incubated at 37°C (50 r/min). At selected time points (0 h, 1 h, 2 h, 4 h, 8 h, 12 h and 24 h), 0.4 mL aliquot of the dialysis medium was withdrawn for further 48 h incubation with the same amount of 2 μM DBCO-Cy3 solution for HPLC measurement, and the same amount of fresh medium was added. The rest of DBCO-Cy3 left was detected via HPLC and the concentration of azide groups from liposomes was calculated by reverse-titrage method (Figure 1D).

![Graph](image.png)

**Figure S6.** Determination of free DBCO-Cy3 left via HPLC. The red line was the fitted standard curve of free DBCO-Cy3, plotted as the integrated absorbance signal of free DBCO-Cy3 at 220 nm versus the concentration of free DBCO-Cy3.
**Biological evaluations**

*In vitro* DBCO-Cy5 labeling of Ac₄ManNAz derivative lipid-treated MDA-MB-231 cells. [1-2]

MDA-MB-231 cells were seeded onto coverslips in a 6-well plate with a cell density of 50 k per well and allowed to attach for 12 h. Ac₄ManNAz derivative lipid-loaded liposome (10 μM in sugar equivalent) or blank PBS was then added. Cells were incubated at 37°C in a humidified 5% CO₂ incubator for 2 h or 12 h. The medium was removed and washed with PBS for three times. DBCO-Cy5 in Opti-MEM was added to the final concentration of 10 μM per well and the cells were incubated for another 1 h. Then the medium was removed, and the cells were washed with PBS for three times. The cells were stained for 10 min with Hoechst 33342 (1.0 μg/mL) for nucleus and CellMask Orange C10045 (0.5 μg/mL) for membrane, followed by fixing for 10 min in 4% paraformaldehyde (PFA) solution. The coverslips were mounted on microscope slides and the prepared samples were stored in the dark for confocal microscope imaging (Figure 1C).

For the quantification of DBCO-Cy5 labelled MDA-MB-231 cells, flow cytometry analysis based on the fluorescence intensity of the Ac₄ManNAz derivative lipid-loaded liposome treated MDA-MB-231 cells (Figure 2B). MDA-MB-231 cells were seeded onto coverslips in a 6-well plate with a cell density of 50 k per well and allowed to attach for 12 h. Ac₄ManNAz derivative lipid-loaded liposome (10 μM in sugar equivalent) or blank PBS was then added. Cells were incubated at 37°C in a humidified 5% CO₂ incubator for 2 h or 12h. The medium was removed and washed with PBS for three times. DBCO-Cy5 in Opti-MEM was added to the final concentration of 10 μM per well and the cells were incubated for another 1 h. Then the medium was removed, and the cells were washed with PBS for three times and confluent MDA-MB-231 cells were suspended using 0.05% Trypsin/0.53 mM EDTA. Cells were placed in microcentrifuge tubes for flow cytometer (Figure 2A).

**SDS-PAGE analysis for azide groups of Ac₄ManNAz derivative lipid-loaded liposome MDA-MB-231 cells**

To observe incorporation of azide groups in glycoproteins in vitro, MDA-MB-231 cells were seeded into 6-well plates of 50 k per well with Ac₄ManNAz derivative lipid-loaded liposome (10 μM in sugar equivalent) for 2 h or 12 h at 37 ℃ in a humidified 5% CO₂ incubator. Then, the MDA-MB-231 cells were washed with PBS for three times. DBCO-Cy5 in Opti-MEM was added to the final concentration of 10 μM per well and the cells were incubated for another 1 h. The cells were lysed using RIPA buffer (Thermo Fisher Scientific Inc., USA) with 1% protease inhibitor (Sigma-Aldrich). Lysates were centrifuged at 10000 rpm for 15 min at 4°C to removed cell debris. The total soluble protein concentration was determined by bicinchoninic acid (BCA) assay and adjusted to 1.0 mg/mL. A loading buffer was added to each sample and samples were loaded onto a 10% SDS-PAGE gel after heating at 100°C for 5 min. After running the gel at 120 V for 120 min, the gel was detected directly under the 365 nm UV by LAS 4010 Luminescent image analyzer and the total protein was visualized by Comassies brilliant blue stain (Figure 2B and 2C).
NMR and MS spectra

$^1$H NMR for 2

$^{13}$C NMR for 2
MS for 2

HRMS for 2
$^1$H NMR for 3

\[ \text{AcO} \overset{\text{HN}}{\text{N_3}} \]

$^{13}$C NMR for 3

\[ \text{AcO} \overset{\text{HN}}{\text{N_3}} \]
$^{1}H$ NMR for 4

$^{13}C$ NMR for 4
MS for 4

HRMS for 4
$^1$H NMR for 5

$^{13}$C NMR for 5
MS for 5

HRMS for 5