GNeosomes: Highly Lysosomotropic Nanoassemblies for Lysosomal Delivery

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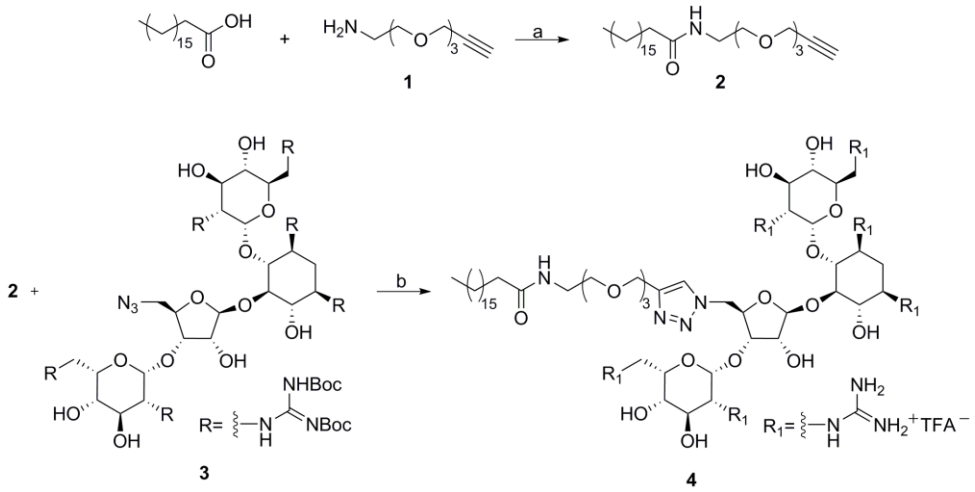
Materials

Materials obtained from commercial suppliers were used without further purification. Chemicals and reagents were obtained from Sigma Aldrich. DOPC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine) and DOPE (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine) were purchased from Avanti Polar Lipids. Deuterated NMR solvents were purchased from Cambridge Isotope Laboratories. PBS (Dulbecco's phosphate buffered saline), F-12 Nutrient Mixture (Ham), DMEM phenol red-free, Streptavidin-Cy3, Fluorescein di-β-D-galactopyranoside (FDG), LysoTracker® Green DND-26, LysoTracker® Deep Red, LysoSensor™ Dextran Blue/Yellow and nuclear stain Hoechst 33342 were purchased from Life Technologies (San Diego, CA, USA). Trypsin/EDTA was purchased from VWR (Mediatech, Manassas, VA, USA). Costar 3524 (Corning) 24-well plates were used. 35 mm glass bottom culture dishes were purchased from MatTek (Ashland, MA, USA).

Instrumentation

NMR spectra were recorded on either a Varian Mercury 400 MHz or 500 MHz spectrometers. Mass spectra were recorded at the UCSD Chemistry and Biochemistry Mass Spectrometry Facility; low resolution mass spectrometry (LR-MS) analysis was performed on a Thermo LCQdeca mass spectrometer using electrospray ionization (ESI) as the ion source. An Agilent 6230 time of flight mass spectrometer (TOFMS) was employed for high resolution MS (HR-MS) analysis using ESI as the ion source. Reversed phase HPLC purification (CLIPEUS, C_{18}, 5µm, 10×250 mm, Higgins analytical) and analysis (Eclipse, XDB-C_{18}, 5µm, 4.6×150 mm) were carried out on an Agilent 1200 series instrument. Fluorescence spectroscopy measurements have been performed on a Horiba fluorimeter. Flow-cytometry studies were performed on a BD FACSCalibur. Particle size (diameter, nm), polydispersity, and surface charge (zeta potential, mV) of the lipid vesicles were measured by dynamic light scattering (DLS) on a Zetasizer Nano ZS (model ZEN3600 from Malvern Instruments) and on a Wyatt Dynapro Nanostar (particle size). Confocal laser scanning microscopy was performed using a Nikon A1R inverted fluorescence microscope with z-stepping motor. Images were processed and analyzed using Nikon Imaging Software Elements and ImageJ (NIH)

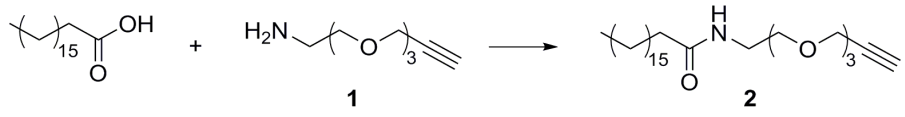
Scheme S1. Synthesis of Stearyl-GNeo (compound **4**)



