

Protocells by spontaneous reaction of cysteine with short-chain thioesters

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Materials and Methods

General information

All reagents obtained from commercial suppliers were used without further purification unless otherwise noted. Acetylthiocholine chloride, octanoic acid, heptanoyl chloride, hexanoyl chloride, *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC·HCl), 4-(dimethylamino)pyridine (DMAP), sodium 2-mercaptoethanesulfonate (MESNA), tris(2-carboxyethyl)phosphine hydrochloride (TCEP·HCl), Cys-Gly (CG), triethylamine (TEA), dichloromethane (DCM), tetrahydrofuran (THF), *N,N*-dimethylformamide (DMF), methanol (MeOH), ethanol (EtOH), toluene, (3-aminopropyl)triethoxysilane (APTES), octadecyltrimethoxysilane (ODTMS), 1-lauroyl-*rac*-glycerol (*C*₁₂-*rac*-glycerol), 1-decanoyl-*rac*-glycerol (*C*₁₀-*rac*-glycerol) and 1-octanoyl-*rac*-glycerol (*C*₈-*rac*-glycerol), Nile Red, 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS) were purchased from Sigma Aldrich (USA). Lauroyl chloride, magnesium chloride and sodium hydroxide were purchased from Acros (USA). Myristoleic acid, 2-methyl-*L*-cysteine (2-Me-Cys), 6-dodecanoyl-2-dimethylaminonaphthalene (Laurdan), propidium iodide (PI), sodium phosphate monobasic, sodium phosphate dibasic, sodium 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethane-1-sulfonate (HEPES sodium salt), sodium chloride and potassium chloride were purchased from Thermo Fisher Scientific (USA). Calcium chloride was purchased from J.T.Baker (USA). Octanoyl chloride was purchased from TCI (Japan). *L*-Cysteine (Cys) and *L*-homocysteine (HCy) were purchased from Chem-Impex (USA). Peptides were purchased from GenScript (USA). Deuterated solvents were purchased from Cambridge Isotope Laboratories (USA). 5 μm-sized silica microspheres were purchased from Bangs Laboratories (USA).

Analytical thin layer chromatography (TLC) was carried out on pre-coated glass silica gel plates with F-254 indicator, and the spots were visualized under 254 nm UV irradiation and/or staining by KMnO₄ solution. Flash column chromatography was performed on silica gel using an appropriate eluent system using a Teledyne ISCO CombiFlash EZ Prep.

¹H and ¹³C NMR spectra were recorded on a Varian VX-500 500 MHz spectrometer (500 MHz for ¹H and 125 MHz for ¹³C) and a Bruker Avance AVA-300 spectrometer (300 MHz for ¹H). ¹H chemical shifts were referenced from the chemical shifts of residual solvent peaks (7.26 ppm for CDCl₃, 3.31 ppm for CD₃OD and 2.50 ppm for DMSO-*d*₆). ¹³C NMR spectra were recorded with complete proton decoupling. ¹³C chemical shifts were referenced from the chemical shifts of CDCl₃ (77.16 ppm), CD₃OD (49.00 ppm) or DMSO-*d*₆ (39.52 ppm). Recorded spectral data were processed with MestReNova (Version 14.2.3). Chemical shifts were reported in parts per million and the following abbreviations (or combinations thereof) were used to explain multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, brs = broad singlet, app s = apparent singlet. Coupling constants, *J*, were reported in Hertz unit (Hz).

LCMS analysis was performed using an Agilent 1260 Infinity LC System coupled with an Agilent 6120 quadrupole mass spectrometer. The LC system was equipped with a diode array detector (DAD), an evaporative light scattering detector (ELSD), and an Eclipse Plus C₈ analytical column. The analysis employed a mixed gradient of solvents as follows: Phase A: H₂O with 0.1% formic acid; Phase B: MeOH with 0.1% formic acid. Following LCMS method was used unless otherwise noted: injection volume of 20 μL, gradient of 0–10 min with 50–100% Phase B, flow rate: 1.0 mL/min.

High resolution mass spectroscopy (HRMS) analyses were performed at the UCSD Molecular Mass Spectrometry Facility using an Agilent 1260 Infinity Binary LC coupled with a 6230 Accurate-Mass TOFMS system for HPLC-HR-ESI/APCI-MS analysis.

