Supplementary Materials:

Materials and Methods

Figures S1-S18

References

Supplementary Materials:

Materials

Tripropargylamine (98%), hydroquinone (≥99%), pentynoic acid (95%), 1-bromododecane (97%), 1-bromotetradecane (≥97%), 1-bromohexadecane (≥97%), oleyl bromide (≥99%), N-[Tris(hydroxymethyl)methyl]-2 aminoethanesulfonate sodium salt (TES) (≥99%), and 3-(N Morpholino)propanesulfonate hemisodium salt (MOPS) (≥99%) were purchased from Sigma-Aldrich. Sodium azide (99%) was purchased from Alfa Aesar. Copper (II) sulfate pentahydrate (98%) was purchased from Acros Organics. 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine (>99%) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) (>99%) were obtained from Avanti Polar Lipids. All materials were used as received.

Methods

The synthesis of alkyne lysolipid (3-(palmitoyloxy)-2-(pent-4-ynoyloxy)propyl (2-(trimethylammonio)ethyl) phosphate) has previously been reported (1).

Synthesis of lauryl azide (1-azidododecane) and palmityl azide (1-azidohexadecane)

Cl, n = 10, 14 → (NaN_3; DMF, 60°C, overnight, 95-97%) → N_3, n = 10, 14

General procedure: Sodium azide (162.5 mg, 2.5 mmol) was added to a stirred solution of alkyl chloride (1.0 mmol) in DMF (3 mL), and the resulting solution was heated to 60 °C and stirred overnight. The reaction was worked up by addition of 20 mL H_2O followed by extraction with EtOAc/Hexanes (3 × 20 mL, v/v = 1:1). The combined organic extracts were washed with water (3 × 60 mL) to remove any remaining DMF, dried over Na_2SO_4, filtered, and concentrated in vacuo to afford alkyl azide (1 mmol) as a pale yellow oil. The azide was obtained in nearly quantitative yield and used as is for all experiments.

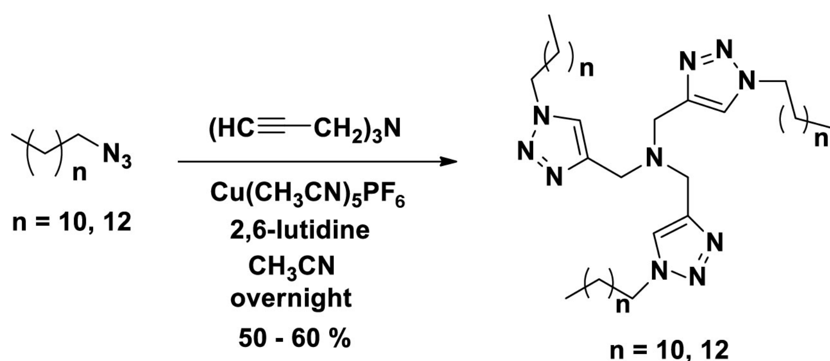
Lauryl azide (n = 10):

Colorless Oil, yield 97%. ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.87 (3H, t, *J* = 7.5 Hz), 1.25-1.35 (18H, m), 1.59 (2H, m), 3.24 (2H, t, *J* = 7.5 Hz); ^{13}C (100 MHz, $CDCl_3$) δ 14.2, 22.8, 26.8, 28.9,

29.3, 29.5, 29.6, 29.72, 29.74, 32.0, 51.6. HRMS $[[M-N_2+H]^+$ m/z calcd. for $[C_{12}H_{26}N]^+$ 184.2060, found 184.2061.

Palmityl azide (n = 14):
Colorless Oil, yield 95%. ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.87 (3H, t, *J* = 5.0 Hz), 1.25-1.35 (26H, m), 1.59 (2H, m), 3.25 (2H, t, *J* = 7.5 Hz); ^{13}C (100 MHz, $CDCl_3$) δ 14.2, 22.8, 26.8, 28.9, 29.3, 29.5, 29.58, 29.65, 29.73, 29.75, 29.76, 29.78, 29.79, 29.80, 32.0, 51.6. HRMS $[M-N_2+H]^+$ m/z calcd. for $[C_{16}H_{34}N]^+$ 240.2686, found 240.2688.

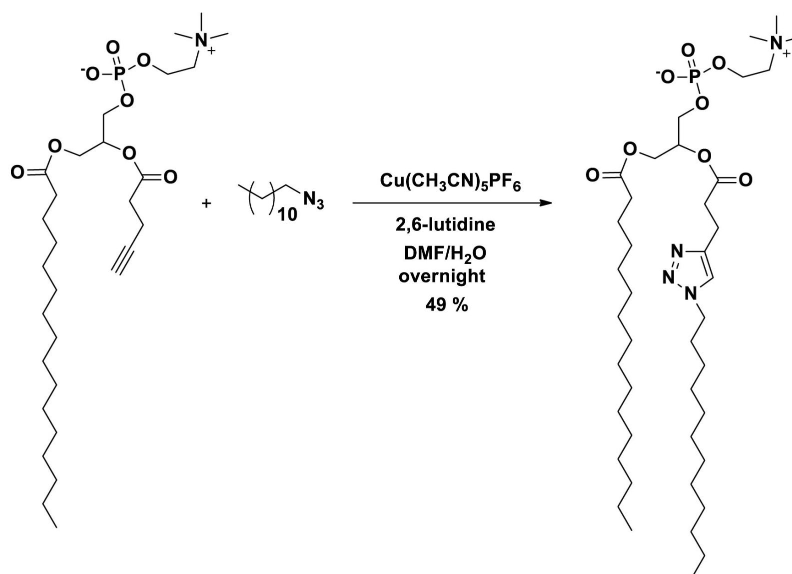
Synthesis of oligotriazole ligand TLTA (Tris-(Lauryl-Triazole-methyl)-Amine) (tris((1-dodecyl-1 H-1,2,3-triazol-4-yl)methyl)amine)



2,6-lutidine (4.0 mg, 0.038 mmol) and alkyl azide (0.171 mmol) were added to a stirred solution of tripropargylamine (5.0 mg, 0.038 mmol) in CH_3CN (1.0 mL), followed by tetrakis(acetonitrile)copper(I) hexafluorophosphate (1.0 mg, 0.0027 mmol). The resulting solution was stirred at room temperature, and the product was formed as a white precipitate. After 12 hours, the reaction was worked up by filtering the precipitate and washing twice with acetonitrile (2 × 2 mL). The white solid was collected and dried in vacuo to afford the pure products, which were used as is for all experiments.

TLTA (n = 10, Tris-(Lauryl-Triazole-methyl)-Amine)
White solid, yield 51%. ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.86 (9H, t, *J* = 7.5 Hz), 1.25-1.30 (60H, m), 1.90 (6H, bs), 4.34 (6H, bs), 7.79 (3H, bs); ^{13}C (100 MHz, $CDCl_3$) δ 14.4, 22.9, 26.8, 29.2, 29.55, 29.62, 29.74, 29.82, 30.5, 32.1, 47.1, 50.7, 124.2, 144.0. HRMS $[M+H]^+$ m/z calcd. for $[C_{45}H_{85}N_{10}]^+$, found 765.6953, 765.6954.

Synthesis of C12-triazole-phospholipid (1-hexadecanoyl-2-(3-(1-dodecyl-1*H*-1,2,3-triazol-4-yl)propanoyl)-*sn*-glycero-3-phosphocholine)



2,6-lutidine (3.0 mg, 0.028 mmol) and 1-azidododecane (7.0 mg, 0.03 mmol) were added to a stirred solution of 3-(palmitoyloxy)-2-(pent-4-ynoyloxy)propyl (2- (trimethylammonio)ethyl) phosphate (15.0 mg, 0.026 mmol) in DMF/H_2O (2.0 mL, v/v = 1:1) followed by tetrakis(acetonitrile)copper(I) hexafluorophosphate (1.0 mg, 0.0027 mmol). The resulting solution was stirred at room temperature for 24 hours. The reaction was worked up by addition of brine (10 mL) and extraction with EtOAc; a small amount of methanol was added to break the emulsification. The organic layer was dried over Na_2SO_4, filtered, and concentrated in vacuo to yield the crude product. The crude product was purified by preparative TLC (GF254, 0.25 mm, 20 × 20 cm, CH_2Cl_2/MeOH/H_2O = 3/1/0.1) to afford 10 mg of product with a yield of 49 %.

White solid, 49%. ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.86 (6H, t, J = 7.5 Hz), 1.23-1.28 (38H, m), 1.54 (2H, bs), 1.84 (2H, bs), 2.24 (m, 2H), 2.73 (bs, 2H), 2.97 (bs, 2H), 3.31 (bs, 9H), 3.76 (bs, 2H), 3.93 (bs, 2H), 4.09 (bs, 1H), 4.27-4.33 (4H, m), 5.20 (1H, bs), 7.40 (1H, bs); ^{13}C (100 MHz, $CDCl_3$) δ 14.2, 21.0, 22.8, 24.9, 26.7, 29.2, 29.3, 29.45, 29.46, 29.48, 29.56, 29.67, 29.73, 29.74, 29.78, 29.79, 29.83, 30.48, 32.01, 32.02, 33.7, 34.1, 50.4, 54.5, 59.5, 63.8, 71.1, 121.3, 146.1, 172.3, 173.7. HRMS $[M+H]^+$ m/z calcd. for $[C_{41}H_{80}N_4O_8P]^+$ 787.5708, found 787.5707.

Vesicle Preparation

The protocol for vesicle formation was adapted from a previously published method (2). Vesicles were prepared by sonication following hydration of a dried lipid film. To prepare lipid films, POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine) in chloroform and TLTA in chloroform were added to a glass vial. The vial was then placed under a gentle stream of filtered nitrogen and dried. Dried films were hydrated in a buffer containing 40 mM MOPS and 140 mM to 600 mM TES (pH 8.45 - 8.5). The vesicle solution was briefly vortexed and then placed in a room-temperature bath sonicator (3510 Branson), which was set to sonicate with heat for 85 minutes (final temp: 56~62°C). Vesicle preparations were allowed to cool to room temperature and then transferred to Eppendorf tubes and sonicated for 20-25 additional minutes at room temperature without heat. Vesicle batches were stored on a shaker at room temperature and used for a period of up to a week.

Preparation of Reactive Precursors

Emulsions of alkyl azide and alkyne lysolipid were prepared by adding 21.6 μL of 30 mM alkyl azide in chloroform and 26 μL of 15 mM alkyne lysolipid in chloroform, followed by drying the chloroform off with filtered nitrogen. 600 μL of buffer with 40 mM MOPS and a TES concentration, matching the vesicle population to be used for the experiment, was added to the film to create emulsions. The solution was briefly vortexed and sonicated without heat for 15 - 30 minutes.

Cu-ligand catalyzed cycloaddition reactions

Copper and hydroquinone were prepared daily with H_2O obtained from a Milli•Q Water Purification System. Tripropargylamine was prepared in 100% EtOH. Vesicles were always added to a reaction mix after every other component had been mixed together, but prior to the addition of copper. With the exception of vesicles, each component was briefly vortexed before addition to the reaction mix. All reactions were placed on a shaker, rotating ~120 rev/min at room temperature. Periodically, 15 μL aliquots of the reaction would be removed for liquid chromatography/mass spectrometry/evaporative light scattering detection (LC/MS/ELSD) sampling or microscopy.

***In situ* Vesicle Synthesis of Triazole Phospholipid**

To prepare vesicles, 50 μL of 100 mM POPC in chloroform was added to each of four LCMS vials, and 0, 7.5, 20 or 50 μL of 10 mM TLTA in chloroform was also added. Vials were then dried and hydrated with 1 mL of 140 mM TES and 40 mM MOPS. Vials were placed in a bath sonicator at room temperature and sonicated with heat for 80-90 minutes. Emulsions were prepared by dehydrating 27 μL of 60 mM lauryl azide and 32.4 μL of 30 mM alkyne lysolipid under nitrogen. The azide and alkyne mix was then hydrated with 1.5 mL of 140 mM TES and 40 mM MOPS, briefly vortexed and sonicated. For all reactions, 2.25 μL of 4 mM hydroquinone was added to 115 μL of emulsion, containing 1.08 mM lauryl azide and 650 μM alkyne lysolipid. Next, 30 μL of vesicles (5 mM POPC + 0, 300, 400 or 500 μM TLTA) were added to the reaction, and the contents were gently mixed together. After addition of 2.25 μL of 8 mM $CuSO_4$, the reaction was mixed together once more, placed on a shaker, and 15 μL were extracted for a LC/MS/ELSD time point. Concentrations were reported based on a calibration curve, created using known concentrations of triazole phospholipid.

Initial reaction conditions were as follows: 830 μM lauryl azide, 498 μM alkyne lysolipid, 0, 15, 40 or 100 μM TLTA, 3.8 mM POPC, 120 μM $CuSO_4$, 60 μM hydroquinone, 140 mM TES and 40 mM MOPS.

***In situ* Vesicle TLTA Autocatalysis**

Vesicles were prepared by hydration as described above, except lauryl azide was also added before hydration. The lipid film for vesicle preparation was prepared with 6.08 μL of 60 mM lauryl azide, 35 μL of 100 mM POPC and 0, 5 or 35 μL of 1 mM TLTA. The film was hydrated with 1 mL of 140 mM TES and 40 mM MOPS. For all reactions, 3.2 μL of 5 mM tripropargylamine was added to 190.8 μL of vesicles (3.5 mM POPC, 365 μM lauryl azide and 0, 5 or 30 μM TLTA). Hydroquinone (3.0 μL of a 4 mM stock) was then added, followed by 3 μL of 8 mM $CuSO_4$. The reaction was placed on a shaker and 15 μL aliquots were periodically extracted for LC/MS/ELSD analysis (Fig S3-4). Concentrations were reported based on a calibration curve created using known concentrations of TLTA.

Initial reaction conditions were as follows: 350 μM lauryl azide, 0, 5 or 35 μM TLTA, 3.3 mM POPC, 120 μM $CuSO_4$, 60 μM hydroquinone, 140 mM TES and 40 mM MOPS.

***In situ* TLTA Autocatalysis Accelerates Triazole Phospholipid Production**

For emulsions, 27 μL of 60 mM lauryl azide in chloroform and 32.4 μL of 30 mM alkyne lysolipid in chloroform were mixed together, dried, hydrated in 1.5 mL of 350 mM TES and 140 mM MOPS and sonicated for 30 min without heat. Vesicles were prepared by dehydrating 15 μL of 100 mM POPC in chloroform and 0 or 15 μL of 10 mM TLTA in chloroform, hydrated in 1.5 mL of 350 mM TES and 40 mM MOPS and sonicated with heat for 90 min. Vesicles were allowed to cool, transferred to Eppendorf tubes and sonicated for an additional 30 minutes at room temperature. For all reactions, stocks were added with the volume and the order shown in the following table:

Order of addition	Component
1	108 μL emulsion (1080 μM alkyl azide + 650 μM alkyne lysolipid) in 350 mM TES + 40 mM MOPS
2	2 μL 3.35 mM tripropargylamine in 100% EtOH
3	2 μL 4 mM hydroquinone in water
4	20 μL vesicles (5 mM POPC + 0 or 500 μM TLTA in 350 mM TES + 40 mM MOPS
5	2 μL 8 mM $CuSO_4$ in water

Initial reaction conditions: 870 μM lauryl azide, 525 μM alkyne lysolipid, 0 or 50 μM tripropargylamine, 0 or 75 μM TLTA, 120 μM $CuSO_4$, 60 μM hydroquinone, 750 μM POPC, 350 mM TES and 40 mM MOPS.

The reaction was placed on a shaker and 15 μL aliquots were periodically extracted for LC/MS/ELSD analysis (Fig S4, S5, S7).

