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The chemical modification of liposome surfaces via a copper-mediated [3+2] azide-alkyne cycloaddition monitored by a colorimetric assay

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S1. Experimental section

General materials and methods. All reagents and solvents were commercial products purchased from Aldrich or Biosolve ltd and used as received. Lipids were purchased from Lipoid and Avanti Polar Lipids, Rink Amide resin (0.78 mmol/g), PyBOP and FmocLys(Mtt)-OH were purchased from Novabiochem. LC-MS spectra were recorded on a JASCO RP-HPLC system, with simultaneous UV detection at 214 and 254 nm, coupled to a PE/SCIEX API 165 mass spectrometer equipped with a custom-made electronspray interface (ESI). HPLC analysis was performed on a Shimadzu system connected to a ELSD-detector. For LC-MS an analytical Vydac C4 column (Grace Vydac, 4.6 mm x 250 mm, 5 µm particle size, flow 1 ml/min) was employed. Buffers: A: 25% (v/v) H_2O in CH_3OH; B: CH_3CN and C: 1% (v/v) TFA in CH_3OH. A linear gradient with increasing percentage of B was applied in 5 column volumes (CV) and 10% of buffer C was used. For RP-HPLC a preparative Vydac C4 column (Grace Vydac, 22 mm x 250 mm, 10 µm particle size, flow 25 ml/min) and the same buffer system used for LC-MS were employed. For HPLC an analytical Alltech Silica Column (Alltech, Altima Silica Column 250 x 4.6 mm, Silica 5 µm particle size, flow 1 ml/min) was employed. Buffers: A: 60% $CHCl_3$, 34.5% CH_3OH, 5% H_2O, 0.5% ammonia (25%). B: CH_3OH. A linear gradient of 75 $\rightarrow$ 0% B was applied in 5 CV. Milli-Q water with a resistance of more than 18.2 MΩ/cm was provided by a Millipore Milli-Q filtering system with filtration trough a 0.22 µm Millipak filter. FRET spectra (exciting λ = 470 nm) were measured with a Luminescence Specrtometer LS 55, Perkin Elmer Instruments and UV spectra (λ = 472 nm) were recorded using a Varian Cary 3 Bio. The size distributions of the liposome solutions was measured by Photon Correlation Spectroscopy (PCS) using a Zetasizer 3000 HS_A, Melvern Instruments. Temperature = 25.0 °C, Viscosity = 0.890 cP; Angle = 90.0 deg RI medium = 1.33; RI particle 1.50 + Abs. 0.00.

^{1}H-NMR and ^{13}C-NMR spectra were measured with a Bruker AC-200 (200 and 50.1 MHz respectively). Chemical shifts are reported in ppm downfield from internal tetramethylsilane (0.00 ppm). In the case of the ^{13}C spectra, the solvent peak was used as a reference ($CDCl_3$: 77.7 ppm). Abbreviations used are s = singlet, d = doublet, dd = doublet of doublets, m = multiplet, br = broad.