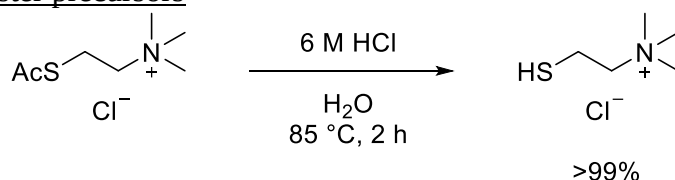
Water contact angles on the glass surfaces were measured using a Ramé-Hart™ Model 200 goniometer.

Phase contrast microscopy imaging was performed using an Olympus BX51 optical microscope equipped with a phase contrast condenser (PH3) and a 100x oil immersion objective. Images were captured with an ORCA-spark Digital CMOS camera (C11440-36U, Hamamatsu) using Olympus cellSens 3.1 imaging software. To obtain the fluorescence microscopy images, a fluorescence mercury light source (U-LH100HGAP0, Olympus) was used, coupled with a FITC (for HPTS) or Texas Red (for Nile Red and PI) filter cube. Confocal fluorescence microscopy imaging was conducted using a Zeiss Axio Observer Z1 motorized inverted fluorescence microscope (Carl Zeiss Microscopy) with a CSU-X1 confocal scanner unit (Yokogawa). A 63x, 1.40 NA oil immersion objective was employed. The respective fluorophores were excited using diode lasers at wavelengths of 405 nm, 488 nm, 561 nm, and 640 nm. Images were captured with an ORCA-Flash4.0 V2 Digital CMOS camera (Hamamatsu) using ZEN Blue 2.3 imaging software (Carl Zeiss Microscopy). In general, samples were prepared by dropping 2 μ L of the sample on a microscope glass slide (Fisherbrand), and were then covered with a glass coverslip (VWR). The obtained images were further analyzed with ImageJ software.

UV-Vis absorbance measurements were performed with a NanoDrop 2000c spectrophotometer (Thermo Scientific) using polystyrene cuvettes with a path length of 10 mm.

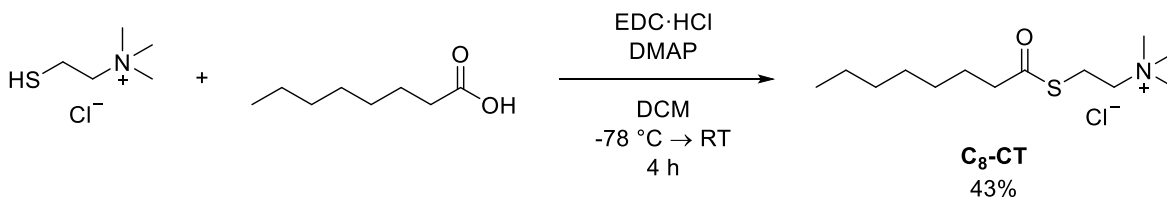
Chemical synthesis

Synthesis of the thioester precursors



Thiocholine chloride

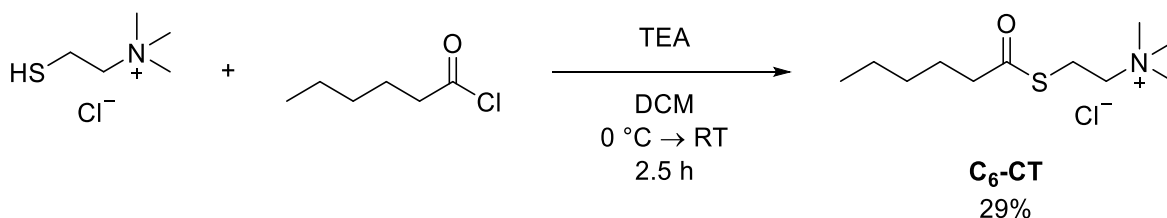
Acetylthiocholine chloride (500 mg, 2.50 mmol) was dissolved in aqueous 6 M HCl solution (5.00 mL), then the reaction mixture was placed in an oil bath and stirred at 85 $^{\circ}$ C. After 2 h of reaction, the reaction mixture was concentrated under reduced pressure, then dried *in vacuo* to afford thiocholine chloride as a white powder (404 mg, >99%). ^1H NMR (300 MHz, CD_3OD): δ 3.60 – 3.48 (m, 2H), 3.16 (s, 9H), 2.99 – 2.88 (m, 2H). Spectral data match those previously reported¹.



