Supporting Information For

Clickosomes–Using Triazole-linked Phospholipid Connectors to Fuse Vesicles

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All the reactions were carried under a N$_2$ atmosphere except those where water was involved (solvent or reactant). Starting compounds and solvents were purchased from Sigma-Aldrich/Fluka or Acros and were used without further purification. m-CPBA provided by Sigma was 77% pure. Column chromatographic separations were carried out using 230-400 mesh silica gel. TLC plates were developed with KMnO$_4$. $^1$H, $^{13}$C and $^{31}$P NMR spectra were recorded (as indicated) on either a Bruker 300 MHz, 400 MHz or 500 MHz spectrometer and are reported as chemical shifts (δ) in ppm relative to TMS (δ = 0). Spin multiplicities are reported as a singlet (s), doublet (d) or triplet (t) with coupling constants (J) given in Hz, or multiplet (m). Broad peaks are marked as br. HRESI-MS were performed on QSTAR Pulsar (AB/MDS Sciex) spectrometer and are reported as mass-per-charge ratio m/z. IR spectra were recorded on a Perkin Elmer Spectrum One FT-IR spectrometer (ATR, Golden Gate). Transmission electron microscopy was performed on a FEI Tecnai G2 Sphera microscope. DLS and zeta potential measurements were performed using a Zetasizer Nano ZS from Malvern Instruments.

1,3-bis(hexadecyloxy)propan-2-ol (2)

Synthesized according to the procedure of Hawthorne and coworkers.1

Sodium hydride (60% in mineral oil) (525 mg, 13.1 mmol, 0.430 ml) was added in small portions (3 times) to 1-hexadecanol (1) (6.63 g, 27.3 mmol, 8.11 ml) at 100 °C. After stirring for 0.5 h, epichlorohydrin (0.25 ml, 3.19 mmol) was added dropwise to the hot mixture and maintained at 100 °C overnight, after which water (2 ml) was added to quench the reaction. The filtrate (obtained in CH$_2$Cl$_2$ extraction of the crude reaction mixture) was washed with water (3 x 150 ml), THF was added to facilitate the separation and solvent was removed under reduced pressure and water by vacuum. Purification on silica gel column (Ethyl Acetate /Pentane 5:95) after removing the solvent under reduced pressure. Pet-OH-Pet (2) (1.06 g, 1.96 mmol, 61%) was isolated as a white solid.

$^1$H NMR (400 MHz, CDCl$_3$) δ 4.07 – 3.78 (m, 1H), 3.64 – 3.15 (m, 8H), 2.60 – 2.11 (m, 1H), 1.65 – 1.39 (m, 4H), 1.25 (s, 54H), 0.88 (t, J = 6.6 Hz, 6H).

$^{13}$C NMR (126 MHz, CDCl$_3$): δ 73.38, 73.33, 71.52, 70.81, 66.50, 59.26, 58.40, 54.50, 31.94, 29.76, 29.69, 29.60, 29.38, 26.16, 22.70, 14.13.

HRMS (Turbo Spray) $^{35}$Cl$_2$O$_3$ [M + H$^+$]: Calculated 541.5565 measured 541.5554.

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2-(N-methyl-N-prop-2-yn-1-ylamino)ethanol

We followed the procedure of Hennequin.\textsuperscript{2} To a solution of 2-methylaminoethanol (3.05 mL, 37.8 mmol) in dioxane and propargyl bromide solution (2.80 mL, 26.0 mmol) in toluene, NaOH (8.40 mL, 6 M in H\textsubscript{2}O, 50.4 mmol) was slowly added at 0 °C. The solution was left stirring at room temperature for 12 h. The solution was then partitioned between H\textsubscript{2}O and EtOAc and the aqueous phase was washed three times with ethyl acetate. The resulting solvent phases were dried over MgSO\textsubscript{4}. The solvent was removed under reduced pressure and the crude oil was purified on a silica gel column (CH\textsubscript{2}Cl\textsubscript{2}/MeOH 95:5) to give the product as slightly yellow oil (1.33 g, 11.7 mmol, 45%).