Synthesis of Stearyl-GNeo: a) EDC, DIEA, DCM, RT, overnight, 81%; b) *i)* $CuSO_4.H_2O$, Ascorbic acid, TBTA, DCM, H_2O, 18 hours; *ii)* TFA, TIS, DCM, RT, 12 h, 51% (two steps)

Compounds **1** and **3** were prepared according to reported procedures.[1,2]

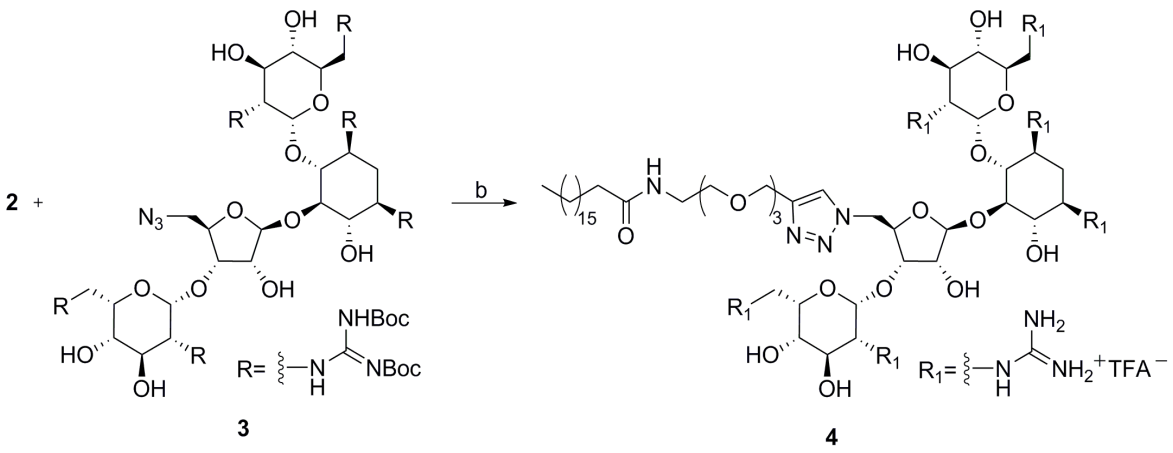
Compound 2



To a solution of stearic acid (250 mg, 0.87 mmol) in dichloromethane (DCM) (4 mL), was added EDC (222 mg, 1.2 mmol) and the solution was stirred at room temperature for 30 minutes. Compound **1** (109 mg, 0.58 mmol) and DIEA (103 µL, 0.58 mmol) were dissolved in DCM (2 mL) and added to the reaction. After stirring overnight at room temperature the reaction was diluted with DCM (30 mL) and washed with aqueous citric acid (5%, 30 mL) and brine. The organic phase was dried over sodium sulfate and evaporated. The residue was purified by flash chromatography (Hexane to 50% Hexane in Ethyl Acetate) to afford the desired compound as a white amorphous powder. Yield: 81%, 213 mg.

^{1}H NMR (400 MHz, $CDCl_3$): δ 6.1 (s, 1H), 4.2 (d, *J*=2.4 Hz, 2H), 3.7 (m, 4H), 3.63 (m, 4H), 3.55 (t, *J*=4.9 Hz, 2H), 3.45 (m, 2H), 2.43 (t, *J*=2.4 Hz, 1H), 2.17 (t, *J*=7.4 Hz, 2H), 1.63 (m, 2H), 1.24-1.27 (s+m, 30H), 0.87 (t, *J*=6.7 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$): δ 173.31, 79.45, 74.69, 70.49, 70.33, 70.14, 69.96, 69.07, 58.41, 39.08, 36.75, 31.92, 29.70, 29.69, 29.67, 29.66, 29.64, 29.53, 29.40, 29.37, 29.35, 25.77, 22.70, 14.15. HRMS: Calculated $[M+H]^+$ 454.3891, found: 454.3893

Compound 4 (Stearyl-GNeo)



Compounds **2** (5.8 mg, 12.7 µmol) and **3** (30 mg, 14 µmol) were dissolved in DCM (200 µL) and TBTA (0.3 mg, 0.64 µmol) was added. $CuSO_4.5H_2O$ (0.2 mg, 0.64 µmol) and sodium ascorbate (0.4 mg, 1.9 µmol) were dissolved in water (200 µL) and added to the organic solution. The mixture was vigorously stirred at room temperature for 18 hours and then diluted with DCM (5 mL) and water (5 mL). The organic phase was washed twice with aqueous EDTA (0.1 M, 5 mL), aqueous KCN (5%, 5 mL) and brine (5 mL). The organic phase was dried over sodium sulfate and evaporated. The residue was dissolved in DCM (0.5 mL) and triethylsilane (50 µL) and trifluoroacetic acid (0.5 mL) were added. The reaction was stirred 12 hours at room temperature, concentrated under vaccum and coevaporated three times with toluene. The residue was dissolved in 5% aqueous acetonitrile (600 µL) and purified on reversed phase HPLC to obtain the desired compound as an amorphous fluffy white powder. Yield: 51%, 13 mg (over two steps).