N,N,N-Trimethyl-2-(octanoylthio)ethan-1-aminium chloride (C₈-CT)

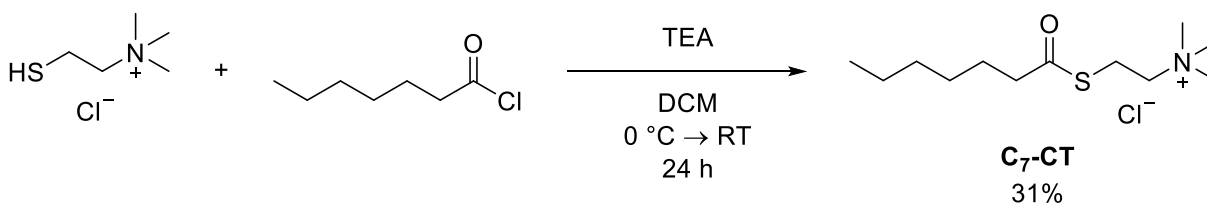
A procedure was adapted from the previous literature² as follows: To a stirred solution of EDC·HCl (211 mg, 1.10 mmol, 1.10 eq.) and DMAP (12.3 mg, 0.100 mmol, 0.100 eq.) in DCM

(8.00 mL), octanoic acid (0.176 mL, 1.10 mmol, 1.10 eq.) was added, then the reaction mixture was stirred at -78 °C under Ar atmosphere. After 30 min, thiocholine chloride (156 mg, 1.00 mmol, 1.00 eq.) was added, then the reaction mixture was further stirred at RT under Ar atmosphere. After 4 h reaction, the reaction mixture was concentrated under reduced pressure. Choline thioester C₈-CT was afforded (122 mg, 43%) as a pale yellow solid after purification by flash column chromatography (SiO₂, DCM/MeOH 90:10 → DCM/MeOH 65:35). ¹H NMR (500 MHz, DMSO-*d*₆): δ 3.38 – 3.29 (m, 2H), 3.27 – 3.20 (m, 2H), 3.10 (s, 9H), 2.64 (t, *J* = 7.3 Hz, 2H), 1.61 – 1.52 (m, 2H), 1.31 – 1.18 (m, 8H), 0.85 (t, *J* = 6.8 Hz, 3H). ¹³C NMR (125 MHz, DMSO-*d*₆): δ 198.1, 63.7, 52.2, 43.3, 31.2, 28.4, 28.2, 25.0, 22.1, 21.3, 14.1. HRMS (ESI-TOF) calculated for C₁₃H₂₈ClNOS [M-Cl]⁺: 246.1886, found 246.1888.



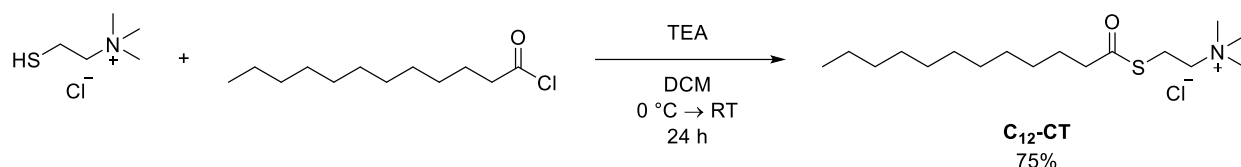
2-(Hexanoylthio)-N,N,N-trimethylethan-1-aminium chloride (C₆-CT)

To a stirred solution of thiocholine chloride (77.8 mg, 0.500 mmol, 1.00 eq.) and TEA (0.077 mL, 0.550 mmol, 1.10 eq.) in DCM (5.00 mL), hexanoyl chloride (0.108 mL, 0.750 mmol, 1.50 eq.) was added at 0 °C, then the reaction mixture was stirred for 2.5 h. The reaction was quenched by addition of 1.00 mL of MeOH, then the mixture was concentrated under reduced pressure. Choline thioester C₆-CT was afforded (36.7 mg, 29%) as a colorless gel after purification by flash column chromatography (SiO₂, DCM/MeOH 90:10 → DCM/MeOH 60:40). ¹H NMR (500 MHz, CD₃OD): δ 3.52 – 3.43 (m, 2H), 3.32 – 3.28 (m, 2H), 3.23 (s, 9H), 2.65 (t, *J* = 7.4 Hz, 2H), 1.76 – 1.58 (m, 2H), 1.42 – 1.25 (m, 4H), 0.97 – 0.85 (m, 3H). ¹³C NMR (125 MHz, CD₃OD): δ 199.6, 66.1, 53.5, 44.6, 32.1, 26.2, 23.4, 22.6, 14.2. HRMS (ESI-TOF) calculated for C₁₁H₂₄ClNOS [M-Cl]⁺: 218.1573, found 218.1573.