R\textsubscript{f}: 0.21 (in CH\textsubscript{2}Cl\textsubscript{2}/MeOH 95:5).

\textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3}): δ 3.73–3.53 (m, 2H), 3.41 (d, J = 2.4, 2H), 2.99 (s, 1H), 2.68–2.59 (m, 2H), 2.37 (s, 1H), 2.32–2.24 (t, J = 2.4, 3H).

\textsuperscript{13}C NMR (101 MHz, CDCl\textsubscript{3}): δ 78.31, 73.34, 58.60, 57.02, 45.71, 41.23.

IR: 3290.85, 2944.67, 2851.72, 2800.63, 2160.73, 1130.06, 1032.12.

\textsuperscript{2} L. F. Hennequin, PCT Int. Appl., 2003064413, 07 Aug 2003
Benzyl (1,3-bis(hexadecyloxy)propan-2-yl) (2-(methyl(prop-2-yn-1-yl)amino)ethyl) phosphate (3)

Pet-OH-Pet (2) (402 mg, 0.74 mmol, 1.0 eq) dissolved in 20 mL dry THF was added to benzyloxy)dichlorophosphine (466 mg, 2.23 mmol, 0.36 mL) and dry triethylamine (654 mg, 6.47 mmol, 0.90 mL) in 20 mL of dry THF. The addition occurred at 0 °C during 160 min. The resulting solution was stirred at 20 °C for 100 min. Then 2-(N-methyl-N-prop-2-yn-1-ylamino)ethanol (589 mg, 5.20 mmol) was added at 0 °C and stirring continued for 90 min. The solution was stored at -20 °C overnight. Then the phosphorus(III) was oxidized using m-CPBA (918 mg, 5.32 mmol) at 0 °C. The mixture was left stirring for 20 minutes. Then the crude product was consecutively partitioned between 110 mL Na$_2$S$_2$O$_3$ 10%, 30 mL NaHCO$_3$ sat. and 3 x100 mL of CH$_2$Cl$_2$. After purification over silica gel columns (MeOH/CH$_2$Cl$_2$ 5:95) and then Ethyl Acetate /Pentane (1:1), the product (3) (410 mg, 0.51 mmol, 69%) was obtained as a yellow wax.

R$_f$: 0.41 (Ethyl Acetate/Pentane 1:1).

$^1$H NMR (500 MHz, CDCl$_3$): δ 7.40 – 7.27 (m, 5H), 5.13 (d, J = 10 Hz, 2H), 4.63-4.59 (m, 1H), 4.15 - 4.12 (m, 2H), 3.60 – 3.58 (m, 4H), 3.44 – 3.43 (m, 4H), 3.37 (s, 2H), 2.73 (t, J = 5 Hz, 2H), 2.34 (s, 3H), 2.21 (s, 1H), 1.55 – 1.52 (m, 4H), 1.29 (s, 52H), 0.89 (t, J = 5 Hz, 6H).

$^{13}$C NMR (101 MHz, CDCl$_3$) : δ 136.21, 136.15, 128.49, 128.33, 127.78, 78.23, 76.50, 76.45, 73.37, 71.71, 70.12, 70.08, 69.10, 69.05, 65.35, 65.31, 55.00, 54.94, 45.86, 41.87, 31.94, 29.72, 29.67, 29.66, 29.64, 29.37, 22.70, 14.13.

$^{31}$P NMR (121 MHz, CDCl$_3$): δ -1.38 (s,100%).

IR: 2923.60, 2853.83, 2187.09, 1279.97, 1017.51.

HRMS (Turbo Spray) C$_{48}$H$_{80}$NO$_8$P [M + H$^+$]: Calculated 806.6422, measured 806.6416.
1,3-bis(hexadecyloxy)propan-2-yl (2-(methyl(prop-2-yn-1-yl)amino)ethyl) hydrogen phosphate (4)

The alcohol (2) (236 mg, 0.29 mmol) was dissolved in 3 mL of CH₂Cl₂. Then CF₃COOH (3.00 ml, 40.0 mmol) were added. The solution was left stirring overnight. Then the acid was evaporated by bubbling N₂ through the solution. The crude product was purified on a silica gel column (CH₂Cl₂/MeOH/NH₄OH 87.5:11:1.5) to give the phosphate 4 (203 mg, 0.28 mmol, 97%) as a white powder.