^{1}H NMR (400 MHz, D_2O): δ 7.95 (s, 1H), 5.69 (s, 1H), 5.02 (s, 1H), 4.99 (s, 1H), 4.69 (m, 4H), 4.41 (bs, 1H), 4.31 (bs, 1H), 4.24 (m, 1H), 4.08-4.06 (m, 2H), 3.75-3.29 (m, 30H), 2.14 (m, 3H), 1.64-1.48 (m, 4H), 1.18 (bs, 30H), 0.79 (t, *J*=6.2Hz, 3H); ^{13}C NMR (126 MHz, D_2O): δ 176.07, 163.02, 162.74, 162.46, 162.18, 157.50, 157.09, 157.01, 156.98, 156.85, 156.30, 143.31, 125.59, 119.64, 117.32, 115.00, 112.67, 111.19, 97.37, 95.62, 85.02, 78.58, 77.41, 77.03, 74.21, 72.50, 71.65, 70.76, 69.42, 69.37, 69.29, 69.25, 69.03, 68.98, 68.80, 66.53, 62.95, 55.18, 53.03, 51.75, 50.19, 41.67, 41.56, 38.63, 35.68, 31.82, 31.63, 29.47, 29.41, 29.36, 29.26, 29.11, 28.98, 28.62, 25.49, 22.33, 13.58.
HRMS: Calculated $[M + 2H]^{2+}$: 673.4230, found: 673.4226

	Z-Average (± SD) / nm	PDI (± SD)	Z-potential (± SD) / mV
Plain liposomes	136.0 (3.4)	0.154 (0.030)	0.44 (0.034)
GNeosomes	125 (0.6)	0.163 (0.023)	27.6 (0.493)
DOTAP-M	123.7 (1.8)	0.153 (0.026)	12.0 (0.321)
DOTAP-N	115.3 (2.5)	0.200 (0.017)	33.2 (1.020)

Table S1. Physicochemical haracterization of lipid vesicles. Lipid formulations are as follows: Plain liposomes, DOPC:DOPE:Cholesterol 73:11:16. GNeosomes, DOPC:DOPE:Cholesterol:Stearyl-GNeo 73:11:16:0.9. DOTAP-M, DOPC:DOPE:Cholesterol:DOTAP 73:11:16:0.9. DOTAP-N, DOPC:DOPE:Cholesterol:DOTAP 73:11:16:5.4

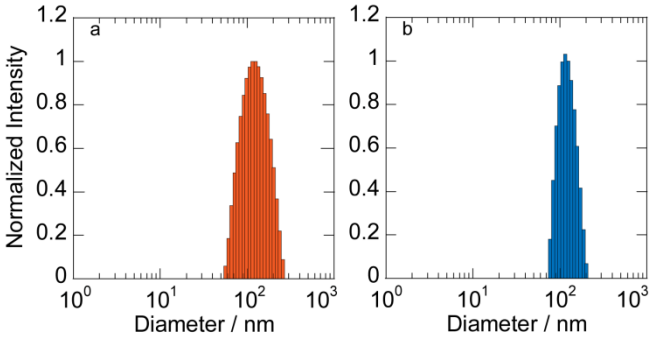


Figure S1. Size distribution (DLS) of a) GNeosomes and b) plain liposomes.

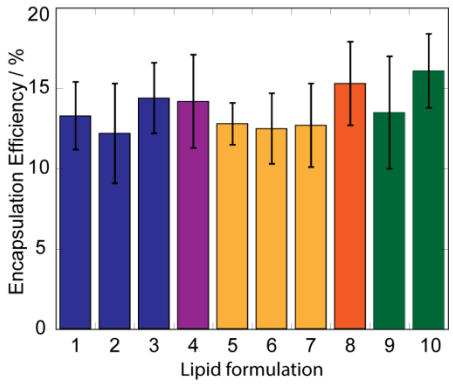


Figure S2. Encapsulation Efficiency. Lipid formulations are as follows: **1**) POPC, **2**) DOPC, **3**) DOPC:DOPE 85:15, **4**) DOPC:DOPE:Cholesterol 73:11:16, **5**) POPC:Stearyl-GNeo, 100:1, **6**) DOPC:Stearyl-GNeo, 100:0.9, **7**) DOPC:DOPE:Stearyl-GNeo 85:15:0.9, **8**) DOPC:DOPE:Cholesterol:Stearyl-GNeo 73:11:16:0.9, **9**) DOPC:DOPE:Cholesterol:DOTAP 73:11:16:0.9, **10**) DOPC:DOPE:Cholesterol:DOTAP 73:11:16:5.4