Serial Transfer Experiments

For emulsions, 27 μL of 60 mM lauryl azide in chloroform and 32.4 μL of 30 mM alkyne lysolipid in chloroform were mixed together, dried and hydrated in 1.5 mL of 350 or 450 mM TES and 40 mM MOPS. Vesicles were prepared by mixing 15 μL of 100 mM POPC in chloroform with 0 or 15 μL of 10 mM TLTA in chloroform, followed by dehydration and hydration with 300 μL of 350 mM or 450 mM TES and 40 mM MOPS. For the first reaction, stocks were added in the order and with the volume shown in the following table:

Order of addition	Component
1	137.7 μL emulsion (1080 μM alkyl azide + 650 μM alkyne lysolipid) in 350 or 450 mM TES + 40 mM MOPS
2	1.8 μL 5 mM tripropargylamine in 100%

	EtOH
3	2.25 µL 4 mM hydroquinone in water
4	6 µL vesicles (5 mM POPC + 0 or 500 µM TLTA in 350 or 450 mM TES + 40mM MOPS
5	2.25 µL 8 mM $CuSO_4$ in water

Initial reaction conditions were as follows: 600 µM alkyne lysolipid, 990 µM lauryl azide, 60 µM tripropargylamine, 0 or 20 µM TLTA, 120 µM $CuSO_4$, 60 µM hydroquinone, 200 µM POPC, 450 mM TES and 40 mM MOPS.

The reaction was placed on a shaker and 15 µL aliquots were periodically extracted for LC/MS/ELSD analysis.

For all subsequent reactions, 10% of the formed vesicles were transferred to a new reaction mix with one of the following two schemes, depending upon the TES concentration:

Order of addition	Component for 350 mM TES Reactions	Component for 450 mM TES Reactions
1	143.7 µL emulsion (1080 µM alkyl azide + 650 µM alkyne lysolipid) in 350 mM TES + 40 mM MOPS	128.7 µL Emulsion (1080 µM alkyl azide + 650 µM alkyne lysolipid) in 450 mM TES + 40 mM MOPS
2	1.8 µL 5 mM tripropargylamine in 100% EtOH	1.8 µL 5 mM tripropargylamine in 100% EtOH
3	2.25 µL 4 mM hydroquinone in water	2.25 µL 4 mM hydroquinone in water
4	16.5 µL vesicles (Taken from previous reaction mix)	15 µL vesicles (Taken from previous reaction mix)
5th	2.25 µL 8 mM $CuSO_4$ in water	2.25 µL 8 mM $CuSO_4$ in water

Excluding any unreacted hydroquinone, tripropargylamine, alkyl azide and alkyne lysolipid carried over from the previous reaction, initial concentrations were as follows:

For 350 mM TES reactions: 932 µM lauryl azide, 561 µM alkyne lysolipid, 0 or 54 µM tripropargylamine, 120 µM $CuSO_4$, 54 µM hydroquinone and 40 mM MOPS

For 450 mM TES reactions: 925 µM lauryl azide, 558 µM alkyne lysolipid, 0 or 60 µM tripropargylamine, 132 µM $CuSO_4$, 60 µM hydroquinone and 40 mM MOPS

Liquid Chromatography/Mass Spectrometry/Evaporative Light Scattering Detection (LC/MS/ELSD)

All experiments were monitored using an Agilent 1260 Infinity Series HPLC with an Agilent Zorbax eclipse plus C8 column, a 380 Varian-Agilent ELSD, and a 6100 Agilent Quadrupole MS. Solvents consisted of HPLC-grade MeOH and H_2O. ACS-grade formic acid (88%) was added to each solvent for a final concentration of 0.1% by volume. For all experiments, 15 µL

aliquots sampled directly from reactions were placed in 200 μL LCMS vials for 10 μL injections. The following method was used to analyze all experiments in this communication.

For HPLC, the flow rate was 1 mL/min. A binary gradient of 50% MeOH and 50% H_2O was ramped to 95% MeOH and 5% H_2O over the first minute and then held constant for the remainder of data collection. A 2.0 minute wash was added to each method to allow the system to equilibrate back to 50% MeOH and 50% H_2O for subsequent reactions (see DAD in Fig S4). For all experiments involving only lauryl azide, a 6.0 min runtime was sufficient to resolve all analytes. Runtimes were extended to 9.0 minutes for experiments utilizing myristyl azide and 15 minutes for any experiments with palmityl azide. The diode array detector was set as follows: peak width > 0.10 min (2.0 s response time) 2.5 Hz, analog zero offset = 5%, analog attenuation = 1000 mAU, spectrum range = 190 nm to 400 nm, spectrum step = 2.0 nm, 210 nm signal bandwidth = 4 nm, reference wavelength = 360.0 nm with bandwidth of 100 nm, and the margin for negative absorbance = 100 mAU.

For ELSD measurements, the nebulization temperature and evaporation temperature were both set to 30 °C, and the evaporative gas flow was 1.60 SLM. The ELSD was auto-zeroed before each run. LED intensity: 100%. Smoothing: 3.0 sec. Gain: 1.0.

The MS was set to the API-ES ionization mode. The mass range scanned masses between 0.1 and 1kD. The fragmentor was set to 70. The drying gas flow rate was 12 L/min, and the drying gas temperature was 350 °C. The nebulization pressure was 35 psig. The step-size was 0.10, the quad temp was 0 °C, and the Vcap was 3000 V.

The settings detailed above resolved all peaks on the ELSD and diode array detector. Each peak found on either detector was aligned with and identified by a corresponding peak on the MS as shown in Fig S4. For most analytes (see the C12 triazole-phospholipid and TLTA ligand shown in Fig S4), a resolved peak with a characteristic retention time was simultaneously visible on all three devices.

Concentrations of analytes were determined by injecting known standards into the LCMS and creating a calibration curve from diode-array detector and/or ELSD data points.

Microscopy

Microscopy of a serial transfer experiment was performed following the addition of $CuSO_4$ to each reaction mixture. 10 μL of reaction mixture was placed on a glass slide and covered with a #1 cover slip, which was subsequently sealed with nail polish to prevent dehydration. For vesicle counting experiments (Fig S14), vesicles were labeled with 0.4 mol% Tx Red-DHPE before addition to the reaction mixture. A 10 μL sample was placed on a glass slide with an EM spacer (120 μM thick), and a 10 x 10 matrix of contiguous frames were acquired in the center of the sample. All photos were collected with a 100x 1.46 NA oil immersion objective on a Zeiss AxioObserver Z1 inverted Microscope outfitted with a Yokogawa CSU10 spinning-disk (Tokyo, Japan) and a photometrics Evolve 512 EMCCD camera (Tucson, AZ). To excite the Texas Red-DHPE, a 561 nm DPSS-laser was used, and the acquired images were processed using ImageJ 1.45j.

Vesicle membrane retention of catalyst

A 20 μL sample of vesicles consisting of 5 mM POPC and 500 μM TLTA was run through a Sephadex G50 column. If TLTA was not retained in vesicular lipid bilayers, loss of ligand would be expected after gel filtration. We tested for such a loss by comparing the ratio of POPC to catalyst before and after separation by Sephadex. Five contiguous fractions containing vesicles, each consisting of ~40 μL, were collected after gel filtration, and a 10 μL aliquot from each fraction was sampled via LCMS. Absorbance was measured at 210 nm for each POPC and TLTA signal by calculating the area under each curve. The data collected from the five samples were averaged together and compared against seven LCMS samples of the same vesicle population that had not been subjected to gel filtration, as shown in Fig S1. Vesicles were prepared as described previously with the following modification: after sonication, vesicles were centrifuged for 3 min at 16K rpm to remove any residual large particles in the suspension.

C12/C16 azide preferential uptake experiments

C12-TPC vesicles with TLTA were prepared in a buffer with 350 mM TES and 20 mM MOPS by two rounds of serial transfer reactions seeded with POPC/TLTA vesicles as described previously, but with one modification: the concentration of 1-azidododecane was 2x (2.16 mM). Negative controls were prepared identically, except the reactions were originally seeded with POPC vesicles containing no TLTA. Seed vesicles were centrifuged after preparation to remove any large residual particles. Selection experiments were performed by incubating negative controls or TLTA containing C12-TPC vesicles in an emulsion mix containing 650 μM alkyne lysolipid, 1.08 mM 1-azidododecane and 1.08 mM azidohexadecane at a TES concentration of either 400 mM or 600 mM.

To deliver the same concentration of the two azides to both the 400 and 600 mM TES emulsions, a concentrated emulsion stock was diluted with one of two different concentrations of buffers as follows. In an LCMS vial, 47.66 μL of 15 mM alkyne lysolipid in chloroform and 39.6 μL of an azide mixture containing 30 mM 1-azidododecane and 30 mM azidohexadecane in chloroform were mixed together and evaporated under nitrogen. The azides and alkyne were hydrated with 550 μL of 550 mM TES and 20 mM MOPS and sonicated for 20 minutes with no heat. The stock was split into two fractions, and each fraction was diluted by 50 % with 20 mM MOPS and either a 250 or 650 mM TES. The reaction was initiated as follows:

Order of addition	Component
1	95.8 μL emulsion (1.08 mM C12 azide, 1.08 mM C16 azide and 650 μM alkyne lysolipid) in 400 or 600 mM TES + 20 mM MOPS
2	1.2 μL 5 mM tripropargylamine in 100% EtOH
3	1.5 μL 4 mM hydroquinone in water
4	11 μL of vesicles from 2nd round of serial transfer reaction
5	1.5 μL 8 mM $CuSO_4$ in water

Excluding any unreacted hydroquinone, tripropargylamine, dodecane azide and alkyne lysolipid carried over from the previous reaction, initial concentrations were as follows: 930 μM dodecane azide, 930 μM hexadecane azide, 566 μM alkyne lysolipid, 0 or 54 μM tripropargylamine, 120 μM $CuSO_4$, 54 μM hydroquinone, 20 mM MOPS and 400 or 600 mM TES.

Dependence of TLTA and triazole phospholipid synthesis on the presence of preexisting phospholipid

For emulsions, 36 μL of 30 mM lauryl azide in chloroform and 43.3 μL of 15 mM alkyne lysolipid in chloroform were mixed together, dried and hydrated in 1 mL of 400 mM TES and 20 mM MOPS. Lipid films were prepared by drying 0 or 35 μL of 100 mM POPC in chloroform and 0 or 35 μL of 10 mM TLTA in chloroform in a glass vial. Films were hydrated with 700 μL of 400 mM TES and 20 mM MOPS. For all reactions, stocks were added with the volume and the order shown in the following table:

Order of addition	Component
1	129 μL emulsion (1080 μM alkyl azide + 650 μM alkyne lysolipid) in 400 mM TES + 20 mM MOPS
2	1.5 μL of 100% EtOH or 5 mM tripropargylamine in 100% EtOH
3	2.25 μL 4 mM hydroquinone in water
4	15 μL of vesicles (0 or 5 mM POPC + 0 or 500 μM TLTA in 400 mM TES + 20 mM MOPS)
5	2.25 μL 8 mM $CuSO_4$ in water

Initial reaction conditions: 560 μM alkyne lysolipid, 940 μM dodecane azide, 120 μM $CuSO_4$, 60 μM hydroquinone, 0 or 50 μM tripropargylamine, 0 or 500 μM POPC and 0 or 50 μM TLTA in 400 mM TES and 20 mM MOPS buffer.

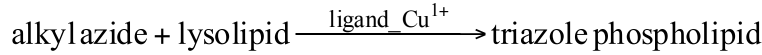
Dependence of Autocatalysis on varying lipid concentrations

Lipid films were prepared by drying the following volumes of chloroform stock solutions in a glass vial under nitrogen: 3.1 μL of 40 mM lauryl azide, 3.15 μL of 5 mM of TLTA and 2.74, 5.48, 10.98 or 13.7 μL of 32.8 mM POPC. Vesicles were then formed upon hydrating the films in 450 μL of 140 mM TES and 40 mM MOPS while sonicating with heat for 85 minutes. Reactions were initiated by mixing 190.8 μL of vesicles with 3 μL of 4 mM hydroquinone, followed by 3.2 μL of 5 mM tripropargylamine and finally, 3 μL of 8 mM $CuSO_4$. At 85 minutes into the reaction, 20 μL of reaction were loaded into an LCMS vial for a 10 μL injection.

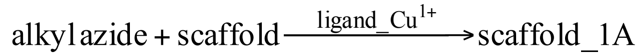
Initial reaction conditions were as follows: 140 mM TES, 40 mM MOPS, 120 μM $CuSO_4$, 60 μM hydroquinone 33 μM TLTA, 263 μM lauryl azide, 80 μM tripropargylamine and 200 μM, 400 μM, 800 μM or 1000 μM POPC

Mathematical modeling

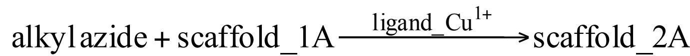
The kinetic model of the autocatalytic synthesis of triazole membranes in the most simplified form includes only five chemical reactions: synthesis of ligand (*L*) from alkyl azide (*A*) and tripropargylamine scaffold (*S*) catalyzed by the Cu^{1+}-ligand complex (*CL*) (3 reactions), formation of Cu-ligand complex from ligand (*L*) and copper (*C*), synthesis of triazole phospholipid (*T*) from alkyl azide and lysolipid (*LL*), also catalyzed by Cu^{1+}-ligand (see Fig 1):



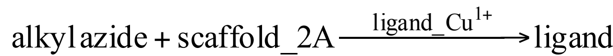
$$\text{alkyl azide} + \text{lysolipid} \xrightarrow{\text{ligand_Cu}^{1+}} \text{triazole phospholipid}$$



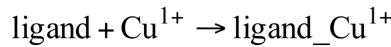
$$\text{alkyl azide} + \text{scaffold} \xrightarrow{\text{ligand_Cu}^{1+}} \text{scaffold_1A}$$



$$\text{alkyl azide} + \text{scaffold_1A} \xrightarrow{\text{ligand_Cu}^{1+}} \text{scaffold_2A}$$

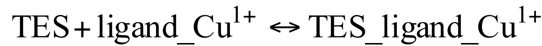


$$\text{alkyl azide} + \text{scaffold_2A} \xrightarrow{\text{ligand_Cu}^{1+}} \text{ligand}$$



$$\text{ligand} + \text{Cu}^{1+} \rightarrow \text{ligand_Cu}^{1+}$$

There is one additional reaction that describes the sequestration of the Cu-ligand complex by TES buffer through reversal binding, which reduces the active concentration of the Cu-ligand complex:



$$\text{TES} + \text{ligand_Cu}^{1+} \leftrightarrow \text{TES_ligand_Cu}^{1+}$$

In the mass action approximation, the differential equations for this model read

$$\frac{d[T]}{dt} = k_T[A][LL][CL]^n$$

$$\frac{d[L]}{dt} = k_L[A][SA_2][CL]^n - k_{CL}[L][C]$$

$$\frac{d[SA_2]}{dt} = k_L[A][SA_1][CL]^n - k_L[A][SA_2][CL]^n$$

$$\frac{d[SA_1]}{dt} = k_L[A][S][CL]^n - k_L[A][SA_1][CL]^n$$

$$\frac{d[CL]}{dt} = k_{CL}[L][C] - k_{TES+}[CL][TES] + k_{TES-}[CLTES]$$

$$\frac{d[CLTES]}{dt} = k_{TES+}[CL][TES] - k_{TES-}[CLTES]$$

where *T* is triazole phospholipid, *A* is alkyl azide, *S* is the tripropargylamine scaffold, SA_1 is scaffold with single alkyl azide chain, SA_2 is scaffold with 2 alkyl azide chains, *L* is the ligand, *LL* is lysolipid, *C* is Cu^{1+}, *CL* is the Cu^{1+}–ligand complex, TES is the effective TES buffer, and CLTES is TES-sequestered Cu^{1+}–ligand complex. Square brackets denote concentrations of these chemicals. k_T, k_L, k_{CL}, k_{TES+}, k_{TES-} are the rate constants of the four reactions and exponent *n* accounts for the non-linear effect of the catalyst.