R_f: 0.19 (CH₂Cl₂/MeOH/NH₄OH 87.5:11.25:1.25).

¹H NMR (500 MHz, CDCl₃): δ 4.37 (br, 1H), 4.12 (s, 2H), 3.76 (s, 2H), 3.58 (s, 4H), 3.44 – 3.41 (m, 4H), 2.99 (s, 2H), 2.62 (s, 3H), 2.41 (s, 1H), 1.54 – 1.51 (m, 4H), 1.29 (s, 52H), 0.89 (t, J = 5 Hz, 6H).

¹³C NMR (127 MHz, CDCl₃): δ 77.59, 73.73, 73.68, 71.57, 70.77, 70.74, 60.95, 55.34, 44.92, 40.96, 31.94, 29.74, 29.74, 29.68, 29.59, 29.38, 22.70, 14.12.

³¹P NMR (121 MHz, CDCl₃): δ 4.09 (s, 100%).

IR: 2917.32, 2850.46, 1467.90, 1223.31, 1064.90, 724.35.

HRMS (Turbo Spray) C₄₁H₇₈NO₇P [M + H⁺]: Calculated 706.6126, measured 706.6109.
1,3-bis(hexadecyloxy)propan-2-yl (2-(dimethyl(prop-2-yn-1-yl)ammonio)ethyl) phosphate (5)

The methylation was done according to a procedure of Eibl and al.\(^3\)

Dimethyl sulfate (265 mg, 2.12 mmol, 8 eq) was added to the phosphate 4 (190 mg, 0.27 mmol, 1 eq) dissolved in 6 mL MeOH and heated to 40 °C. The solution was left stirring for 30 min and then potassium carbonate (293 mg, 2.12 mmol, 8 eq) in 2 mL water was added. The solution was again stirred for 1 hour at 40 °C. Then the solvents were evaporated and the product was purified over silica gel column using (CH\(_2\)Cl\(_2\)/MeOH/NH\(_4\)OH 87.5:11:1.5) then (CH\(_2\)Cl\(_2\)/MeOH/NH\(_4\)OH 75:22:3) to give the alkyne 5 (188 mg, 0.26 mmol, 97%) as a white powder.

R\(_f\): 0.11 (CH\(_2\)Cl\(_2\)/MeOH/NH\(_4\)OH 87.5:11:25:1.25).

\(^1\)H NMR (400 MHz, CDCl\(_3\)): δ 4.69 (s, 2H), 4.31 (m, 3H), 3.88 (s, 2H), 3.54 (s, 4H), 3.44 – 3.41 (m, 10H), 2.92 (s, 1H), 1.50 (s, 4H), 1.25 (s, 52H), 0.88 (t, J = 5 Hz, 6H).

\(^{13}\)C NMR (127 MHz, CDCl\(_3\)): δ 80.72, 72.71, 71.55, 70.61, 59.20, 54.88, 51.53, 31.97, 29.80, 29.79, 29.72, 29.66, 29.42, 26.20, 22.73, 14.16.

\(^{31}\)P NMR (121.5 MHz, CDCl\(_3\)): δ -0.49 (s, 100%).

IR: 3231.69, 2917.42, 2850.46, 2129.00, 1468.21, 1378.09, 1239.22, 1064.52.

HRMS (Turbo Spray) C\(_{42}\)H\(_{85}\)NO\(_6\)P [M + H\(^+\)]: Calculated 730.6109, measured 730.6125.