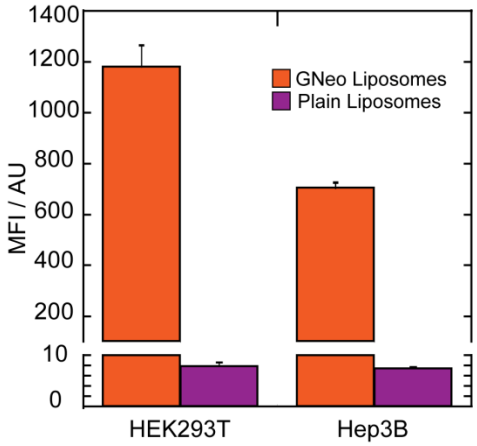


Figure S3. Cellular uptake. HEK293T and Hep3B cells were incubated with the indicated lipid vesicles (0.3 mg mL^{-1}) for 1 h at 37 °C. Mean fluorescence intensity (MFI) was measured by flow cytometry. The background signal from untreated cells was subtracted.

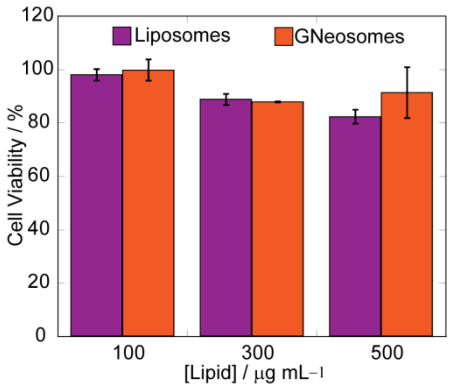


Figure S4. Cell viability. CHO-K1 cells were incubated for 24 hours with plain liposomes or GNeosomes at the indicated concentrations in serum-free medium. Medium was replaced and Cell titer blue was added. Cell viability was calculated by measuring the fluorescence intensity at 530/580.

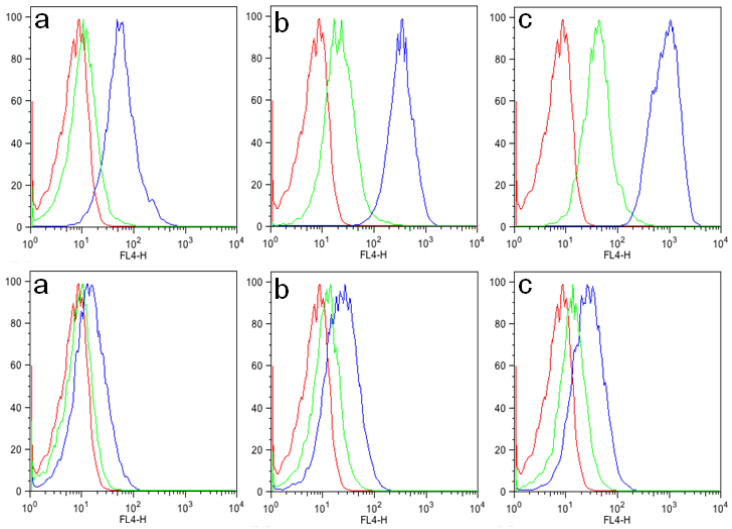


Figure S5. Flow Cytometry of the cellular delivery of Cyanine derivative (Cy5). CHO-K1 and psg-A745 cells were incubated with GNeosomes and plain liposomes loaded with Cy5 for one hour at 37°C and subsequently analyzed by FACS. Upper panels: GNeosomes. Lower panels: Plain liposomes. Untreated CHO-K1 cells (red), treated psg-A745 cells (green) and treated CHO-K1 cells (blue). Liposome concentrations: a) 100 μg/mL, b) 300 μg/mL and c) 500 μg/mL.

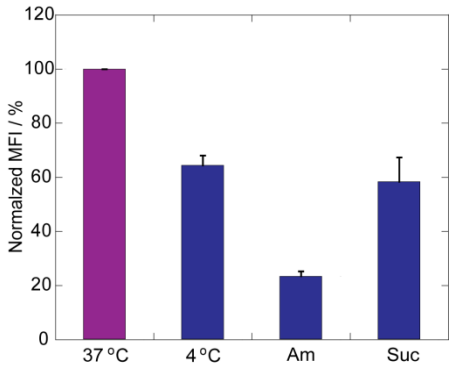


Figure S6. Uptake mechanisms of plain liposomes. CHO-K1 cells were incubated with plain liposomes (300 µg mL^{-1}) at 37 °C and at 4 °C. Cells were treated with amiloride (**Am**, 10 minutes, 5 mM) or sucrose (**Suc**, 1 hour, 400 mM) at 37 °C prior to incubation with liposomes. The background signal from untreated cells was subtracted and the MFI was normalized.