Here we take into consideration that alkyl azide, lysolipid, scaffold, and copper are consumed in the synthesis reactions. From the conservation of mass,

$C_1 = [A]+[SA_1]+2[SA_2]+3[L]+3[CL]+[T]+3[CLTES]$,
$C_2 = [LL]+[T]$,
$C_3 = [S]+2[SA_2]+[SA_1]+[L]+[CL]+[CLTES]$,
$C_4 = [C]+[CL]+[CLTES]$,

where constants

$C_1=[A]_0+[SA_1]_0+2[SA_2]_0+3[L]_0+3[CL]_0+[T]_0+3[CLTES]_0$,
$C_2=[LL]_0+[T]_0$,
$C_3=[S]_0+[SA_2]_0+[SA_1]_0+[L]_0+[CL]_0+[CLTES]_0$,
$C_4=[C]_0+[CL]_0+[CLTES]_0$

and the subscript 0 indicates initial concentrations of the corresponding components. The final asymptotic state will depend on the relative concentrations of these four components. If $[LL]_0+3[S]_0\leq[A]_0$, both lysolipid and scaffold deplete before alkyl azide, and the system will reach a unique final state $[T]=[LL]_0$. In the opposite case ($[LL]_0+3[S]_0>[A]_0$) the amount of alkyl azide is the primary limiting factor, so it will deplete before both lysolipid and scaffold. For such conditions, the stationary state is $[A]_0=[SA_1]+[SA_2]+3[L]+3[CL]+[T]+3[CLTES]$, where the final concentration of $[T]$ is undetermined. It will depend on the initial concentrations of all components and the kinetic parameters of the system.

We fit the kinetic parameters of this system based on the experimental data shown in Fig 2. Specifically, we analyzed the growth of autocatalyst concentration for different initial concentrations of ligand (Fig 2A), the growth of triazole phospholipid concentration for different initial concentrations of ligand (Fig 2B), and the growth of triazole phospholipid concentration with and without the scaffold (Fig 2C). The model describes all these regimes well.

The fitted parameters values associated with this model were: $n=0.87$, $k_T=1.0\cdot10^{-5}\mu M^{-1.87}hr^{-1}$, $k_L=2.3\cdot10^{-3}\mu M^{1.87}hr^{-1}$, $k_{CL}=5.4\cdot10^{-3}\mu M^{-1}hr^{-1}$, $k_{TES+}=0.14\mu M^{-1}hr^{-1}$, $k_{TES-}=7.9\cdot10^{-7}\mu M^{-1}hr^{-1}$. The effective TES concentration for the Fig 2A,B was estimated to be 12.1 μM, while for Fig. 2C it was 73.7 μM.

Image Analysis and Vesicle Tracking

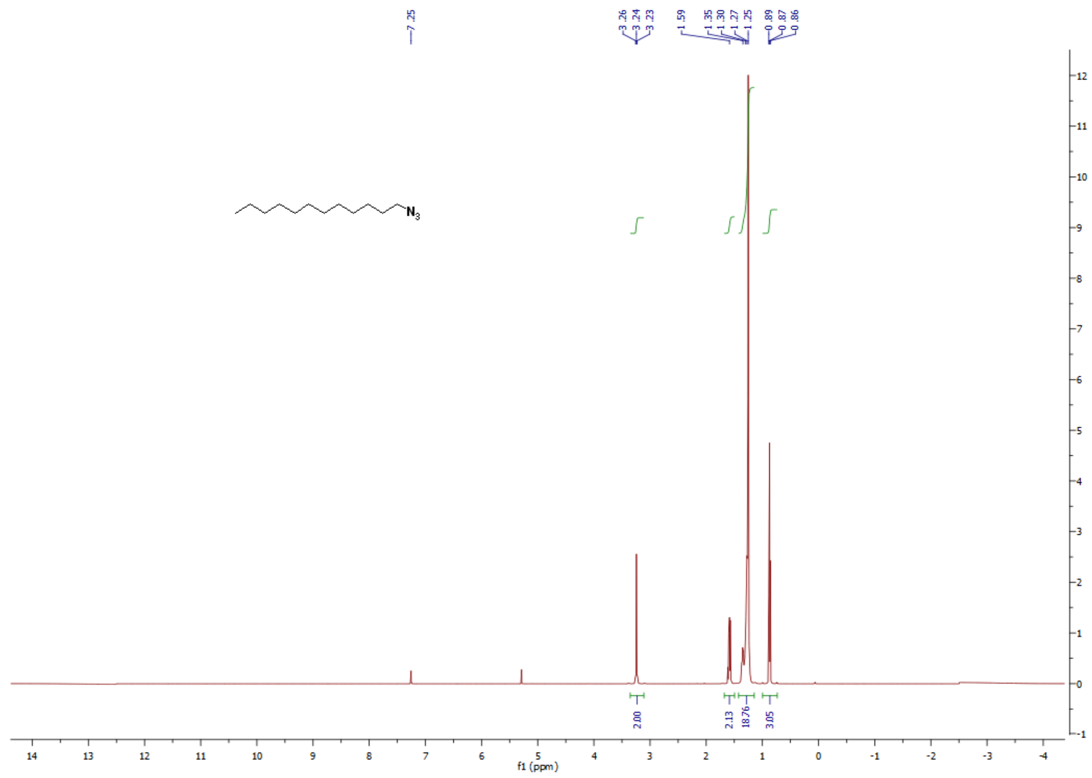
Vesicle growth was monitored using a membrane-staining fluorescent dye (Rh-DHPE) and time-lapse confocal microscopy. We used MATLAB® image toolbox functions to perform image analysis and identify individual vesicle objects. After background (generated using mean filter) subtraction, an Otsu threshold was applied to raw images, followed by filling of holes, and removal of objects that were deemed too small (<10pixels) to be considered vesicles. Vesicle objects were tracked using an automatic vesicle tracking algorithm based on previously developed single-cell tracking algorithm (3). Single vesicle area trajectories were calculated using total pixel number of tracked vesicle objects. Results of single vesicle tracking and

analysis are shown in Fig. 4B and 4C. In addition, vesicles greater than 10 pixels were counted before and after the agitation experiments to show an increase in vesicle number (Fig. S14).

HPTS-labeled Vesicle Experiments

HPTS-labeled MLVs were prepared by modifying an existing protocol (4). Briefly, vesicles hydrated with HPTS were separated from unencapsulated dye by dialysis with a 3-micron filter, and no extrusion step was performed. Sonicated vesicles were also prepared in the presence of HPTS, and unencapsulated dye was separated from vesicles by 10 rounds of washing with buffer using a spin filter.

NMR Spectral Data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3.23
1.59
1.35
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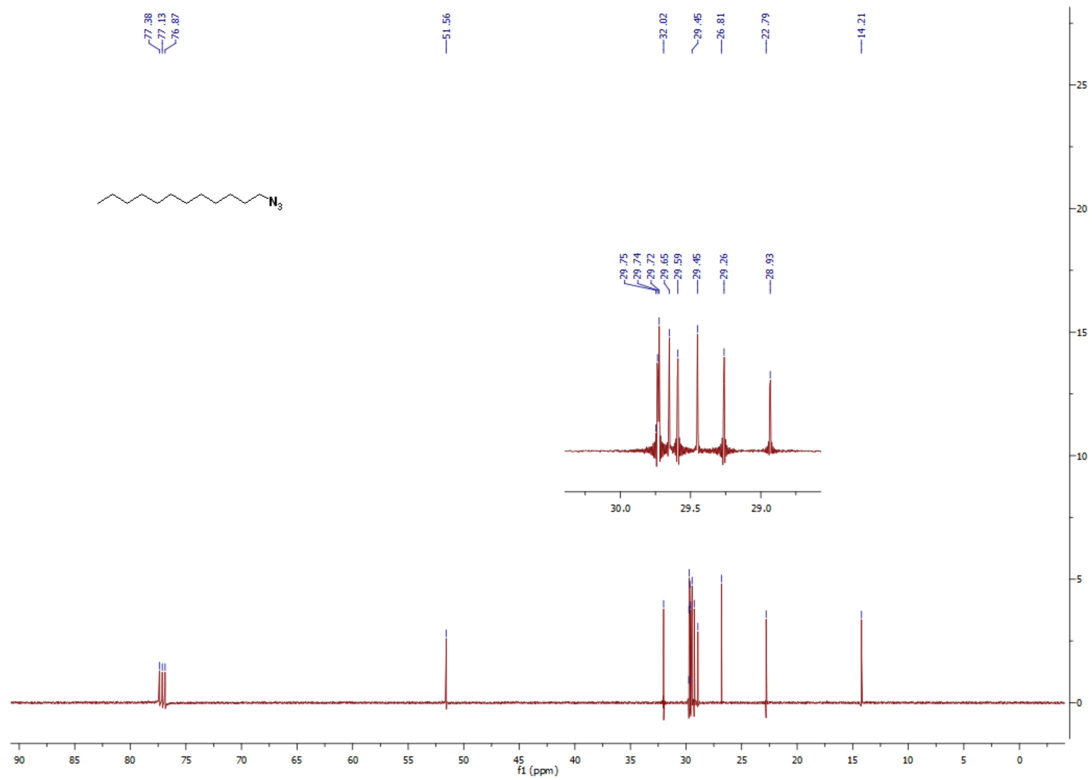
3.05
f1 (ppm)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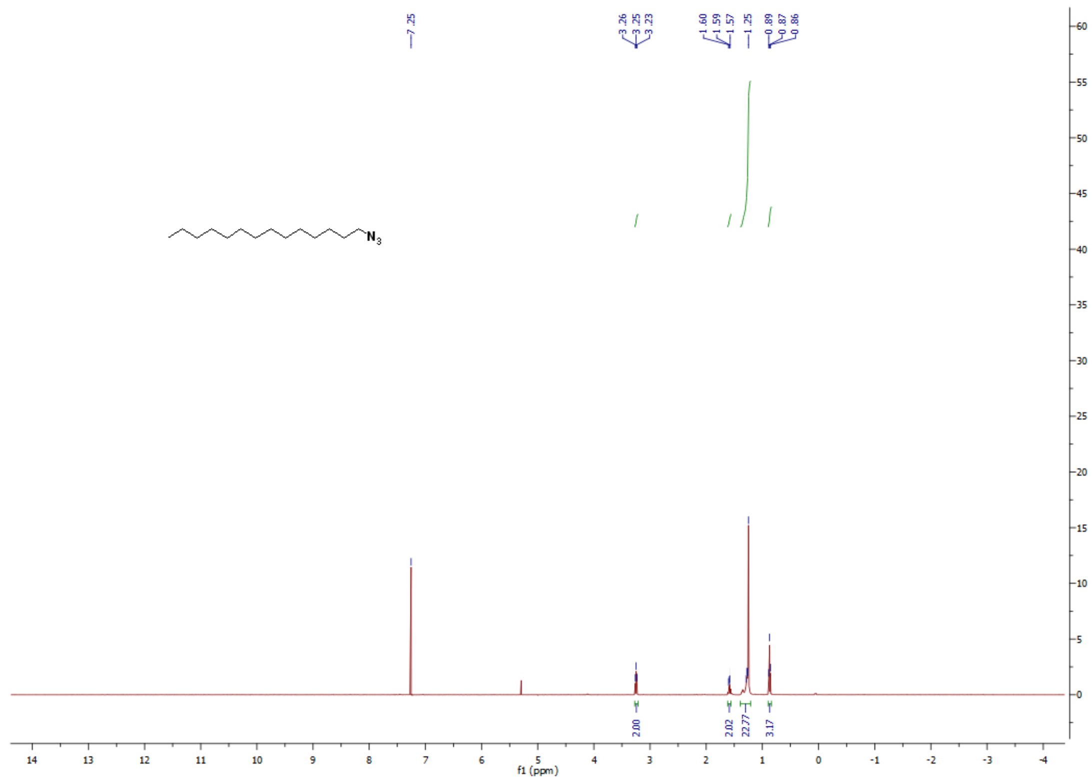
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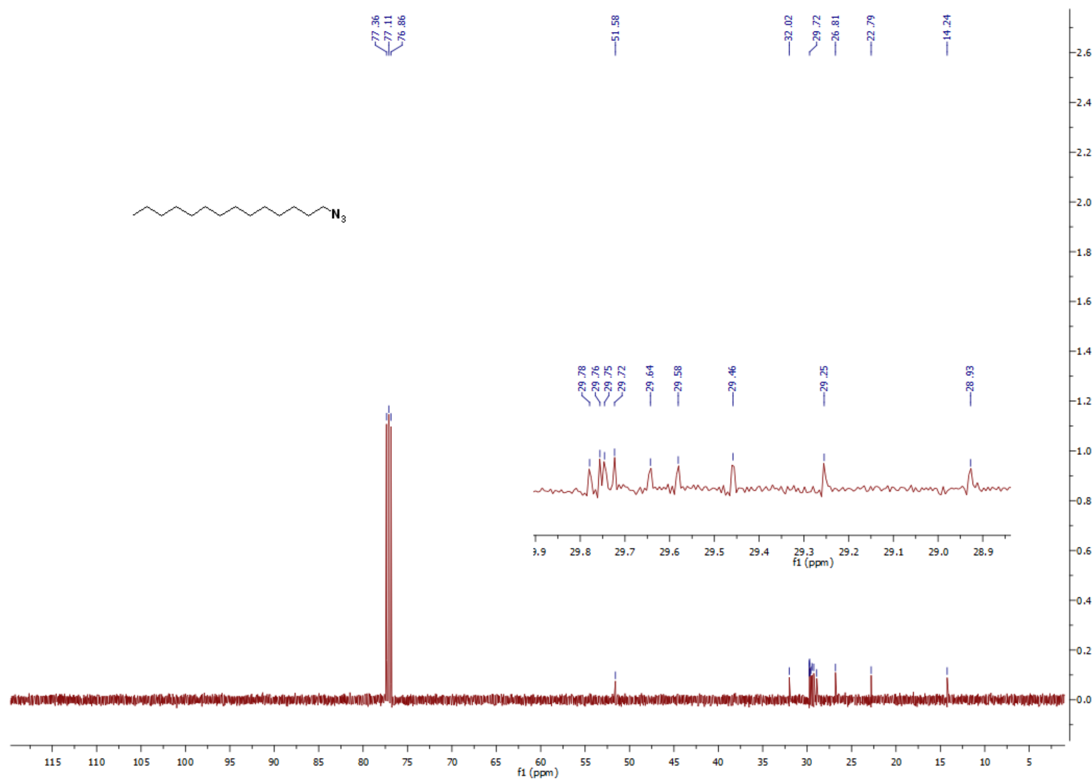
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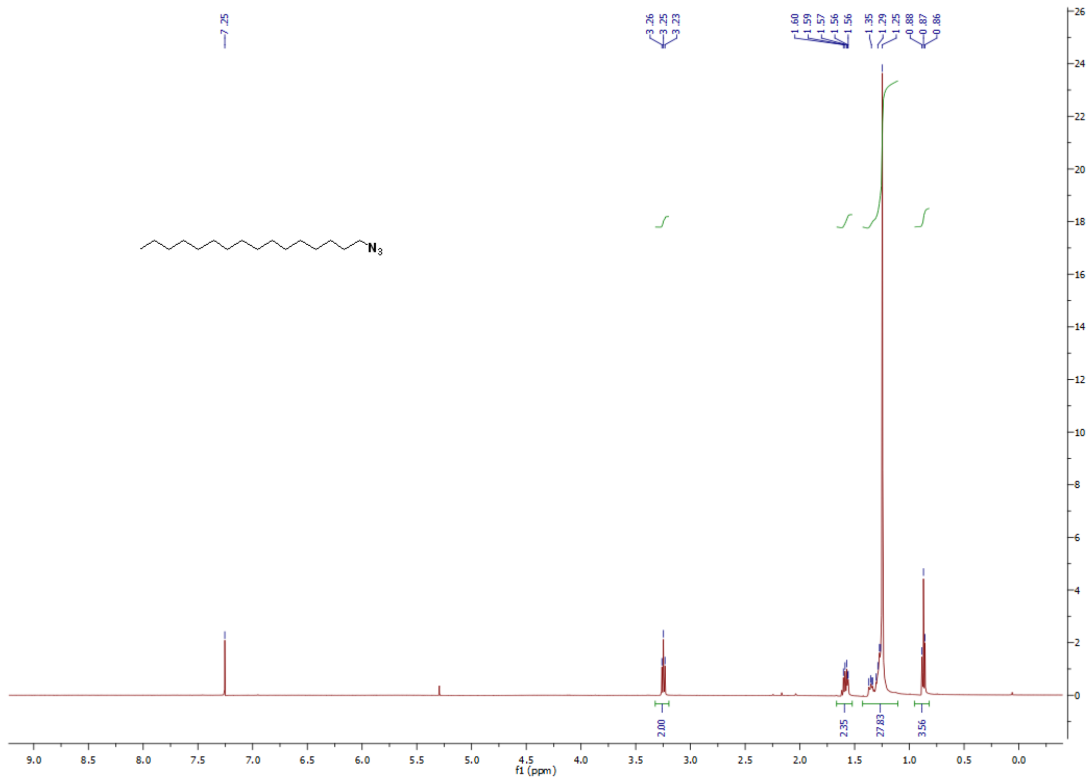