1,2-Dioleoyl-sn-Glycero-3-Phosphoethanolamine-*N*-prop-2'-ynamide (DOPE-COCH≡CH 2). 1,2-Dioleoyl-sn-Glycero-3-Phosphoethanolamine (DOPE) (1.49 g, 2 mmol) in CH_2Cl_2 (10 ml) was treated dropwise with a preactivated solution (0°C) of propiolic acid (3 mmol, 0.2 ml) and *N*-(3-Dimethylaminopropyl)-*N*'-ethylcarbodiimide (EDC, 3 mmol, 288 mg) in CH_2Cl_2 (5 ml). After stirring for 20 hrs at room temperature the reaction mixture was again dropwise treated with a preactivated solution (0°C) of propiolic acid (3 mmol, 0.2 ml) and *N*-(3-Dimethylaminopropyl)-*N*'-ethylcarbodiimide (EDC, 3 mmol, 288 mg) in CH_2Cl_2 (5 ml). After at total of 40 hrs, TLC analysis revealed complete conversion. The mixture was concentrated under reduced pressure and purified by silica gel column chromatography (100/0 → 85/10mv/v CH_2Cl_2/CH_3OH). Evaporation of the solvents yielded 1.52 g (1.9 mmol, 95%) of product as a "sticky" foam. Finally, the product was purified by RP-HPLC using a linear gradient of 0 → 90% B in 5 CV. ESI-MS: R_t 14.4 min, *m/z* = 796.8 $[M+H]^+$, *m/z* = 818.7 $[M+Na]^+$, *m/z* = 1592.6 $[2M+H]^+$; *m/z* = 604. [3] ^{1}H NMR (200 MHz, $CDCl_3$, δ): 7.8 (br, 1H, N*H*), 5.4 (m, 5H, =C*H* and C*H* glycero), 4.5-4.4 (m, 1H, CH_A glycero), 4.2-4.0 (m, 5H, CH_B glycero, $CH_{A'B'}$ glycero, CH_2O), 3.7-3.4 (m, 2H, CH_2N), 3.0-2.9 (m, 2H, ≡C*H*), 2.7-2.5 (m, 4H, CH_2 succinyl), 2.4-2.3 (m, 4H, CH_2CO oleoyl), 2.1-1.9 (m, 8H, $CH_2CH{=}CH$), 1.7-1.5 (m, 4H, CH_2CH_2CO oleoyl), 1.4-1.2 (m, 49H, CH_2 oleoyl, CH_3 TEA), 1.0-0.8 (m, 6H, CH_3 oleoyl). ^{13}C APT NMR (50.1 MHz, $CDCl_3$, δ): 173.6 (*C*O), 153.1 (≡*C*H), 129.7, 129.4 (=*C*H), 70.2 (*C*H glycero), 64.2, 63.6, 62.4 (*C*H_2 glycero, *C*H_2O), 40.2 (*C*H_2N), 33.8-22.4 (*C*H_2 oleoyl), 13.8 (*C*H_3 oleoyl). IR (thin film from CH_2Cl_2, cm^{-1}): 3251 (w, NH stretch, Amide A band), 3008 (CH_3 anti-symmetric stretch), 2923 (CH_2 anti-symmetric stretch), 2852 (CH_2 symmetric stretch), 2109

(CH≡CH stretch), 1736 (C=O stretch), 1652 (C=O stretch, Amide I band), 1538 (coupled NH deformation and C-N stretch, Amide II band).

General protocol for the preparation of small unilamellar liposomes.

A lipid mixture consisting of 50 mol% of DOPE-COCH≡CH (**1**), 1 mol% of DOPE-LR and 49 mol% of DOPC was homogeneously dissolved in $CHCl_3$ (2 ml). The solvent was evaporated under a flow of N_2 and then under vacuum for 10 min. Next, the lipid film was hydrated for 1 h at room temperature with milli-Q water (total lipid concentration 1 mM). The samples were vortexed resulting in a turbid suspension and sonicated for 1 h until a clear solution appeared. The size of the particles was measured by Photon Correlation Spectroscopy (PCS).

Procedure for the "click" reaction.

Fmoc protected Rink Amide resin (0.78 mmol/g, 0.25 mmol, 321 mg) was washed with DCM (15 ml x 3) and NMP (15 ml x 3), followed by removal of the Fmoc group using 20% piperidine in NMP (15 ml x 1 min x 1, 15 ml x 2 min x 2) and washing with NMP (15 ml x 3). *N*-α-Fmoc-*N*-ε-4-methyltrityl-Lys-OH (Fmoc-Lys(Mtt)-OH, 625 mg, 1 mmol, 4 equiv.) in NMP (10 ml) was preactivated with benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP, 4 equiv.)/diisopropylethylamine (DIPEA, 8 equiv.) for 2 min and added to the resin. The reaction vessel was shaken for 2 hrs, then the resin was washed with NMP (15 ml x 3) and the Fmoc group removed as described above. Diazo transfer was performed on the solid support, [1] using an excess of triflyl azide (5 mmol, 20 equiv.) in DCM (23 ml) in the presence of a catalytic amount of $CuSO_4 \cdot 5H_2O$ (5 mg, 20 mmol, 0.8 equiv.) in MeOH (1 ml). The reaction mixture was shaken for 16 hrs at room temperature. After the Kaiser test confirmed completeness of the diazo transfer, the resin was washed first with NMP (15 ml x 3) and subsequently with 0.5% DIPEA in NMP (15 ml x 3), 0.05 M diethyldithiocarbamic acid sodium salt in NMP (15 ml x 3), NMP (15 ml x 5) and finally with DCM (15 ml x 3).