Benzyl (1,3-bis(hexadecyloxy)propan-2-yl) (2-(2-(2-chloroethoxy)ethoxy)ethyl) phosphate (6)

Pet-OH-Pet (2) (350 mg, 0.647 mmol) was dissolved in 20 mL dry THF and was added to (benzylxylo)dichlorophosphine (406 mg, 1.94 mmol, 0.322 ml) and dry triethylamine (524 mg, 5.18 mmol, 0.722 mL) in 25 mL dry THF. The addition occurred at 0 °C during 2 h. Stirring was continued at 20 °C for 2 h. Then triethylene glycol monochlorohydrin was added (675 mg, 3.88 mmol, 0.582 mL) at 0 °C and stirring continued for 30 min. Oxidation of the phosphorus(III) was performed with 3-chloroperbenzoic acid (365 mg, 1.62 mmol) at 20 °C. The mixture was stirred for 45 minutes. The crude product was partitioned consecutively between 30 mL Na$_2$S$_2$O$_3$ 10%, 30 mL NaHCO$_3$ sat. and 50 mL CH$_2$Cl$_2$. The aqueous phase was washed with 3 x 40 mL of CH$_2$Cl$_2$. The crude material was purified over a silica gel column using Ethyl Acetate /Pentane 2:5) to give the chloride 6 (233 mg, 0.272 mmol, 41%) as a white wax.

R$_f$: 0.13 (Pentane/Ethyl acetate 5:2).

$^1$H NMR (500 MHz, CDCl$_3$): δ 7.41 – 7.33 (m, 5H), 5.12 (d, J = 10 Hz, 2H), 4.64-4.59 (m, 1H), 4.21 - 4.18 (m, 2H), 3.72 – 3.57 (m, 18H), 1.58 – 1.51 (m, 4H), 1.29 (s, 52H), 0.88 (t, J = 5 Hz, 6H).

$^{13}$C NMR (101 MHz, CDCl$_3$): δ 136.32, 136.26, 128.63, 128.49, 127.94, 71.84, 71.54, 70.79, 70.24, 69.19, 66.78, 42.85, 32.08, 29.86, 29.52, 26.25, 22.84, 14.27.

$^{31}$P NMR (121 MHz, CDCl$_3$): δ -1.43 (s,100 %).

IR: 2922.73, 2852.96, 1461.80, 1279.62, 1118.89, 733.01, 1013.52.

HRMS (Turbo Spray) C$_{46}$H$_{91}$ClO$_8$P [M + H$^+$]: Calculated 861.6134 measured 861.6127.
1,3-bis(hexadecyloxy)propan-2-yl (2-(2-chloroethoxy)ethoxy)ethyl) hydrogen phosphate (7)

The chloride 6 (84.0 mg, 0.108 mmol, 1.0 eq) was dissolved in 1 mL of CH₂Cl₂. Then CF₃COOH (4.00 mL, 53.3 mmol) was added. The solution was stirred for 3 h at 20 °C. Then the acid was removed by bubbling N₂ through the solution. The crude material was purified over a silica gel column (CH₂Cl₂/MeOH/NH₄OH 87.5:11:1.5) to give the chloride 7 (84 mg, 0.108 mmol, 99%) as a white wax.

R_f: 0.69 (CH₂Cl₂/MeOH/NH₄OH 87.5:11:1.5).

¹H NMR (500 MHz, CDCl₃): δ 4.67 (m, 1H), 4.32 - 4.28 (m, 1H), 4.01 – 3.98 (m, 2H), 3.75 – 3.39 (m, 18H), 1.53 – 1.51 (m, 4H), 1.25 (s, 52H), 0.89 (t, J = 5 Hz, 6H).

¹³C NMR (125.77 MHz, CDCl₃): δ 141.16, 128.44, 127.39, 126.94, 73.57, 73.52, 71.60, 71.23, 70.88, 70.81, 70.37, 70.30, 70.27, 70.05, 64.90, 64.59, 64.55, 50.24, 42.75, 31.93, 29.74, 29.68, 29.60, 29.47, 29.38, 26.13, 26.06, 22.69, 14.10.

³¹P NMR (121 MHz, CDCl₃): δ -0.69 (s, 100 %).

IR: 2916.62, 2850.00, 1467.83, 1357.13, 1236.92, 1098.92, 1069.39.