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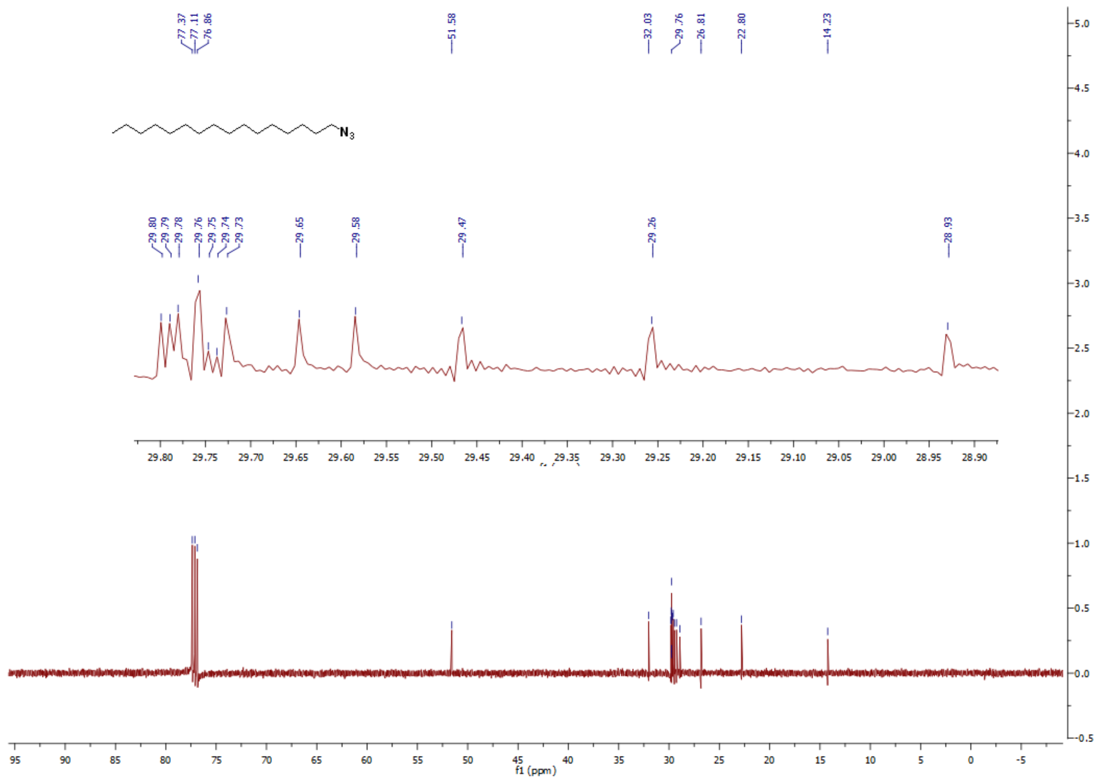

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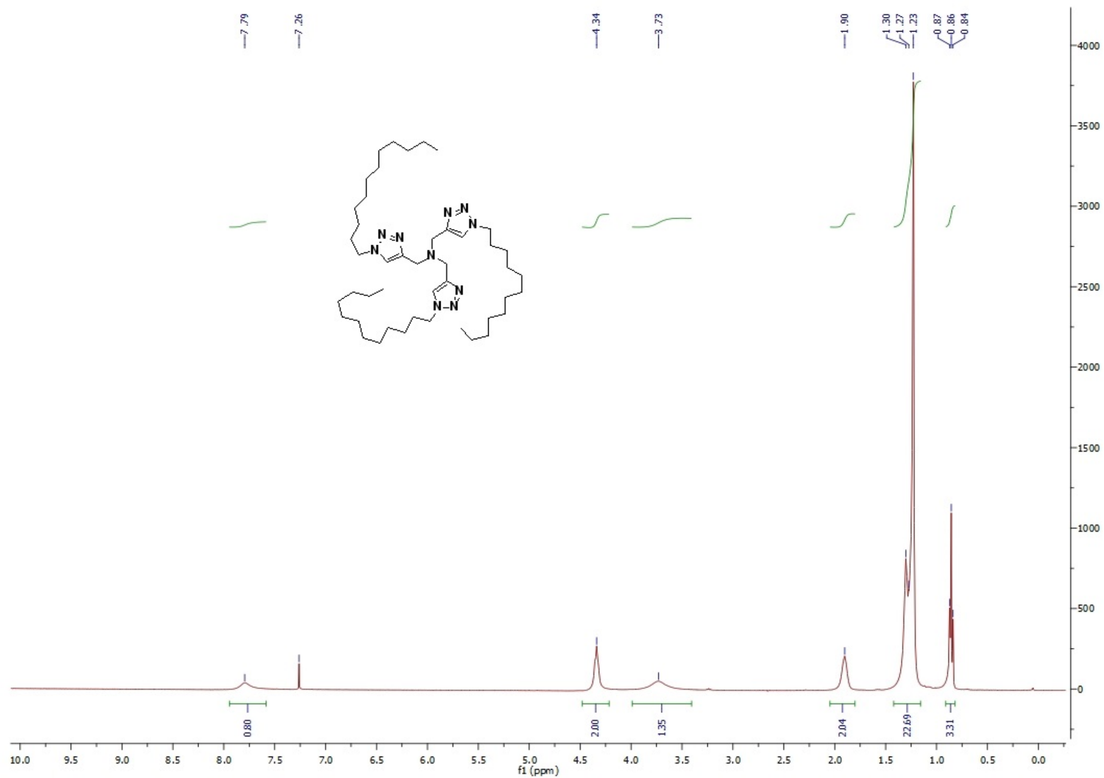

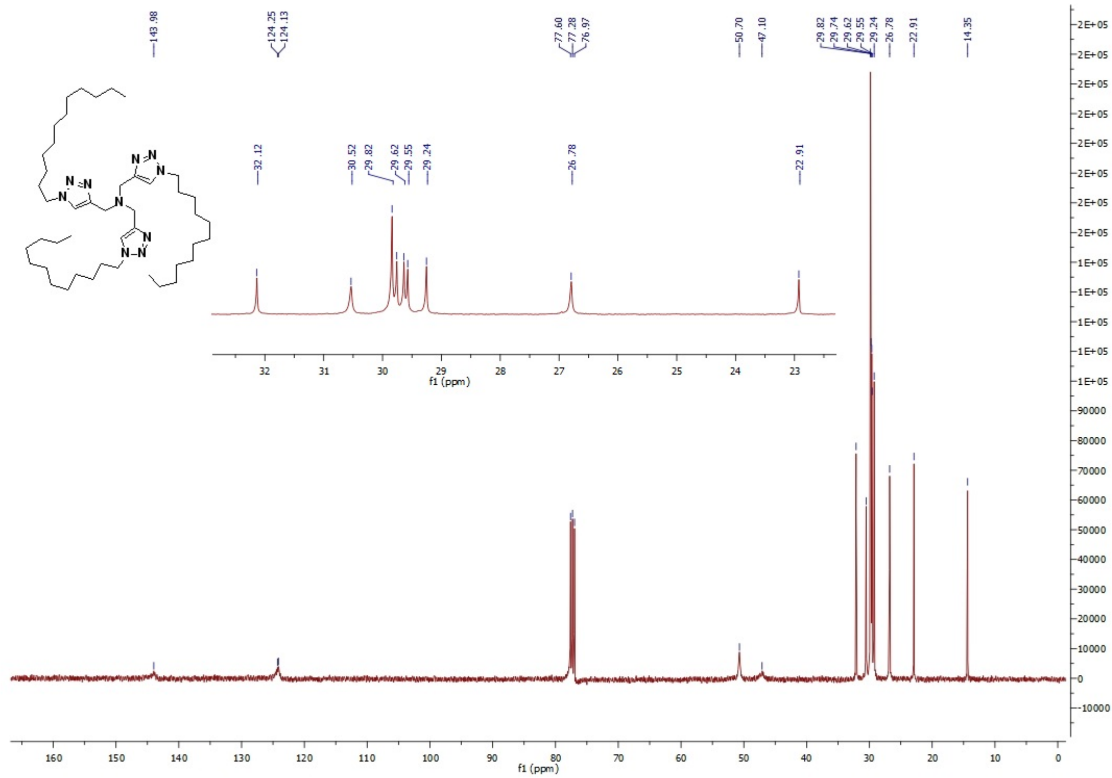

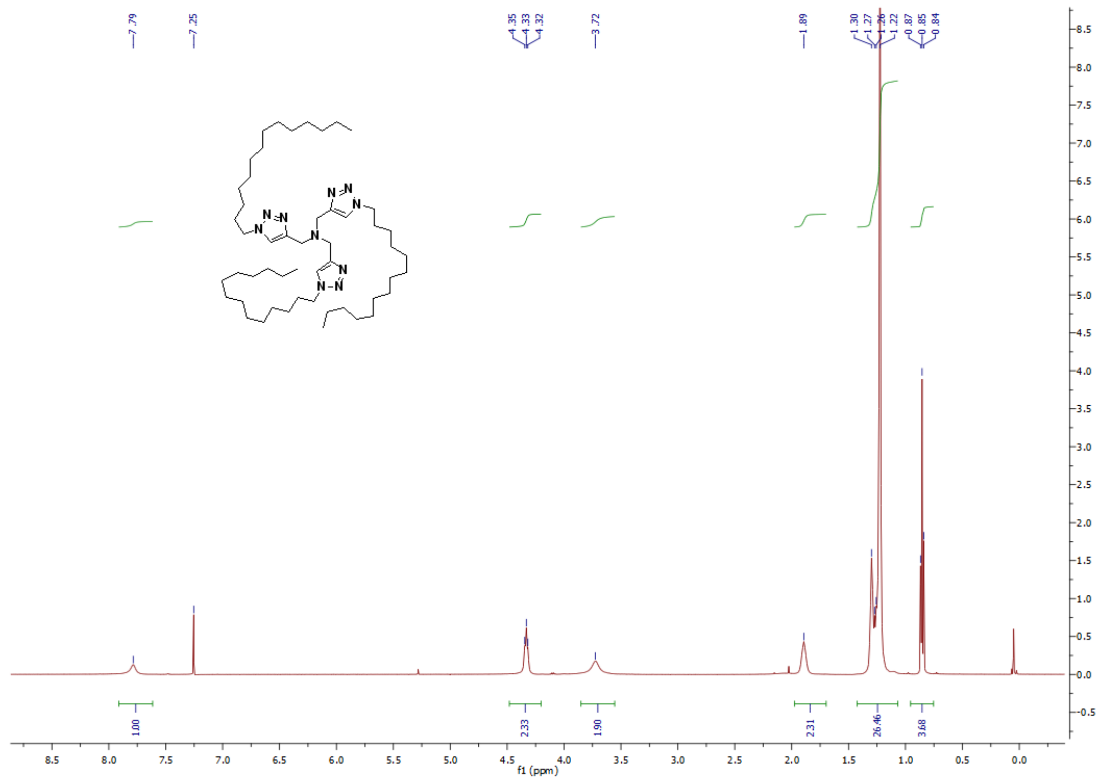

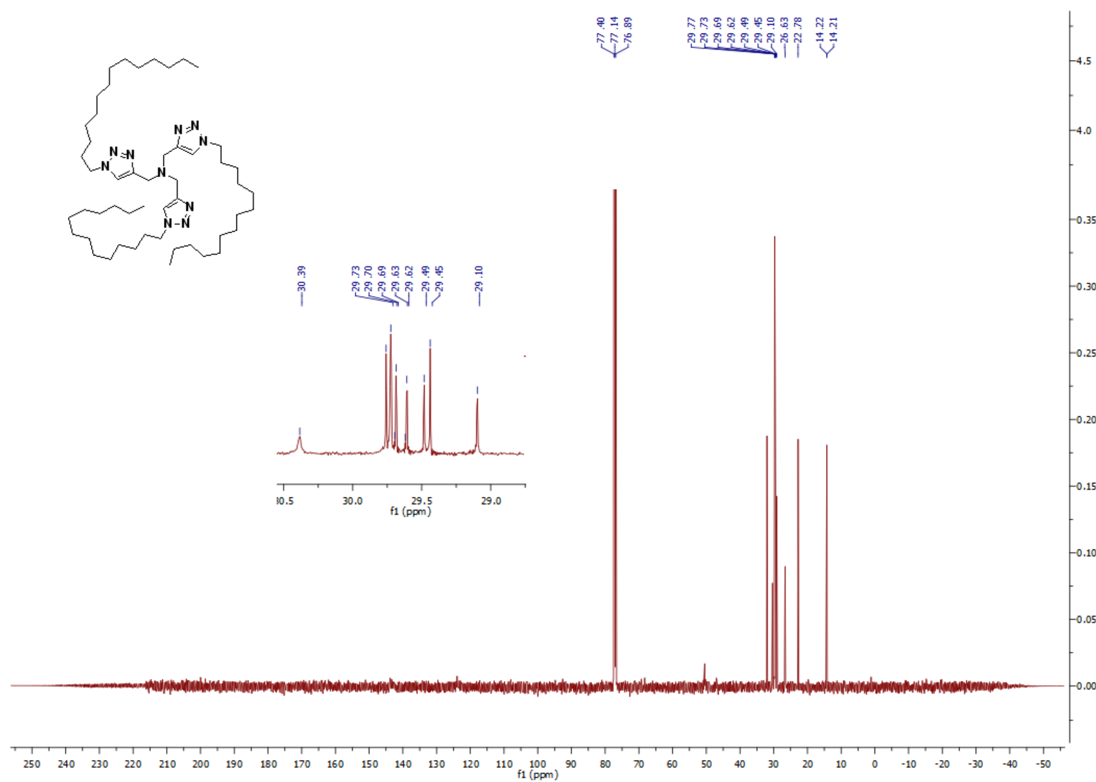

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1.00, 0.80, 3.96, 1.10, 1.80, 1.55, 8.67, 1.77, 1.79, 1.70, 1.51, 1.69, 38.61, 6.52

f1 (ppm)

173.69, 172.28, 146.06, 121.34, 71.05, 63.83, 59.47, 54.49, 50.37, 32.03, 29.67, 29.46, 29.18, 22.80, 20.97, 14.23

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f1 (ppm)

f1 (ppm)

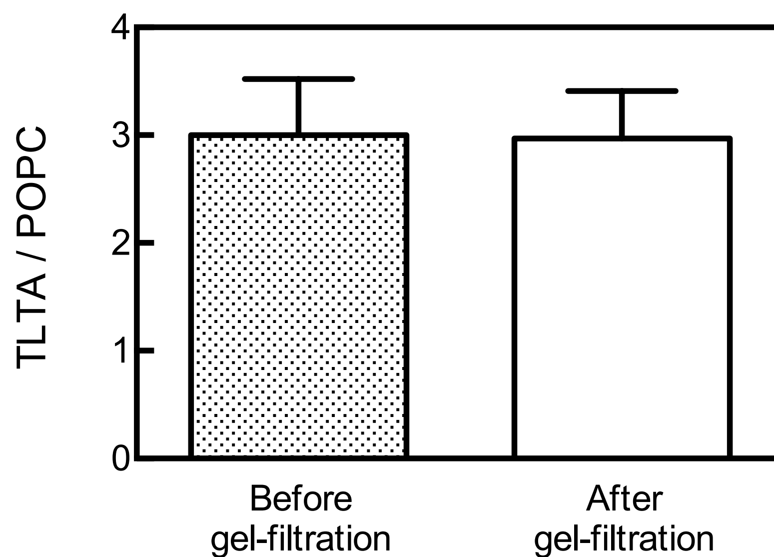


Figure S1. Retention of TLTA in POPC vesicles before and after gel-filtration with a sephadex G50 column. The ratio of TLTA to POPC absorbance at 210 nm before (3.0 ± 0.52 – left bar) and after (2.97 ± 0.44 right bar) sephadex filtration.