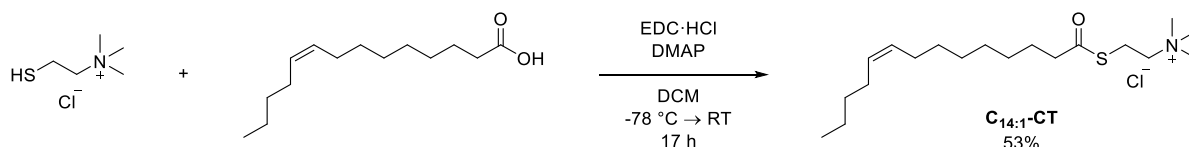
2-(Heptanoylthio)-N,N,N-trimethylethan-1-aminium chloride (C₇-CT)

To a stirred solution of thiocholine chloride (234 mg, 1.50 mmol, 1.00 eq.) and TEA (0.232 mL, 1.65 mmol, 1.10 eq.) in DCM (7.50 mL), heptanoyl chloride (0.352 mL, 2.25 mmol, 1.50 eq.) was added at 0 °C, then the reaction mixture was stirred for 24 h. The reaction was quenched by addition of 1.00 mL of MeOH, then the mixture was concentrated under reduced pressure. Choline thioester C₇-CT was afforded (125 mg, 31%) as a white solid after purification by flash column chromatography (SiO₂, DCM/MeOH 90:10 → DCM/MeOH 60:40). ¹H NMR (500 MHz, CD₃OD): δ 3.55 – 3.44 (m, 2H), 3.37 – 3.34 (m, 2H), 3.27 (s, 9H), 2.70 (t, *J* = 7.4 Hz, 2H), 1.81 – 1.62 (m, 2H), 1.52 – 1.21 (m, 6H), 0.96 (t, *J* = 6.9 Hz, 3H). ¹³C NMR (125 MHz, CD₃OD): δ 199.6, 66.1, 53.6, 44.6, 32.6, 29.6, 26.5, 23.5, 22.5, 14.4. HRMS (ESI-TOF) calculated for C₁₂H₂₆ClNOS [M-Cl]⁺: 232.1730, found 232.1727.



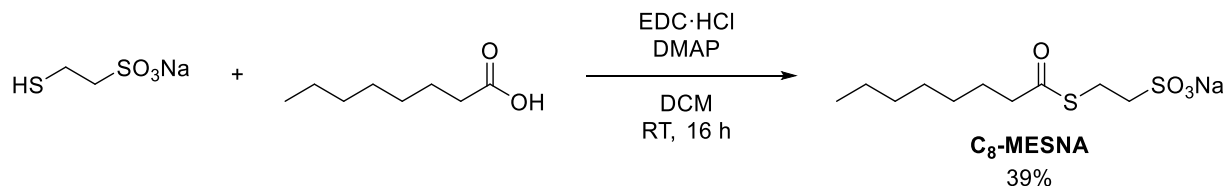
2-(Dodecanoylthio)-N,N,N-trimethylethan-1-aminium chloride (C₁₂-CT)