HRMS (Turbo Spray) C₄₈H₁₅₆ClO₈P [M + H⁺]: Calculated 771.5665 measured 771.5699.
Sodium azide (64.3 mg, 0.984 mmol) was added to a solution of chloride 7 (123 mg, 0.159 mmol) dissolved in 20 mL of dry DMF and heated to 100 °C for 3 h. The crude material was purified over silica gel column (CH$_2$Cl$_2$/MeOH 9:1) then (CH$_2$Cl$_2$/MeOH 3:1) to yield the azide 8 (123 mg, 0.157 mmol, 99%) as a white powder.

R$_f$: 0.69 (CH$_2$Cl$_2$/MeOH/NH$_4$OH 87.5:11:1.5).

$^1$H NMR (500 MHz, CDCl$_3$): δ 4.37 - 4.32 (m, 1H), 4.02 – 4.01 (m, 2H), 3.73 – 3.41 (m, 18H), 1.55 – 1.53 (m, 4H), 1.26 (s, 52H), 0.89 (t, J = 5 Hz, 6H).

$^{13}$C NMR (125 MHz, CDCl$_3$): δ 73.14, 71.57, 71.13, 70.51, 70.33, 70.08, 69.87, 64.40, 50.62, 31.95, 29.79, 29.70, 29.67, 29.60, 29.40, 26.17, 22.71, 14.13.

$^{31}$P NMR (121 MHz, CDCl$_3$): δ -1.58 (s,100%).

IR: 2916.70, 2849.92, 2103.80, 1976.13, 1672.67, 1467.75, 1238.18, 1099.05, 1068.69.

HRMS (Turbo Spray) C$_{41}$H$_{85}$N$_3$O$_8$P [M + H$^+$]: Calculated 778.6068 measured 778.6068.
Triazole-bridged phospholipid (9)

The 1,3 Huisgen cycloaddition was done using the procedure of Musiol and al. ⁴

To 20 mL THF was added the alkyne 5 (50.5 mg, 69.1 µmol, 1.01 eq) and the azide 8 (53.4 mg, 6.43 µmol, 1 eq) together with a Cul salt solution (2.61 mg, 0.0137 mmol, 0.2 eq) and N,N-diisopropylethylamine (0.120 mL, 0.686 mmol). The reaction mixture was stirred 5 h at 20 °C. The crude material was purified with a gradient over silica gel column (CH₂Cl₂/MeOH 9:1) then (CH₂Cl₂/MeOH/NH₄OH 87.5:11:1.5), then (CH₂Cl₂/MeOH/NH₂OH 75:22:3) and (CH₂Cl₂/MeOH/H₂O 65:25:4) to give the product 9 (83 mg, 0.0569 mmol, 83%) as a white powder.

Rᶠ: 0.27 (CH₂Cl₂/MeOH/NH₄OH 87.5:11:1.5).

¹H NMR (500 MHz, CDCl₃): δ 7.40 – 7.27 (m, 5H), 5.13 (d, J = 10 Hz, 2H), 4.61 (m, 1H), 4.15 -4.12 (m, 2H), 3.60 - 3.58 (m, 4H), 3.44 – 3.43 (m, 4H), 3.37 (s, 2H), 2.73 (t, J = 5 Hz, 2H), 2.34 (s, 3H), 2.21 (s, 1H), 1.55 – 1.52 (m, 4H), 1.29 (s, 52H), 0.89 (t, J = 5 Hz, 6H).

¹³C NMR (101 MHz, CDCl₃): δ 135.48, 129.85, 73.90, 73.54, 71.56, 71.04, 70.68, 70.54, 70.34, 69.96, 68.86, 64.72, 60.08, 59.12, 51.13, 50.30, 31.94, 29.75, 29.60, 29.39, 26.11, 22.70, 14.13.

³¹P NMR (121 MHz, CDCl₃): δ -1.38 (s,100%).

IR: 2917.86, 2850.94, 1677.27, 1468.07, 1228.43, 1120.86, 1065.66.

HRMS (Turbo Spray) C₈₃H₁₅₉N₄O₁₄P₂ [M + H⁺]: Calculated 1508.2105 measured 1508.2110.