The methyltrityl (Mtt) group was deprotected by rinsing the resin with 10 ml of DCM/triisopropylsilane (TIS)/trifluoroacetic acid (TFA) 94/5/1 v/v over a period of 2 min (3x) followed by washing with DCM (15 ml x 4) and dry THF (15 ml x 3). A mixture of NBD-Cl (200

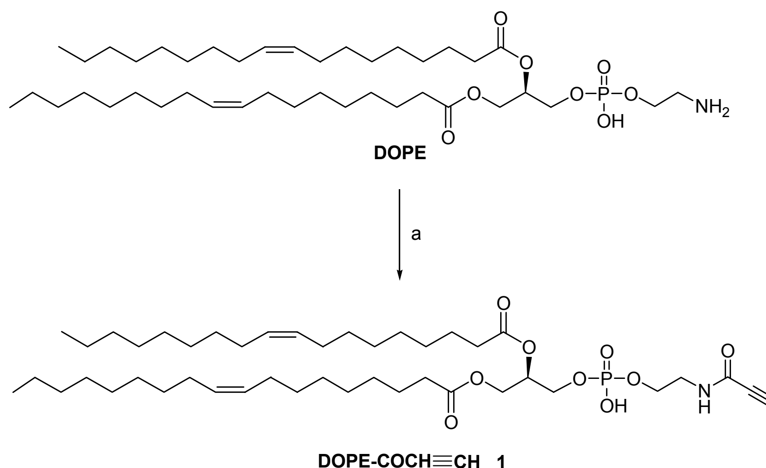
mg, 1 mmol, 4 equiv.) and 0.5% DIPEA in dry THF (20 ml) was added to the resin and shaken for 16 hrs at room temperature. [2] The dark brown resin was rinsed in THF (15 ml x 3), NMP (15 ml x 3) and DCM (15 ml x 3). The *N*-α-azido-Lys(NBD) was cleaved from the solid support by treatment with 10 ml of TFA/H_2O 90/10 v/v for 10 min (3x) at room temperature. After filtration of the resin, the TFA solution was concentrated under reduced pressure to yield a dark brown oil that was directly used in the next step. 2 equiv. (relative to DOPE-COCH≡CH **1**) of crude N_3-Lys(NBD)-NH_2 **2** was weight in a vial and dissolved in 1/1 v/v MeOH/$CHCl_3$ mixture followed by evaporation of the solvents to form a thin film. The vesicle solution (solution **A**) was added to the vial containing **2** and CuBr (0.5 equiv.). Negative control solution **B** did not contain CuBr. The solutions were incubated at 40 °C for 10 min and then under gentile swirling at room temperature. Successively, solutions were dialyzed against water and subsequently lyophilized. The formation of the product was confirmed by LC-MS using the condition described in the general materials and methods. A linear gradient of 40 → 90% B was applied in 5 CV. LC-MS: R_t 12.6 min; ESI-MS: *m/z* = 1130.7 $[M+H]^+$, *m/z* = 1152.6 $[M+Na]^+$, *m/z* = 967.7 $[M\text{-}NBD+H]^+$, *m/z* = 603.9 [3] (DOPE-COCH≡CH: R_t 13.8 min, *m/z* = 796.8 $[M+H]^+$, *m/z* = 818.7 $[M+Na]^+$, *m/z* = 603.9; [3] DOPE-LR: R_t 15.5 min, *m/z* = 1284.6 $[M+H]^+$, *m/z* = 1306.6 $[M+Na]^+$, *m/z* = 603.9 [3] and DOPC: R_t 16.9 min, *m/z* = 786.6 $[M+H]^+$, *m/z* = 808.6 $[M+Na]^+$).

General protocol for the colorimetric assay, FRET and UV measurements.