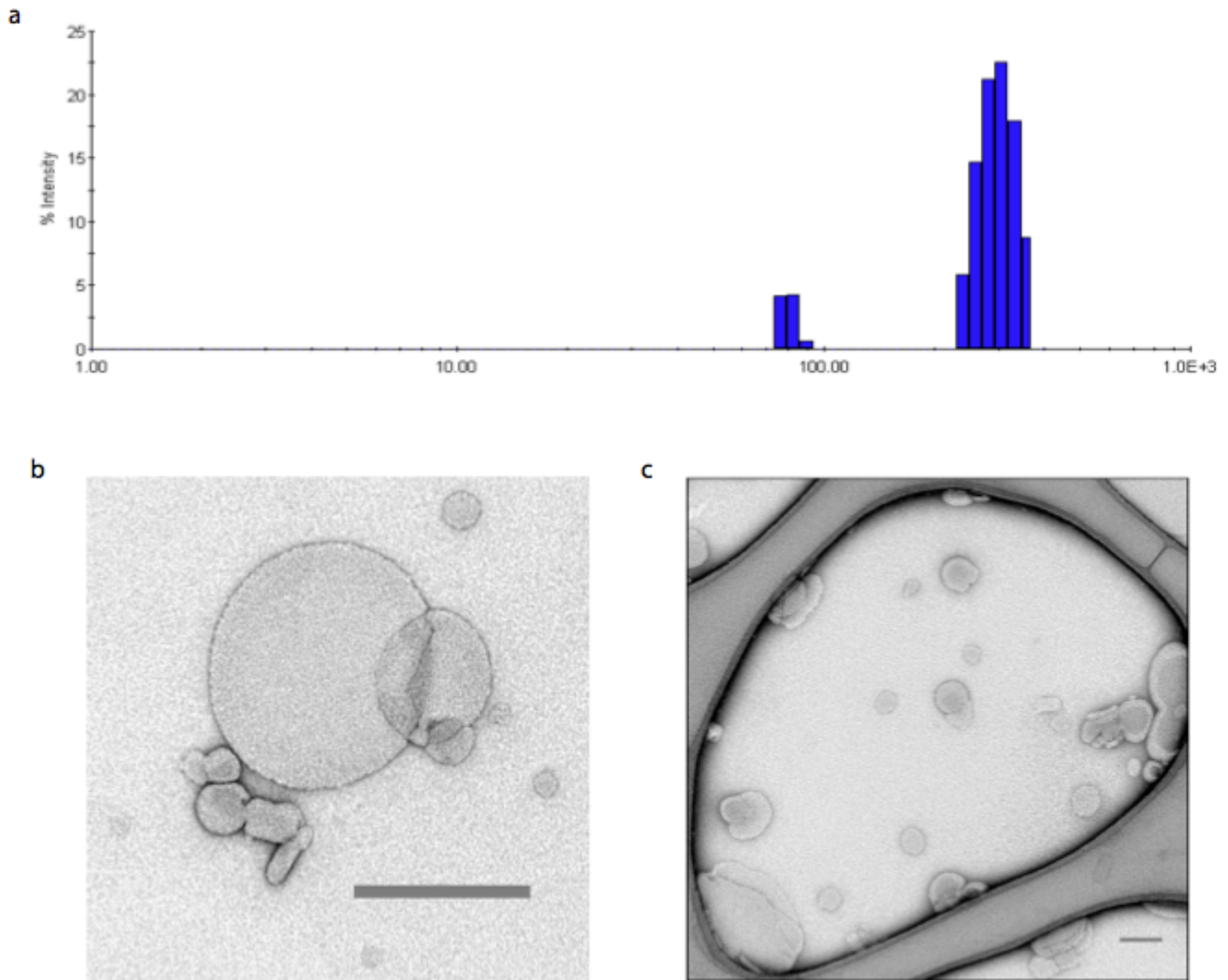


Figure S2. Characterization of POPC vesicles containing the ligand, TLTA. A) DLS histogram of sonicated POPC (500 μM) vesicles containing 10 mol % TLTA. B) TEM images of POPC vesicles containing 10 mol % TLTA. Scale bars = 300 nanometers (left) and 100 nanometers (right).

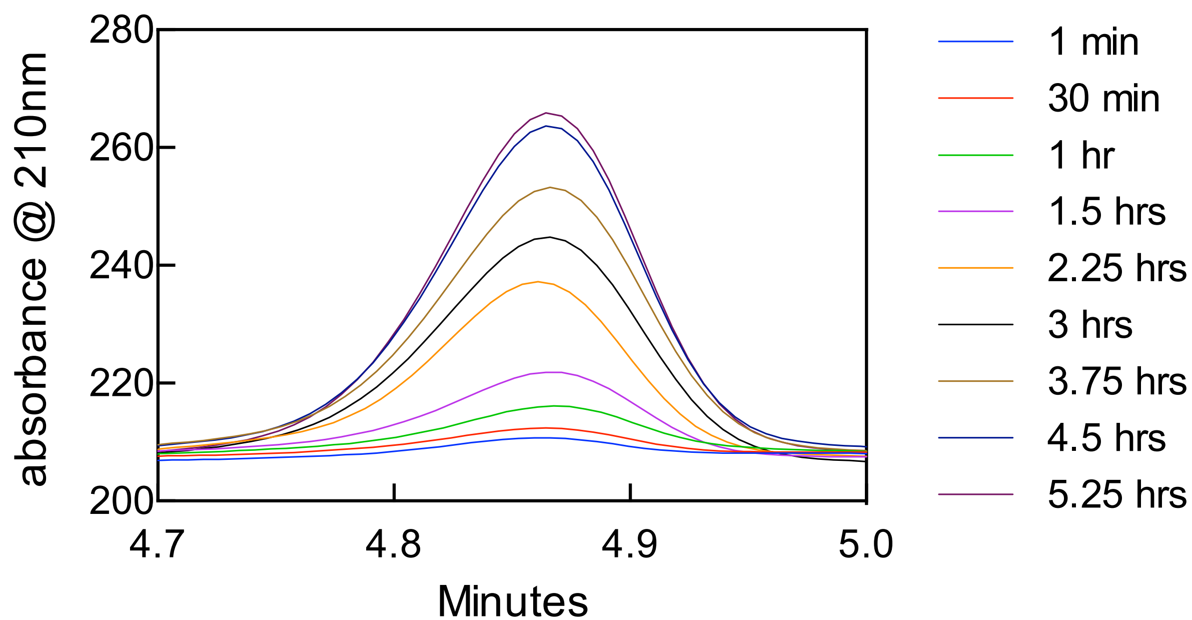


Figure S3. Overlaid HPLC traces monitoring the synthesis of TLTA ligand from the TLTA-autocatalytic experiment depicted in Figure 2a in the main text. Catalytic vesicles contain an initial concentration of 5 μM TLTA. Over ~ 6hrs, catalytic vesicles convert 80 μM tripropargylamine and excess lauryl azide into an additional 68 ± 9 μM TLTA (n=3), yielding a total TLTA concentration of 73 ± 9 μM.

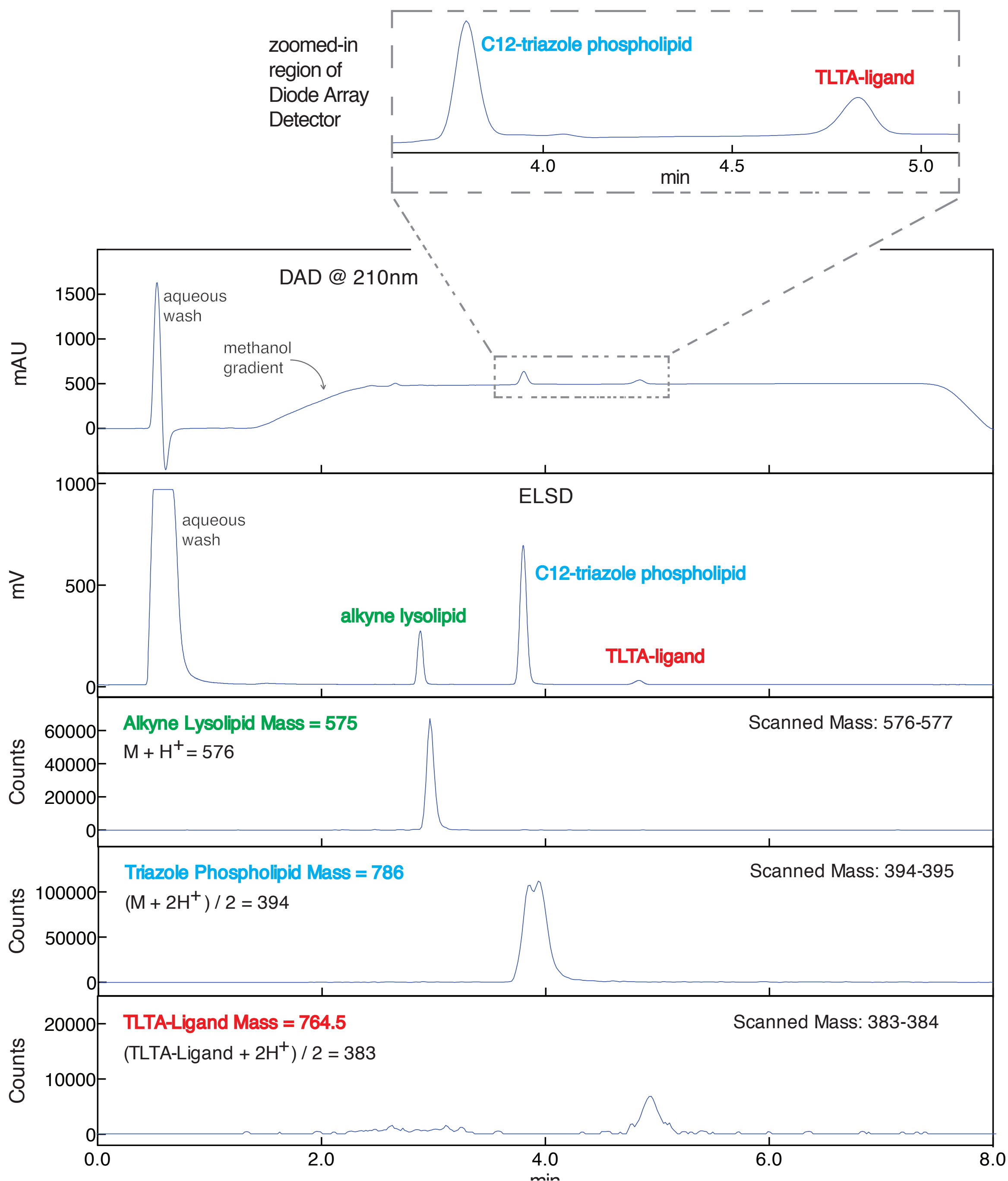


Figure S4. Raw data of a time point taken during the 8th reaction of a serial transfer experiment demonstrating typical LC-MS ELSD data output. The lag in retention times when comparing the DAD to the ELSD or MS reflects additional delay volume an analyte must negotiate after egressing the DAD and traveling to the downstream ELSD and MS detectors.

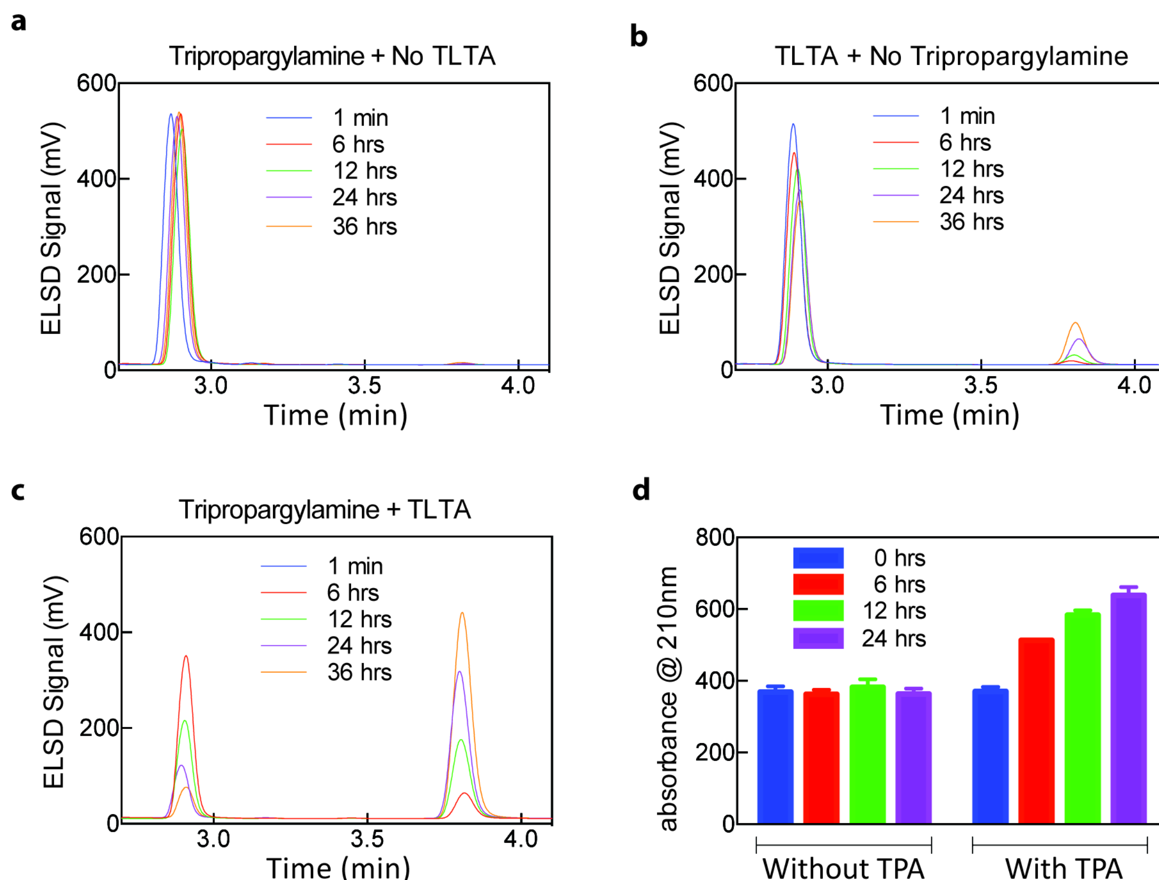


Figure S5. *In situ* autocatalysis promotes triazole phospholipid synthesis. Initial concentrations of alkyne lysolipid, 1-azidododecane, $CuSO_4$, and hydroquinone were 525 μM, 870 μM, 120 μM and 60 μM, respectively. a) In the presence of 50 μM tripropargylamine (TPA), negligible conversion of alkyne lysolipid (retention time = 2.9 min) into triazole phospholipid (retention time = 3.8 min) takes place with vesicles lacking TLTA. b) In the absence of TPA, vesicles loaded with 75 μM TLTA sluggishly convert alkyne lysolipid into triazole phospholipid (retention time = 3.8 min). c) In the presence of 50 μM TPA, vesicles loaded with 75 μM TLTA convert alkyne lysolipid into triazole phospholipid rapidly. d) *In-situ* synthesis of TLTA was monitored with a diode array detector at 210 nm. In the presence of 50 μM TPA, vesicles loaded with 75 μM TLTA synthesize additional TLTA (4 right-most bars – also Fig. S5c). When the same population of vesicles is immersed in reactive precursors with no TPA present, the concentration of TLTA remains constant and no *in situ* synthesis occurs (4 left-most bars – also Fig. S5b). No additional TLTA synthesis was detected in vesicle populations lacking TLTA after exposure to 50 μM TPA (Fig. S5a).