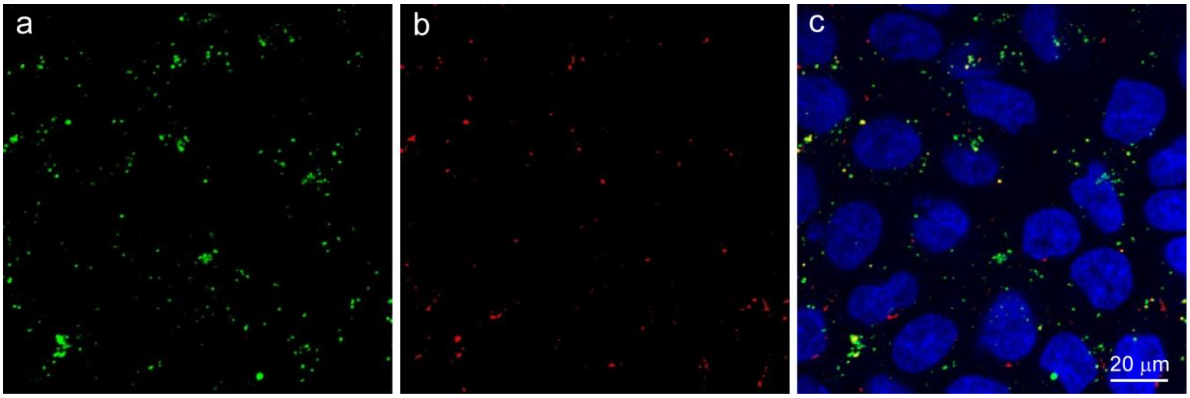


Figure S7. Intracellular localization of GNeosomes. a) LysoTracker Green DND-26, b) Vesicles loaded with Cy5 and c) merged images with nuclear Hoechst dye.

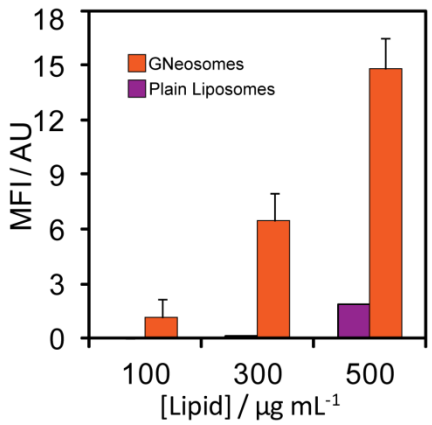


Figure S8. Flow Cytometry of the cellular delivery of Streptavidin-Cy3. CHO-K1 cells were incubated with liposomes (at the indicated concentrations) loaded with Streptavidin-Cy3 for one hour at 37°C and subsequently analyzed by FACS as described above.