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Vesicle Formulation

Large unilamellar vesicles by extrusion technique (LUVET\textsubscript{100}) were prepared as follows:

**Positively charged vesicles with 5 (Mix A)**

Egg yolk phosphatidylcholine (EggPC, Avanti Polar Lipids, 15.9 mg, 21 mmol), alkyne 5 (1.9 mg, 2.6 mmol), and (di-n-dodecyl)dimethylammonium bromide (1.2 mg, 2.6 mmol) were weighed into a round bottomed flask and dissolved in 1 mL chloroform. After evaporation to dryness, the film was dried for 12 h under high vacuum. 1 mL of ultra-pure H\textsubscript{2}O was added to the flask to hydrate the film for 1 min. The suspension was sequentially frozen (liquid nitrogen bath) and melted (water bath at 40 °C) five times, then extruded 11 times using a mini-extruder (Avanti Polar Lipids) and 100 nm filter-size (Whatman). Vesicles were stored at 5 °C in the dark until use.

**Positively charged vesicles without 5 (Mix C)**

Egg yolk phosphatidylcholine (EggPC, Avanti Polar Lipids, 17.9 mg, 24 mmol) and (di-n-dodecyl)dimethylammonium bromide (1.2 mg, 2.6 mmol) were weighed into a round bottomed flask and dissolved in 1 mL chloroform. After evaporation to dryness, the film was dried for 12 h under high vacuum. 1 mL of ultra-pure H\textsubscript{2}O was added to the flask to hydrate the film for 1 min. The suspension was sequentially frozen (liquid nitrogen bath) and melted (water bath at 40 °C) five times, then extruded 11 times using a mini-extruder (Avanti Polar Lipids) and 100 nm filter-size (Whatman). Vesicles were stored at 5 °C in the dark until use.

**Negatively charged vesicles (Mix B)**

Egg yolk phosphatidylcholine (EggPC, Avanti Polar Lipids, 17.9 mg, 24 mmol) and azide 8 (2.0 mg, 2.6 mmol) were weighed into a round bottomed flask and dissolved in 1 mL chloroform. After evaporation to dryness, the film was dried for 12 h under high vacuum. 1 mL of ultra-pure H\textsubscript{2}O was added to the flask to hydrate the film for 1 min. The suspension was sequentially frozen (liquid nitrogen bath) and melted (water bath at 40 °C) five times, then extruded 11 times using a mini-extruder (Avanti Polar Lipids) and 100 nm filter-size (Whatman). Vesicles were stored at 5 °C in the dark until use.

**Reactive Vesicles**

10 µl of each vesicle suspension was added into an Eppendorf tube that was filled with 980 µL of ultra-pure H\textsubscript{2}O. Either 10 mg of CuBr was added or nothing (control). The suspensions were then shaken on a carousel shaker at 20 rpm.

After 1 day or 4 days the vesicle suspension was filtered over a small column of Sephadex G-50 in order to remove traces of CuBr that would interfere with DLS measurements.

Dynamic Light Scattering measurements (all performed at 20 °C) of the vesicles right after their preparation.

DDAB = (di-n-dodecyl)dimethylammonium bromide.
Negative staining of Liposomes for TEM

Vesicles were prepared for TEM using the following protocol.

- evaporate a thin layer of carbon on formvar coated copper grids (200 mesh)
- glow discharge the grid for 30 s
- prepare 2 drops of uranyl acetate (2% in water: 400 µL saturated UA + 600 µL ultrapure water)
- put a 5 µL drop of liposome suspension onto the grid surface and let it adsorb for 30 s
- blot off extra liquid using filter paper while holding the grid vertically
- put the grid onto the staining solution (2% uranyl acetate in water) for 1 s then stain for 30 s
- blot and suck off the remaining liquid with a thin Pasteur pipette
- analyze the sample using a Transmission electron microscope at 120 kV (Tecnai G2, FEI, Eindhoven, Netherlands).
TEM images showing the vesicle suspension (Mix A + Mix B) in the presence of CuBr after 4 days. Scale bar = 1 µm.
TEM images showing the vesicle suspension (Mix B + Mix C) in the presence of CuBr after 4 days. Scale bar = 1 µm. Negative control: without compound 5.