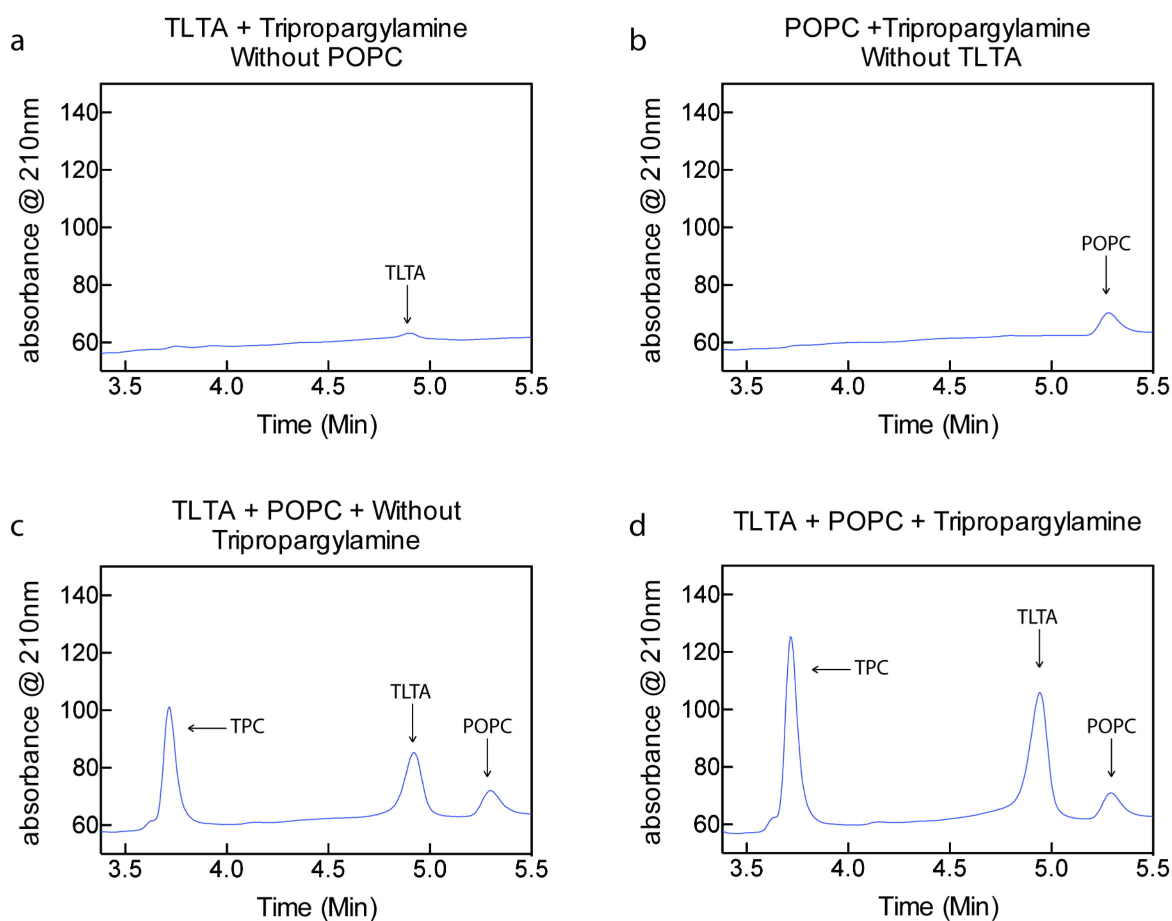


Figure S6. TLTA and triazole phospholipid synthesis require both preexisting TLTA and phospholipid membranes. a) In the absence of POPC, TLTA fails to stay in solution and synthesize either triazole phospholipids or additional TLTA. b) When TLTA is initially absent from POPC membranes, neither triazole phospholipid nor TLTA is synthesized. c) In the absence of tripropargylamine and in the presence of POPC, TLTA is able to synthesize triazole phospholipid, but it is unable to synthesize additional TLTA. d) When POPC and tripropargylamine are both present, TLTA synthesizes both triazole phospholipid and additional TLTA. Conditions for experiments: 560 μM alkyne lysolipid, 940 μM dodecane azide, 120 μM $CuSO_4$, 60 μM hydroquinone, 400 mM TES, 20 mM MOPS buffer, 0 or 50 μM tripropargylamine, 0 or 500 μM POPC, and 0 or 50 μM TLTA. Reactions were sampled with LCMS at 20 hrs.

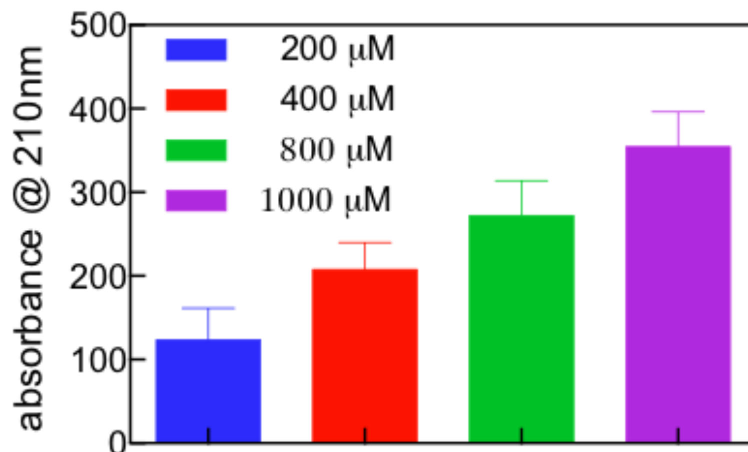


Figure S7. Effect of Phospholipid Concentration on the Formation of Autocatalyst. Additional autocatalyst is synthesized in the presence of increasing concentrations of phospholipid. 33 μM TLTA was solubilized with 263 μM dodecane azide and 200, 400, 800 or 1000 μM POPC, along with 80 μM tripropargylamine, 60 μM hydroquinone, 120 μM $CuSO_4$, 140 mM TES and 40 mM MOPS. Error bars represent standard deviations (n=3).

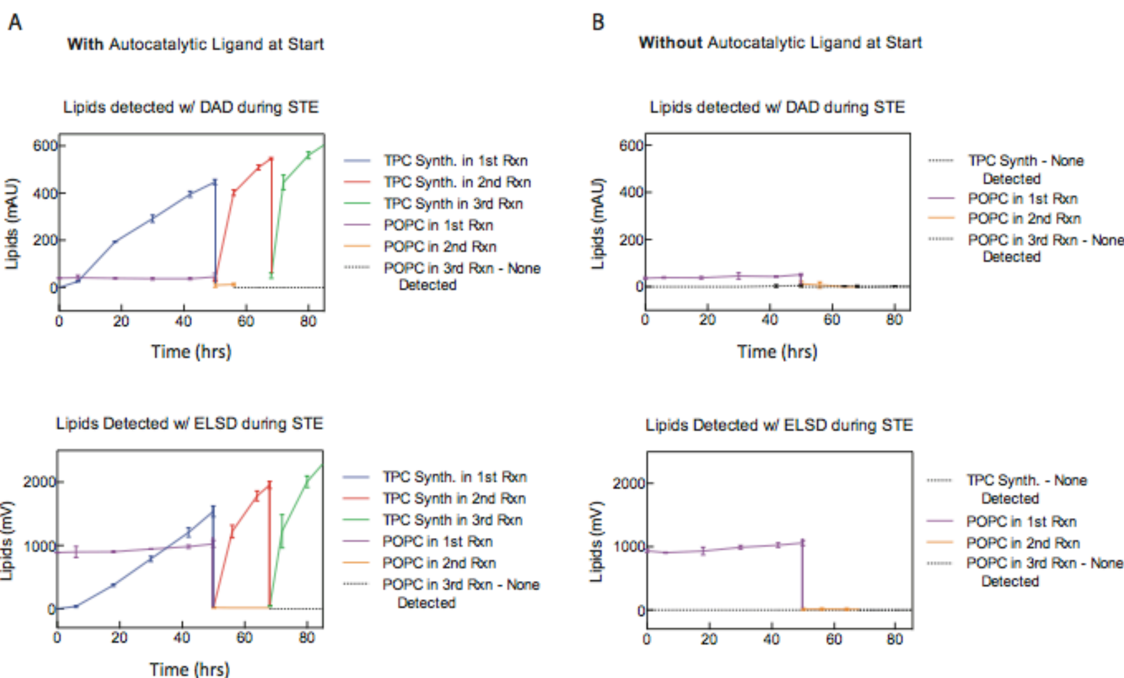
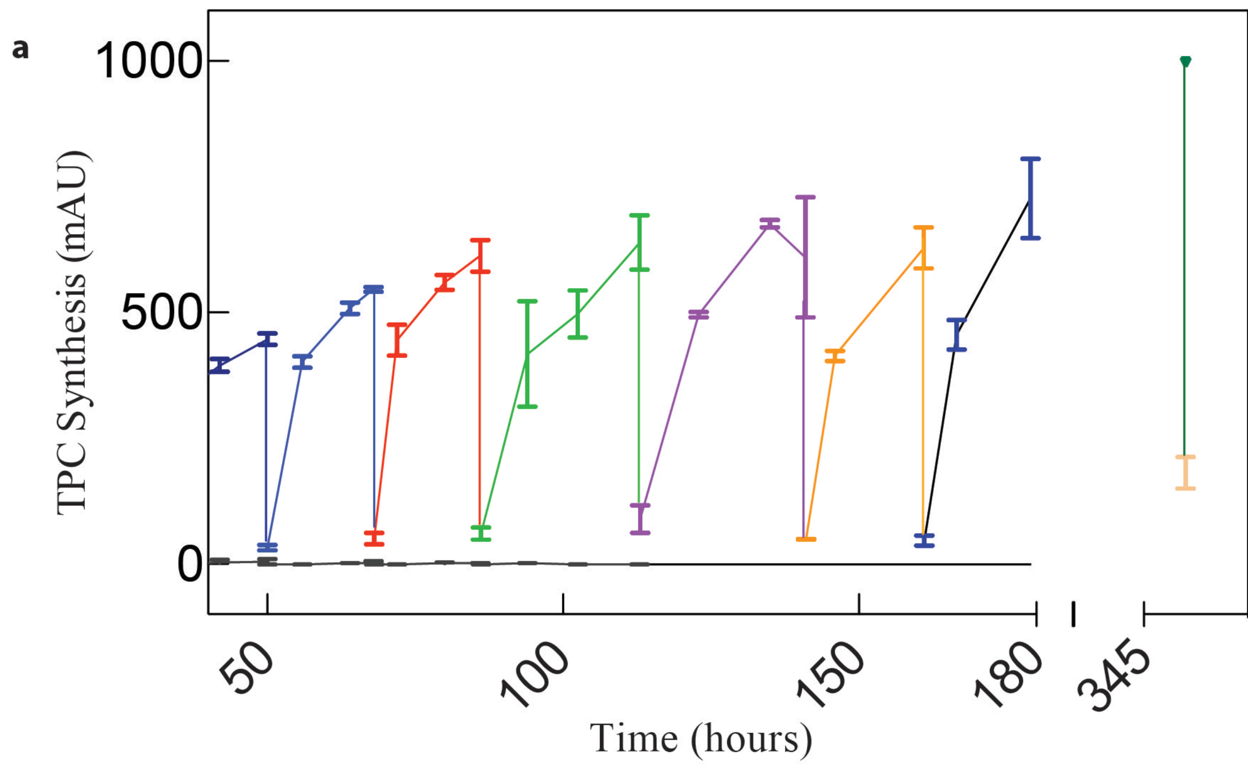


Figure S8. Synthesis and dilution of lipid concentrations during the first three rounds of a serial transfer experiment (STE). Top panel = a Diode Array Detector (DAD) monitors lipid synthesis (in mAU). Bottom panel = an Evaporative Light Scattering Detector (ELSD) measures lipid synthesis (in mV). A) When an initial population of 200 µM POPC vesicles containing 20 µM TLTA is mixed with precursors, triazole phospholipid is synthesized. When 10 % of the 1st reaction (by volume) is used to seed a second reaction, the initial concentrations of POPC and synthesized triazole phospholipid are both diluted by a factor of 10. After the second reaction is transferred in the same way into a third reaction, only 2 µM POPC remains from the initial seed population of 200 µM, and the remaining POPC is no longer detectable by ELSD or DAD. B) When the initial seed population of 200 µM POPC vesicles does not contain the ligand TLTA, no triazole lipid is synthesized, and by the third reaction, only 2 µM of total lipid is detectable. Error bars represent standard deviations (n=3).