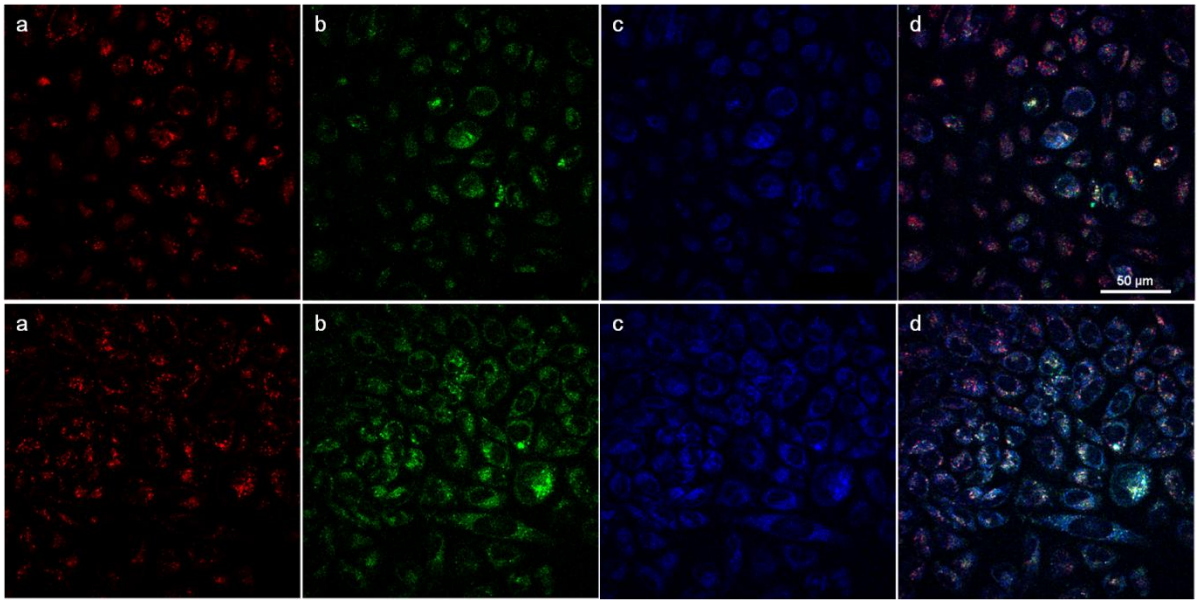


Figure S9. Release of cargo in the lysosomes. CLSM images used to assess the lysosomal release of encapsulated cargo. CHO-K1 cells were incubated with GNeosomes loaded with LysoSensor™ Dextran Blue/Yellow or with unencapsulated LysoSensor™ Dextran Blue/Yellow. Upper panels: unencapsulated LysoSensor™. Lower panels: GNeosomes loaded with LysoSensor™. a) Lysotracker® Deep Red (pseudocolored in red), b) green channel, c) blue channel and d) merged image of **a**, **b** and **c**.

	GNeosomes		Liposomes		GNeosomes / Liposomes	
[Lipid] mg / mL	MFI	SD	MFI	SD	MFI	SD
0.1	4.12	0.03	0.74	0.03	5.57	0.22
0.3	11.7	0.56	1.975	0.035	5.92	0.10
0.5	15.72	0.45	2.205	0.095	7.14	0.37
1	26.77	0.4	2.975	0.165	9.03	0.52

Table S2. Values used to plot **Figure 5a**. Flow cytometry of lysosomal targeting by liposomes loaded with FDG. CHO-K1 cells were incubated for one hour with GNeosomes or plain liposomes at the indicated concentrations. The background signal from untreated cells was subtracted and the ratio between the signals from GNeosomes and plain liposomes was calculated. MFI: Mean Fluorescence Intensity. SD: Standard Deviation.