To a stirred solution of thiocholine chloride (311 mg, 2.00 mmol, 1.00 eq.) and TEA (0.422 mL, 3.00 mmol, 1.50 eq.) in DCM (10.0 mL), lauroyl chloride (0.960 mL, 4.00 mmol, 2.00 eq.) was added at 0 °C, then the reaction mixture was stirred for 24 h. The reaction was quenched by addition of 1.00 mL of MeOH, then the mixture was concentrated under reduced pressure. Choline thioester C₁₂-CT was afforded (510 mg, 75%) as a white solid after purification by flash column chromatography (SiO₂, DCM/MeOH 90:10 → DCM/MeOH 70:30). ¹H NMR (500 MHz, DMSO-*d*₆): δ 3.36 – 3.35 (m, 2H), 3.26 – 3.19 (m, 2H), 3.10 (s, 9H), 2.64 (t, *J* = 7.3 Hz, 2H), 1.59 – 1.52 (m, 2H), 1.29 – 1.17 (m, 16H), 0.85 (t, *J* = 6.8 Hz, 3H). ¹³C NMR (125 MHz, DMSO-*d*₆): δ 198.1, 63.7, 52.2, 43.3, 31.4, 29.1, 28.9, 28.8, 28.8, 28.3, 25.0, 22.2, 21.2, 14.1. HRMS (ESI-TOF) calculated for C₁₇H₃₆ClNOS [M-Cl]⁺: 302.2512, found 302.2512.



(Z)-N,N,N-trimethyl-2-(tetradec-9-enoylthio)ethan-1-aminium chloride (C_{14:1}-CT)

A procedure was adapted from the previous literature² as follows: To a stirred solution of EDC·HCl (211 mg, 1.10 mmol, 1.10 eq.) and DMAP (12.3 mg, 0.100 mmol, 0.100 eq.) in DCM (8.00 mL), myristoleic acid (0.267 mL, 1.10 mmol, 1.10 eq.) was added, then the reaction mixture was stirred at -78 °C under Ar atmosphere. After 30 min, thiocholine chloride (156 mg, 1.00 mmol, 1.00 eq.) was added, then the reaction mixture was further stirred at RT under Ar atmosphere. After 17 h reaction, the reaction mixture was concentrated under reduced pressure. Choline thioester C_{14:1}-CT was afforded (194 mg, 53%) as a pale yellow solid after purification by flash column chromatography (SiO₂, DCM/MeOH 90:10 → DCM/MeOH 75:25). ¹H NMR (500 MHz, DMSO-*d*₆): δ 5.39 – 5.23 (m, 2H), 3.37 – 3.35 (m, 2H), 3.26 – 3.19 (m, 2H), 3.10 (s, 9H), 2.64 (t, *J* = 7.3 Hz, 2H), 2.06 – 1.93 (m, 4H), 1.62 – 1.49 (m, 2H), 1.37 – 1.19 (m, 12H), 0.92 – 0.80 (m, 3H). ¹³C NMR (125 MHz, DMSO-*d*₆): δ 198.1, 129.7, 129.7, 63.7, 52.2, 43.3, 31.4, 29.1, 28.6, 28.5, 28.2, 26.6, 26.4, 25.0, 21.8, 21.3, 13.9. HRMS (ESI-TOF) calculated for C₁₉H₃₈ClNOS [M-Cl]⁺: 328.2669, found 328.2670.

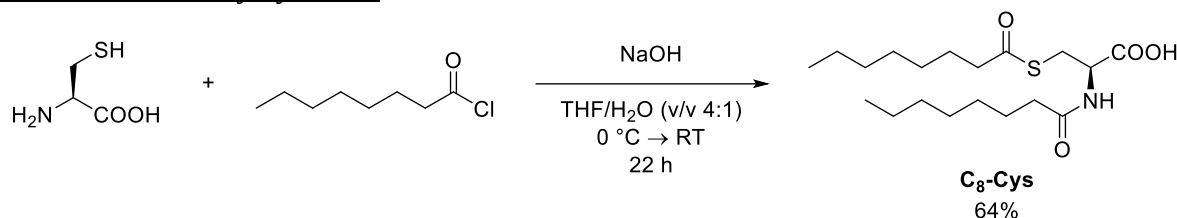


Sodium 2-(octanoylthio)ethane-1-sulfonate (C₈-MESNA)

A procedure was adapted from the previous literature³ as follows: To a stirred solution of octanoic acid (0.352 mL, 2.20 mmol, 1.10 eq.), EDC·HCl (422 mg, 2.20 mmol, 1.10 eq.) and DMAP (24.7 mg, 0.200 mmol, 0.100 eq.) in DCM (10.0 mL), MESNA (335 mg, 2.00 mmol, 1.00 eq.) was added, then the reaction mixture was stirred at RT. After 16 h reaction, the reaction