a



b

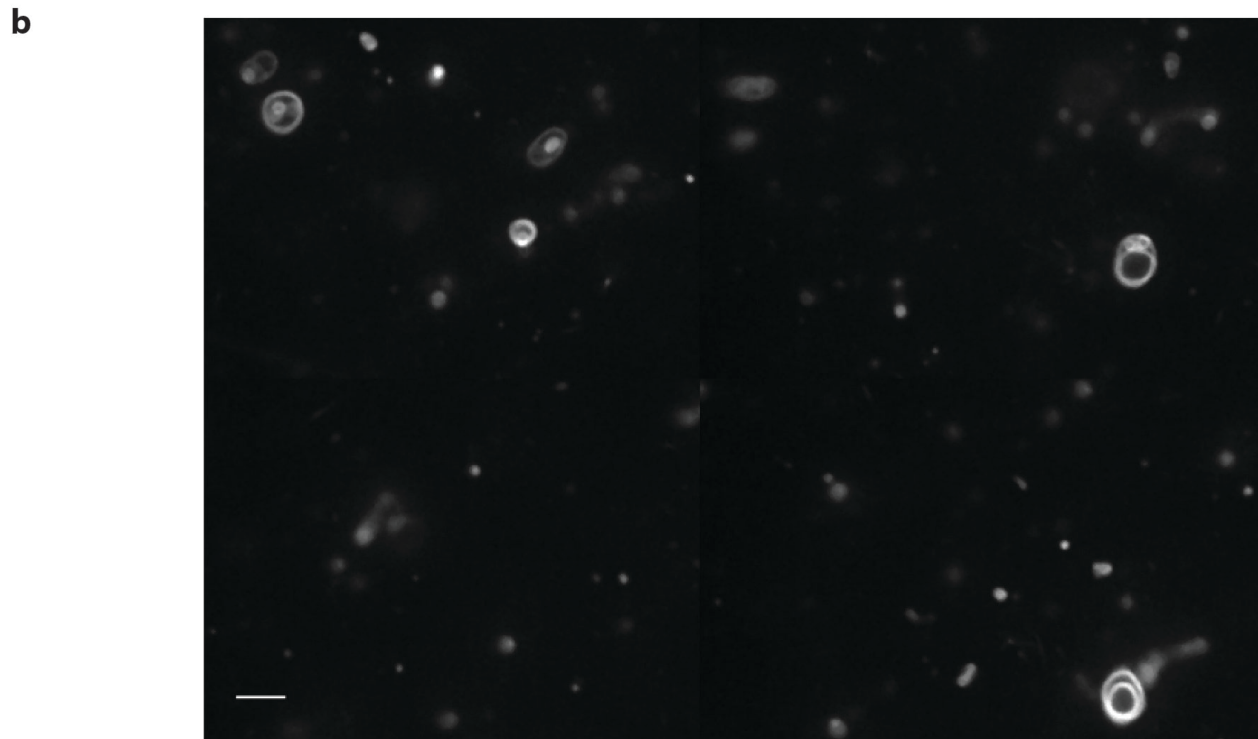


Figure S9. Fifteen rounds of three distinct serial transfer experiments were monitored in parallel with a combination of confocal imaging and HPLC over the course of 500 hours. a**)** The first seven rounds were tracked with HPLC by detecting triazole phospholipid synthesis

using a diode array detector (210 nm). These rounds comprised the first 180 hours of the experiment as shown above. Error bars display the standard deviation of three distinct lineages of serial transfer experiments. Subsequent rounds were monitored by imaging and also periodically by HPLC, as shown by the transition from the 11th to the 12th reaction at 347 hours. Only one lineage was monitored at the last time point for the 11th reaction, represented by the inverted green triangle. b**)** Four contiguous tiles stitched together from the 15th round of a serial transfer experiment at 473 hours after initiation of the first round. Three multilamellar vesicles (upper left corner, lower right corner, upper right region), each roughly five microns in diameter, are clearly visible. Scale bar represents 5 μm.

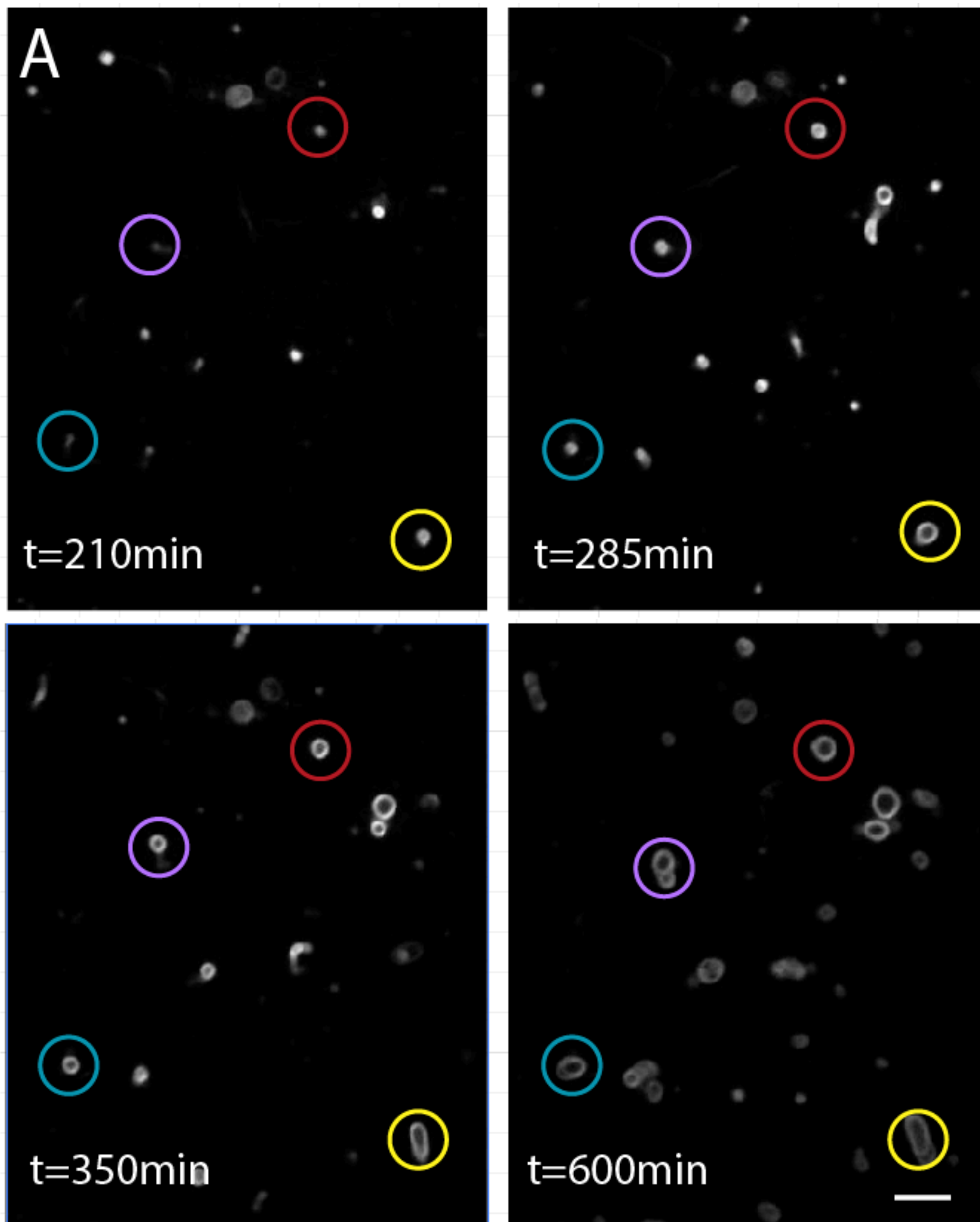


Figure S10. Monitoring growth of continually propagating synthetic membranes. Initial conditions for displayed rounds (excluding unreacted precursors from previous rounds): 132 μM $CuSO_4$, 60 μM hydroquinone, 450 mM TES, 40 mM MOPS, 60 μM tripropargylamine, 558 μM alkyne lysolipid, and 925 μM alkyl azide. Larger vesicles emerge from the growth of smaller catalytic vesicles exposed to ligand and lipid precursors. Vesicle growth could be monitored using a membrane-staining fluorescent dye (Rh-DHPE) and time-lapse confocal microscopy. Vesicles grew in size over several hours and their size and location were tracked using an

automatic vesicle tracking algorithm base on a previously developed single-cell tracking algorithm (3). Scale bar represents 5 μm.

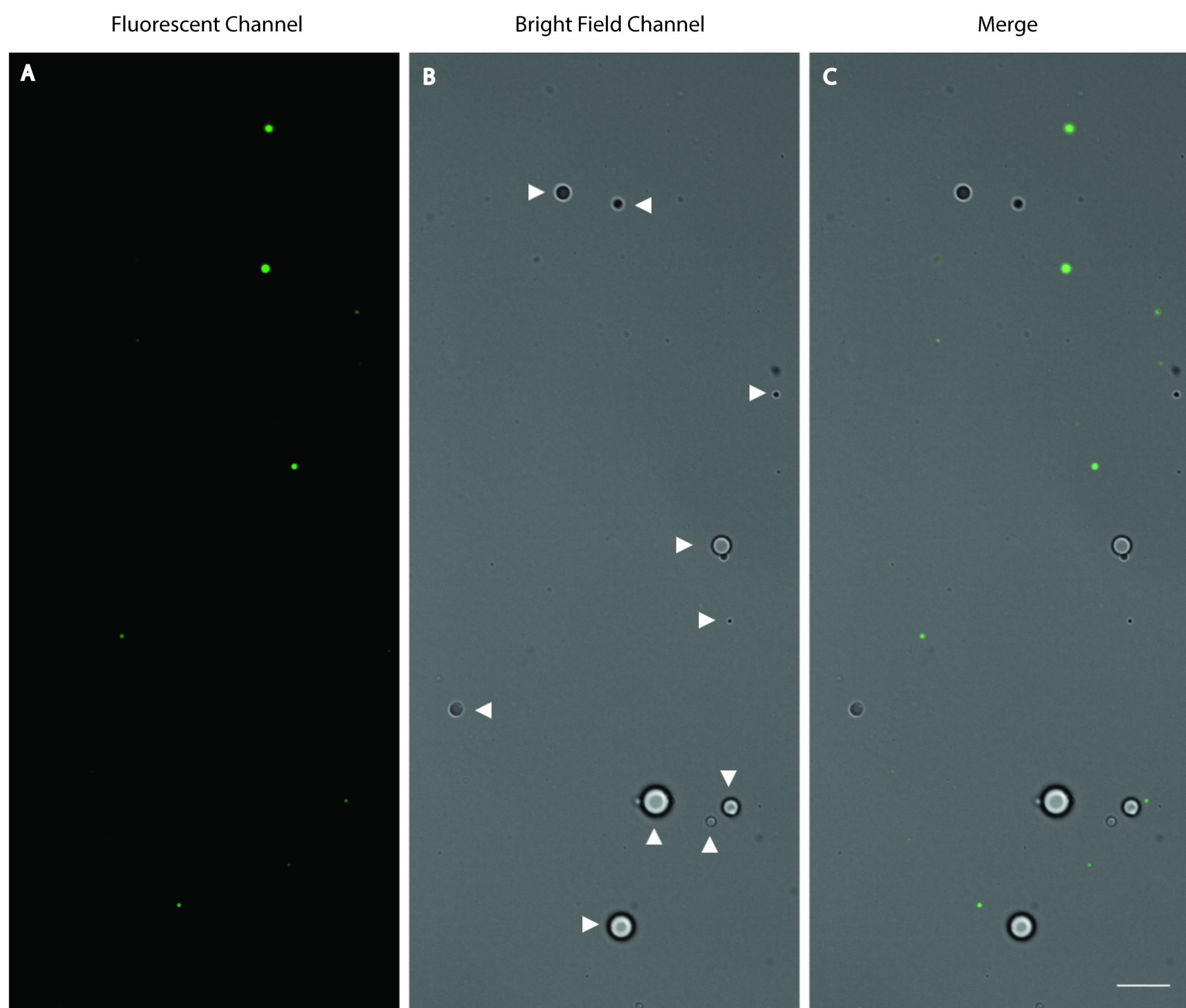


Figure S11. Distinguishing oil droplets from vesicles. A) HPTS-labeled MLVs observed in the fluorescent channel. B) Dodecane azide oil droplets imaged in the bright field channel. Arrows point to oil droplets, which appear as either dark with a white halo when out of plane or as gray/white with a thick black perimeter when in the plane of imaging. In comparison, vesicles are significantly more difficult to observe with bright field microscopy. C) A merged image of fluorescent and bright field channels. No instances of vesicles appearing as oil droplets or oil droplets appearing as vesicles are observed. Scale bar = 6 μm

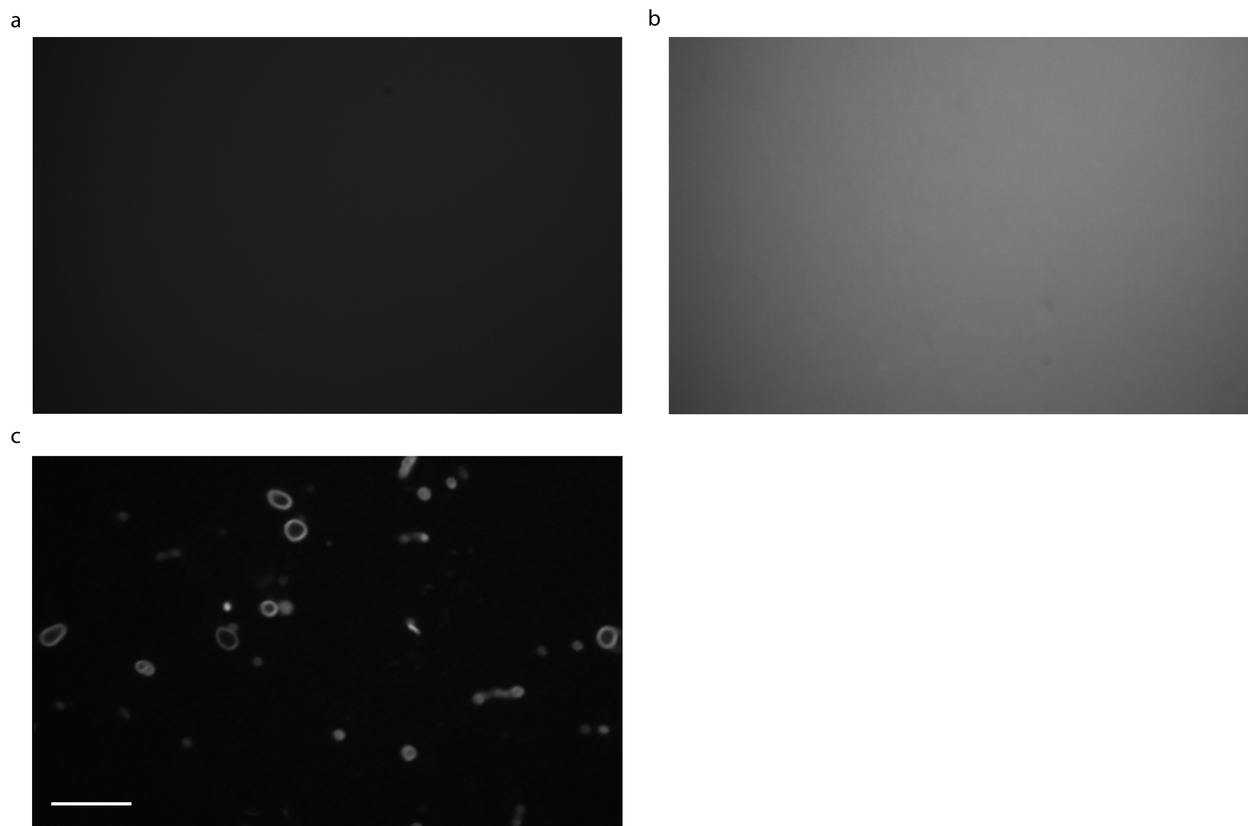


Figure S12. Vesicle growth is not due to POPC aggregation. A) In the absence of copper, sonicated POPC vesicles containing 10 mol % TLTA do not synthesize triazole phospholipid (also supported by HPLC-ELSD-MS) or display aggregation. Time point shown = 50 hrs. B) Increasing laser intensity or exposure time (shown here) does change the result obtained in (A). C) An example of propagating artificial membranes containing both TLTA and copper at 7 hours. Initial conditions: 0 (A and B) or 120 μM (C) $CuSO_4$, 60 μM hydroquinone, 450 mM TES, 40 mM MOPS, 54 μM tripropargylamine, 558 μM alkyne lysolipid, and 925 μM alkyl azide. Scale bar = 10 μm.