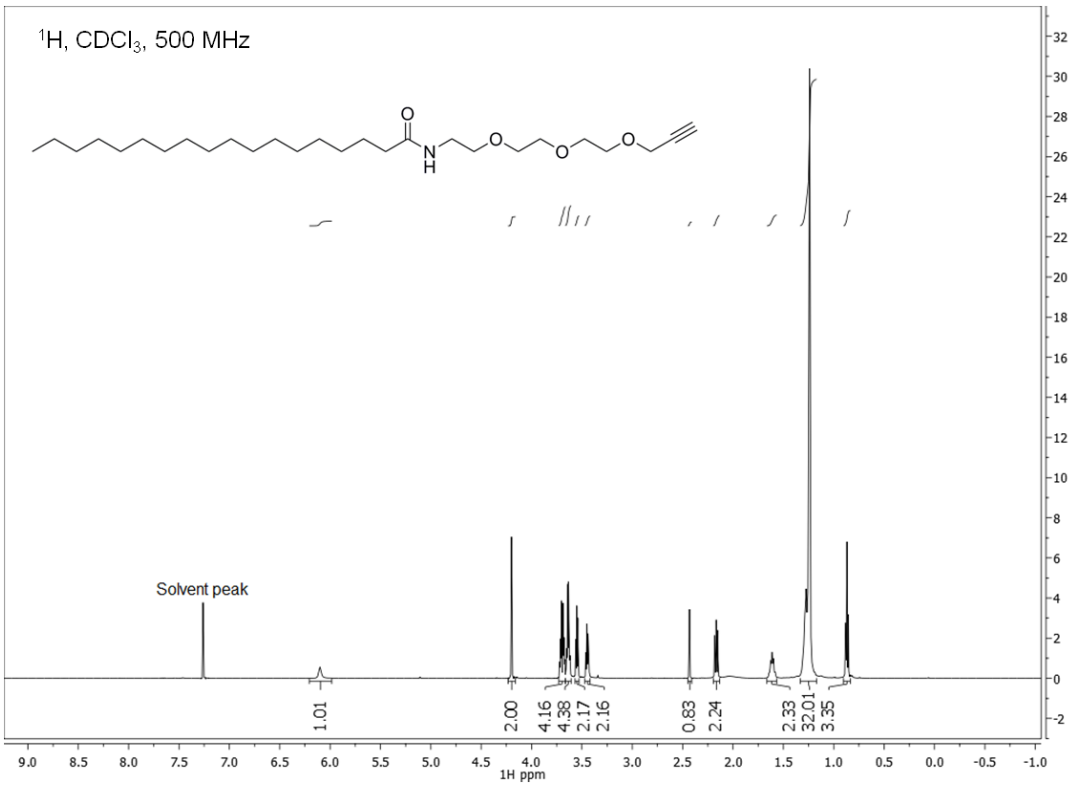


Figure S10. ^{1}H NMR of compound **2**

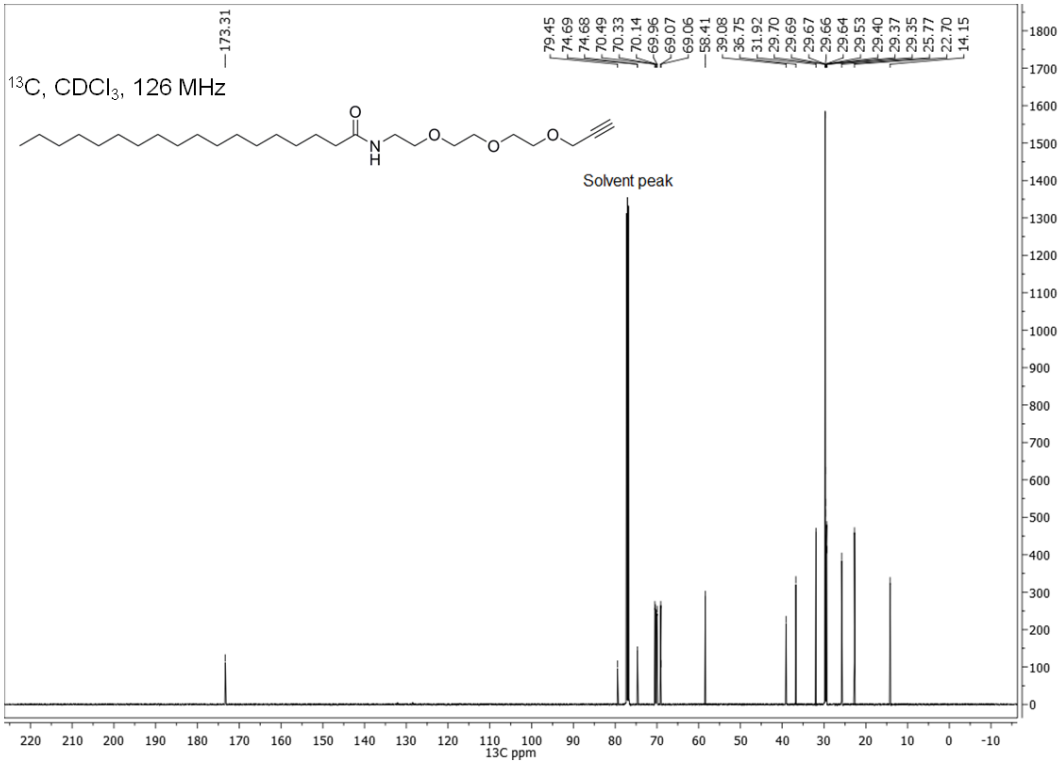


Figure S11. ^{13}C NMR of compound **2**

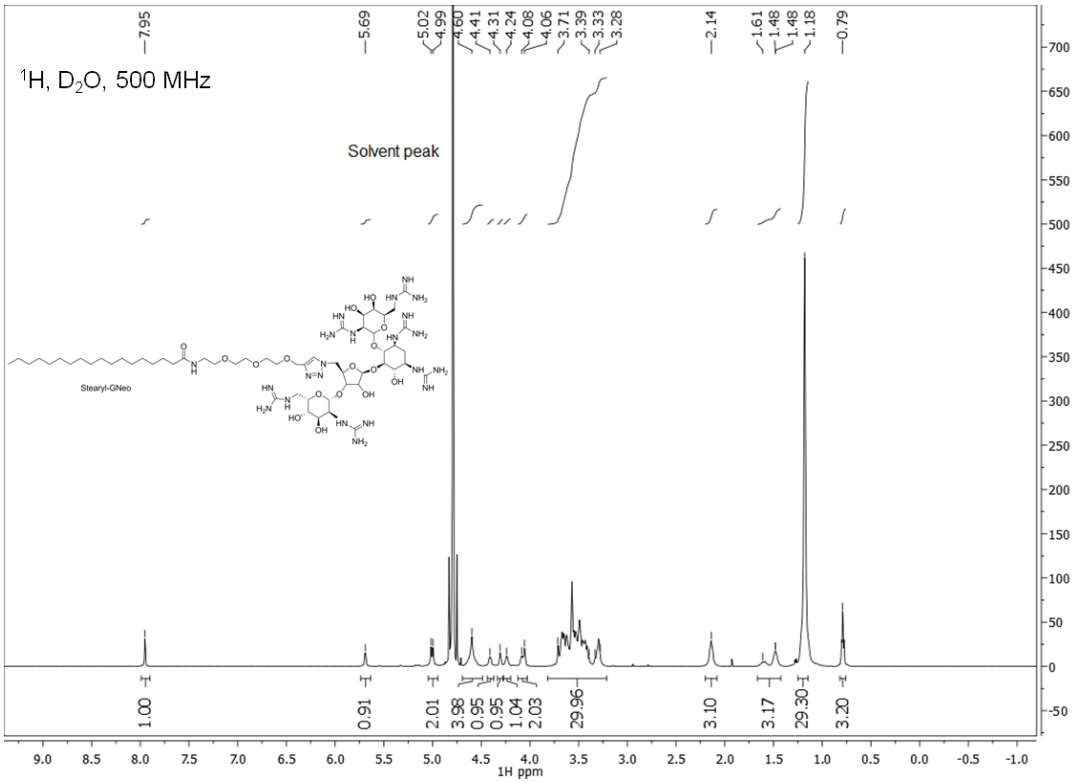


Figure S12. ^{1}H NMR of compound **4** (Stearyl-GNeo)

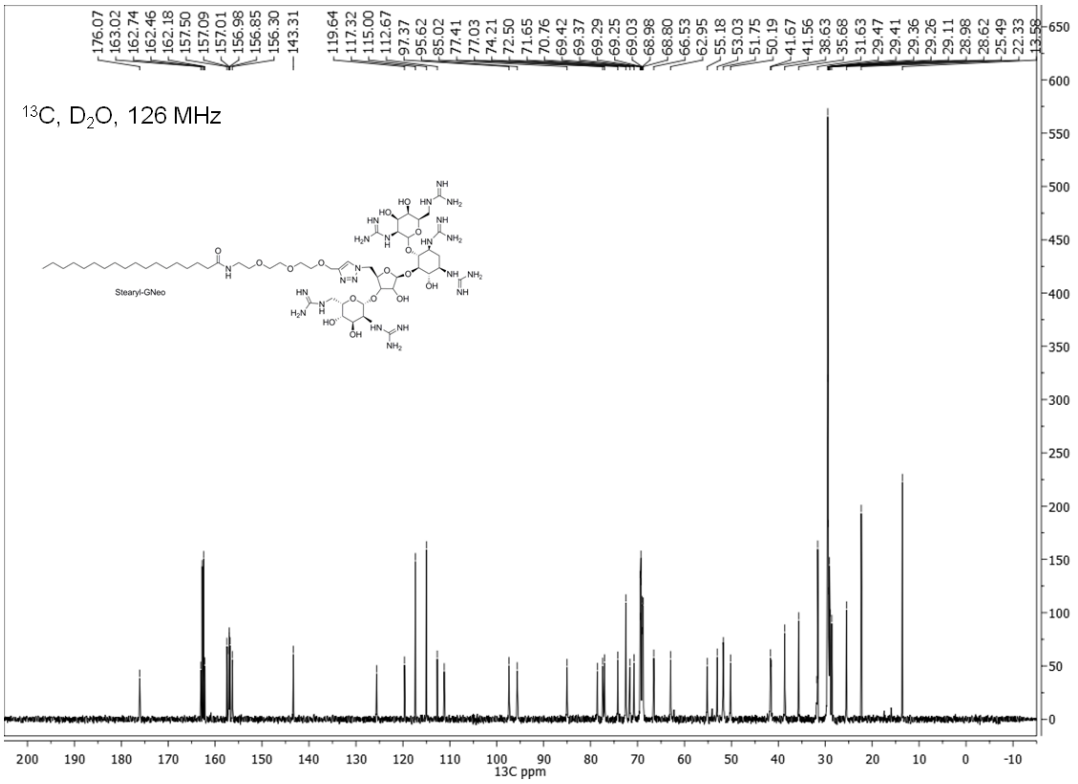


Figure S13. ^{13}C NMR of compound **4** (Stearyl-GNeo)

Supporting References

(1) Inoue, M.; Tong, W.; Esko, J. D.; Tor, Y. Aggregation-Mediated Macromolecular Uptake by a Molecular Transporter. *ACS Chem. Biol.* **2013**, *8*, 1383-1388.

(2) Natarajan, A.; Du, W.; Xiong, C. Y.; DeNardo, G. L.; DeNardo, S. J.; Gervay-Hague, J. Construction of di-Scfv through a Trivalent Alkyne-Azide 1,3-Dipolar Cycloaddition. *Chem. Commun.* **2007**, *7*, 695-697.