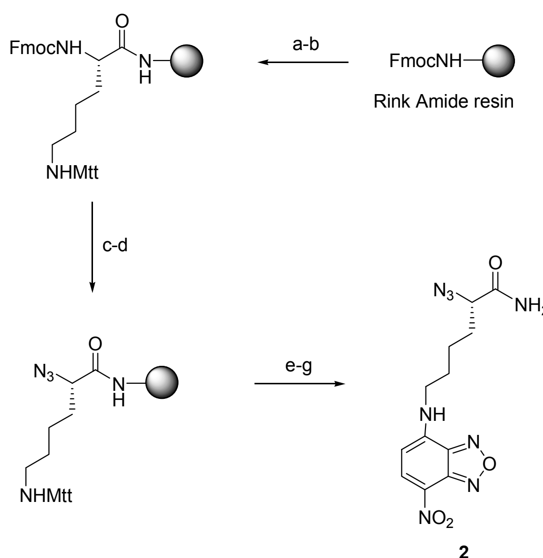
Samples (300 μL) were taken at several time intervals (t = 0, 0.5, 1, 2, 4 and 20 hrs) and dialyzed against water. After which FRET (exciting λ = 470 nm) and UV (λ = 472 nm) spectra were measured.

HPLC analysis.

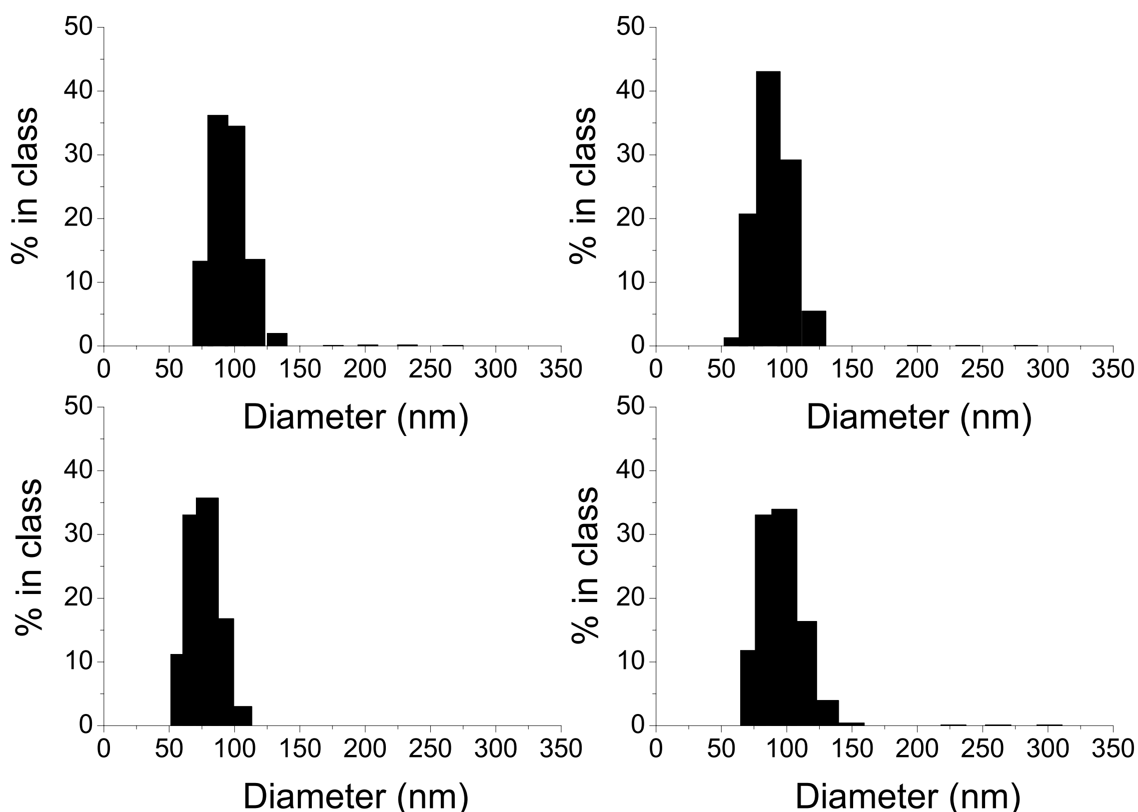
All samples used in the colorimetric assay were analyzed by HPLC. Standard lipid solutions (c = 1 mg/ml) were used to assigne the peaks. R_t DOPE-LR ~ 21 min; R_t DOPE-COC≡CH ~ 22 min and R_t DOPC ~ 29 min.



Scheme S2. Reagents and conditions: a) CH≡CHCOOH (3 equiv.), EDC (3 equiv.) in DCM.