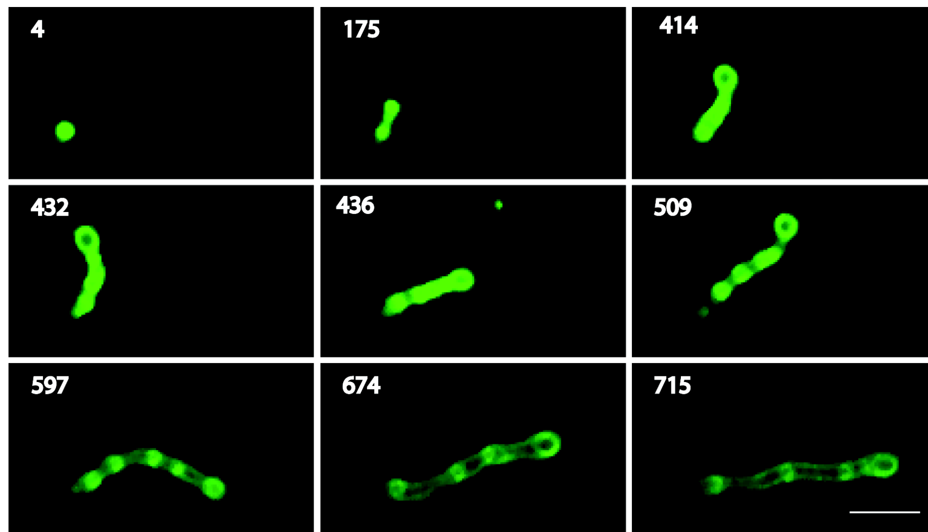


Figure S13. Vesicle growth over a 12-hour time period results in a tubular morphology that resembles a pearling instability. Conditions: 132 μM $CuSO_4$, 60 μM hydroquinone, 450 mM TES, 40 mM MOPS, 60 μM tripropargylamine, 558 μM alkyne lysolipid, and 925 μM alkyl azide. Window leveling, brightness and contrast are the same for all images and were adjusted to allow for vesicle visualization at later time points (bottom row). As a result, earlier time points (top two rows) appear over exposed. Scale bar represents 5 μm.

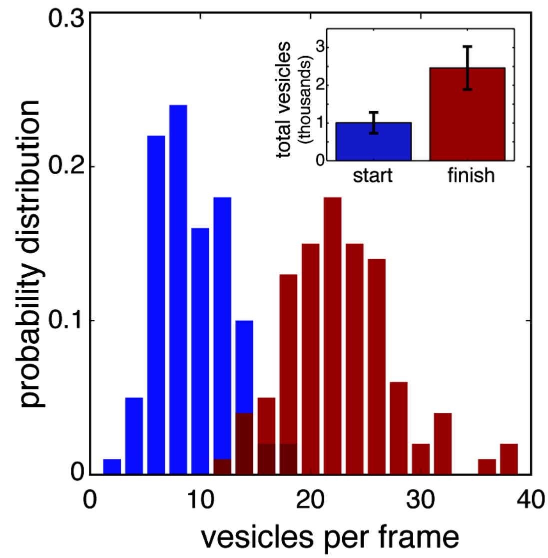


Figure S14. Observable large (>100pixels) vesicles before (blue) and after (red) reaction with lipid precursors in a 0.1mm by 0.1mm frame. Inset shows total number of vesicles in 100 frames (3 replicates, error bars show standard deviation.). Membranes were stained with Texas Red-DHPE, exposed to reactive lipid precursors, and agitated for 49 hours.

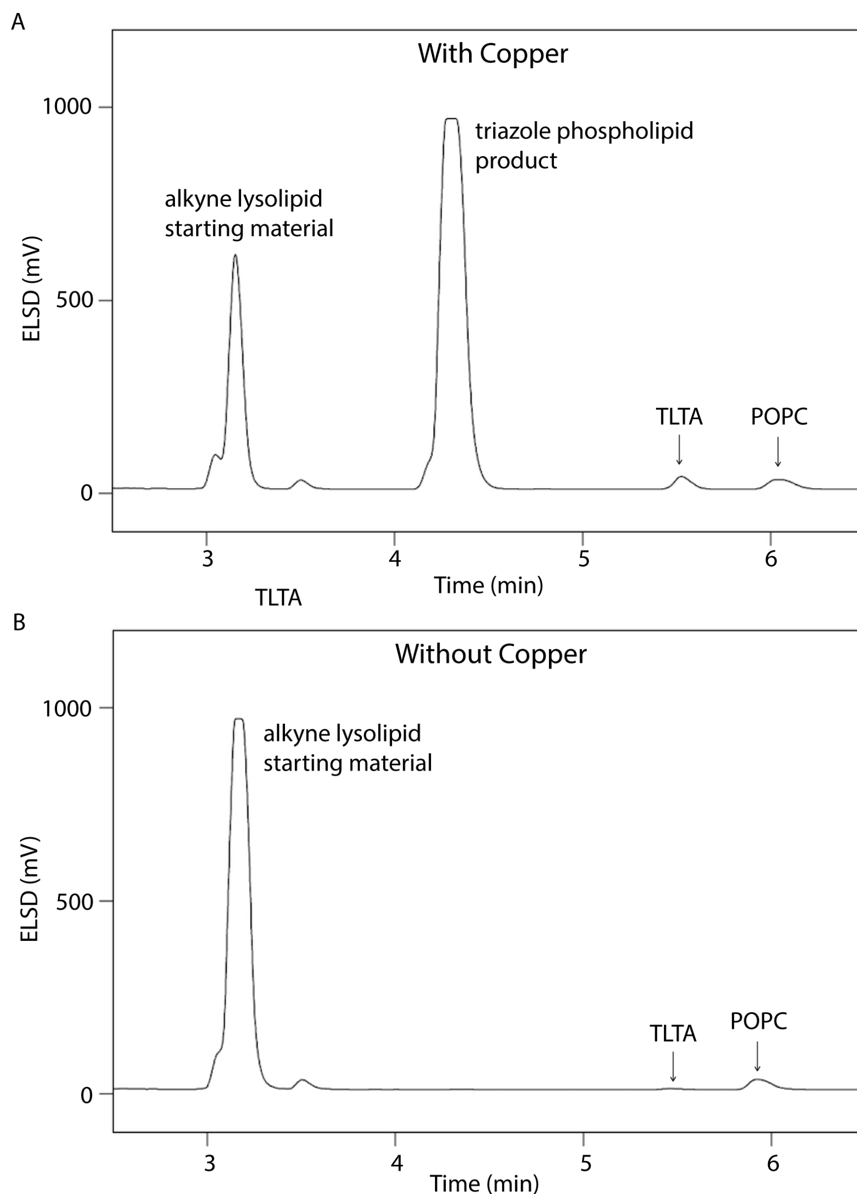


Figure S15. Monitoring the progress of triazole phospholipid and TLTA synthesis during MLV counting experiments (Fig. 5). Catalytic MLVs containing 10 mol percent TLTA and HPTS were added to a solution containing 60 µM hydroquinone, 350 mM TES, 60 µM tripropargylamine, 615 µM alkyne lysolipid, and 1.01 mM alkyl azide. The solution was split into two vials, and to the first vial, 2 µL of 8 mM copper sulfate in water was added to give a final reaction concentration of 120 µM copper sulfate (A). To the second vial, 2 µL of water was added in place of copper (B). When copper is present, triazole phospholipid and additional TLTA is synthesized.

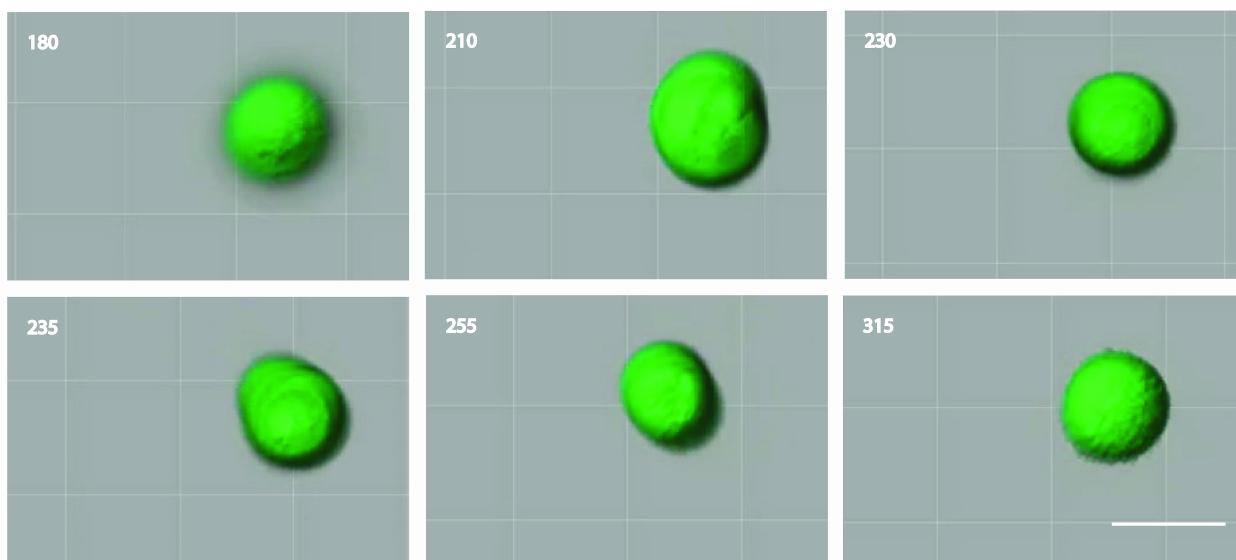


Figure S16. Large multilamellar vesicles (MLVs) containing TLTA and HPTS do not divide in the absence of copper. MLVs with encapsulated HPTS were incubated in 0 μM $CuSO_4$, 60 μM hydroquinone, 350 mM TES, 60 μM tripropargylamine, 615 μM alkyne lysolipid, and 1.01 mM alkyl azide. Numbers represent minutes after beginning of experiment. The elliptical appearance of the MLV at 210, 235, 255 minutes is due to MLV movement during acquisition of multiple cross sectional images. Scale bar represents 5 μm.

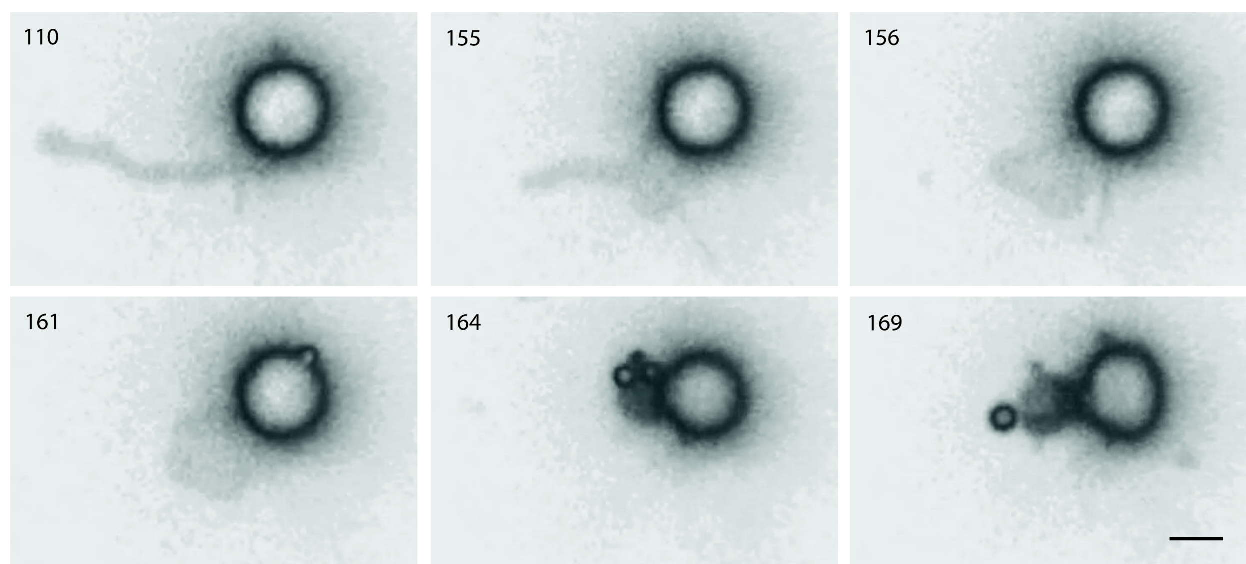


Figure S17. In some cases, vesicle division is preceded by tubule collapse and reorganization. Multiple cross sectional images from the MLV shown in Fig. 6 were removed to improve the signal to noise ratio of a tubule which was only observed in three contiguous cross sectional slices (shown here) of the tomogram. Tubule formation begins at 110 minutes, and tubule collapse occurs at 155 minutes. MLVs with encapsulated HPTS were incubated in 120 μM $CuSO_4$, 60 μM hydroquinone, 350 mM TES, 60 μM tripropargylamine, 615 μM alkyne lysolipid, and 1.01 mM alkyl azide. Visualization of the tubule was achieved by eliminating most cross-sections of the MLV tomogram shown in Fig. 6, which improves tubule signal-to noise, and re-leveling tomogram brightness and contrast. Scale bar = 3 μM.

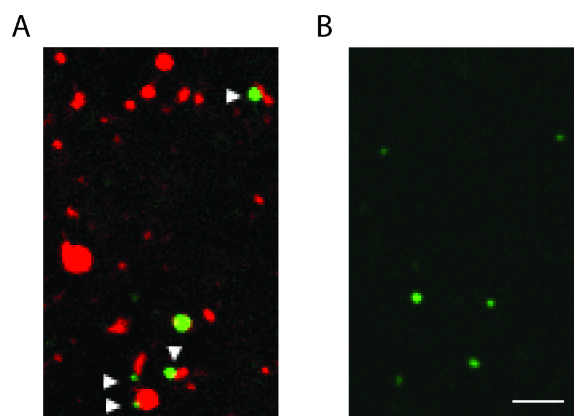


Figure S18. Catalytic vesicles trigger nucleation of new vesicles. (A) Vesicles with TLTA and encapsulating the dye HPTS (shown in green) catalyze lipid synthesis, resulting in the formation of new vesicles, labeled with Texas Red-DHPE. In several cases, the vesicles adjoin the TLTA catalytic vesicles (arrows point to catalytic vesicles that give rise to adjoining Texas Red-labeled membranes). The adjoining membranes do not contain HPTS, indicating that vesicle contents have not been shared and division likely did not occur. (B) Identical vesicles with TLTA and HPTS are unable to synthesize additional membrane in the absence of copper. Scale bar represents 4 μm. Conditions: 0 or 120 μM $CuSO_4$, 60 μM hydroquinone, 350 mM TES, 60 μM tripropargylamine, 615 μM alkyne lysolipid, and 1.01 mM alkyl azide.

1. Budin I & Devaraj NK (2012) Membrane assembly driven by a biomimetic coupling reaction. *J Am Chem Soc* 134(2):751-753.
2. Johnson SM, Bangham AD, Hill MW, & Korn ED (1971) Single Bilayer Liposomes. *Biochimica et biophysica acta* 233(3):820-&.
3. Mondragon-Palomino O, Danino T, Selimkhanov J, Tsimring L, & Hasty J (2011) Entrainment of a Population of Synthetic Genetic Oscillators. *Science* 333(6047):1315-1319.
4. Zhu TF & Szostak JW (2009) Preparation of large monodisperse vesicles. *PLoS One* 4(4):e5009.