Scheme S3. Reagents and conditions: a) 20% piperidine in NMP. b) Fmoc-Lys(Mtt)-OH (4 equiv.) PyBOP (4 equiv.) and DiPEA (8 equiv.) in NMP. c) 20% piperidine in NMP. d) $CF_3SO_2N_3$, $CuSO_4{\cdot}5H_2O$ (cat.) in DCM. e) 94/1/5 v/v DCM/TFA/TIS. f) NBD-Cl (4 equiv.), 0.5% DiPEA in dry THF. g) 90% TFA in H_2O.



S4. Size distribution of liposomes determined by Photon Correlation Spectroscopy (PCS). Vesicle solution **A** (top left) before reaction, cumulant Z average = 122.9 nm; polydispersity = 0.359. (top right) after reaction, cumulant Z average = 133.4 nm; polydispersity = 0.257. Size distributions for negative control solution **B** (bottom left) before addition of N_3-Lys(NBD)-NH_2 **2**, cumulant Z average = 111.5 nm; polydispersity = 0.373. (bottom right) after addition of **2**. cumulant Z average = 129.6 nm; polydispersity = 0.325.

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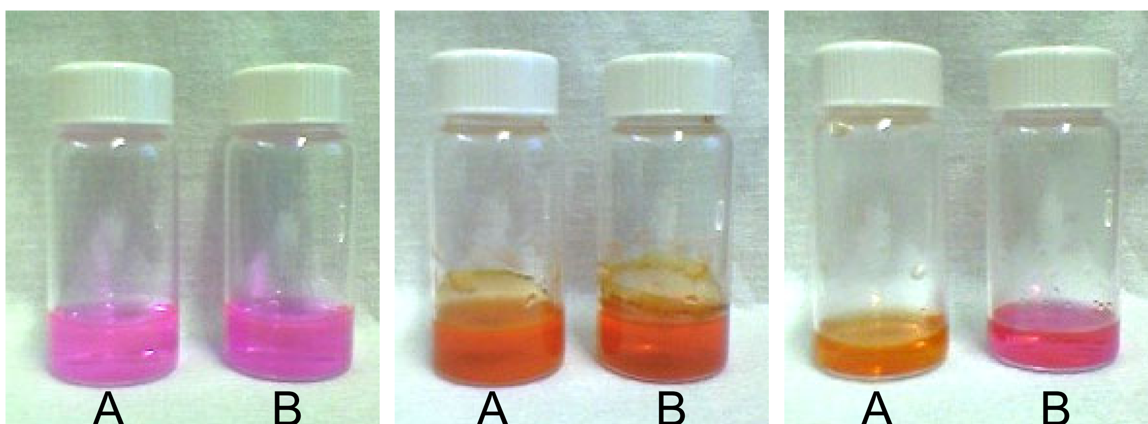
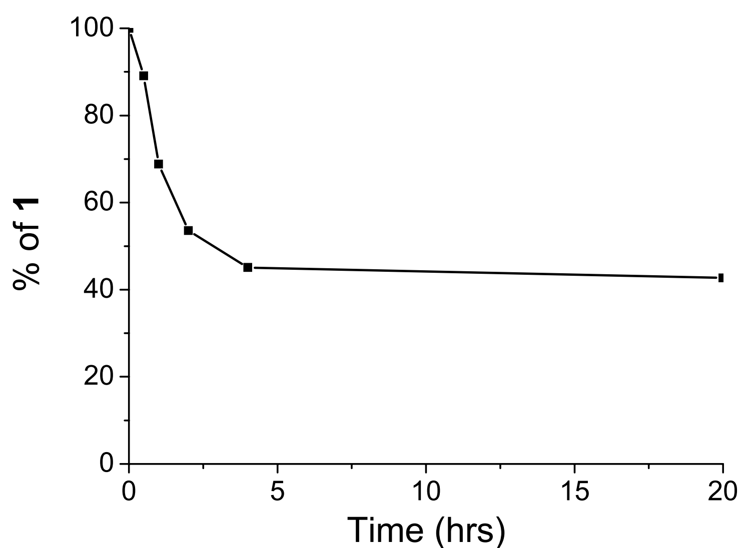


S5. Images of the color change (left) before reaction. (middle) during reaction. (right) after reaction.

In all cases A corresponds to the vesicle solution **A** and B to the negative control solution **B**.



S6. Normalized HPLC data. Decrease of DOPE-COCH≡CH (**1**) as a function of time.

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