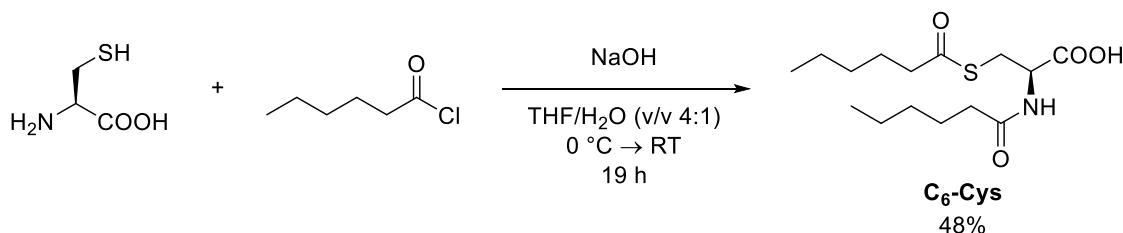
mixture was concentrated under reduced pressure, diluted with water, then washed with EtOAc 3 times. Subsequently, the aqueous layer was collected and concentrated under reduced pressure. The resulting residue was washed with MeCN and DCM, then dried *in vacuo* to afford C₈-MESNA (228 mg, 39%) as a white solid. ¹H NMR (500 MHz, DMSO-*d*₆): δ 3.05 – 2.97 (m, 2H), 2.60 – 2.55 (m, 2H), 2.54 (t, *J* = 7.4 Hz, 2H), 1.59 – 1.49 (m, 2H), 1.33 – 1.15 (m, 8H), 0.92 – 0.78 (m, 3H). ¹³C NMR (125 MHz, DMSO-*d*₆): δ 198.8, 51.0, 43.4, 31.1, 28.4, 28.2, 25.2, 24.4, 22.1, 14.0. HRMS (ESI-TOF) calculated for C₁₀H₁₉NaO₄S₂ [M-Na]⁻: 267.0730, found 267.0733.

Synthesis of the diacylcysteines



N,S-di-octanoyl-*L*-cysteine (C₈-Cys)

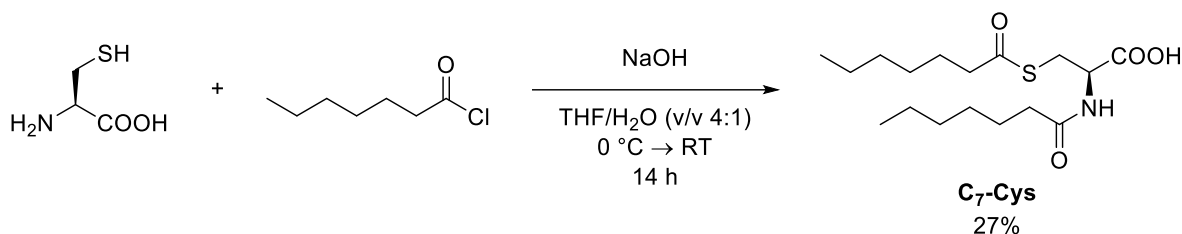
A procedure was adapted from the previous literature⁴ as follows: To a stirred solution of Cys (485 mg, 4.00 mmol, 1.00 eq.) and NaOH (320 mg, 8.00 mmol, 2.00 eq.) in water (2.00 mL), THF (8.00 mL) was added, then the reaction mixture was stirred at 0 °C. Octanoyl chloride (1.52 mL, 8.80 mmol, 2.20 eq.) was added dropwise, then the reaction mixture was further stirred for 22 h. After the reaction, THF was removed under reduced pressure, then the mixture was acidified by addition of aqueous 6 M HCl solution. The mixture was extracted with EtOAc 3 times, dried over anhydrous MgSO₄, filtered, then concentrated under reduced pressure. Diacylcysteine C₈-Cys was afforded (951 mg, 64%) as a white powder after purification by flash column chromatography (SiO₂, hexane/EtOAc 90:10 → hexane/EtOAc 50:50). ¹H NMR (500 MHz, DMSO-*d*₆): δ 7.71 (brs, 1H), 4.15 (td, *J* = 7.4, 4.6 Hz, 1H), 3.37 (dd, *J* = 13.1, 4.7 Hz, 1H), 3.01 (dd, *J* = 13.1, 7.5 Hz, 1H), 2.53 – 2.50 (m, 2H), 2.12 – 1.99 (m, 2H), 1.57 – 1.48 (m, 2H), 1.48 – 1.39 (m, 2H), 1.32 – 1.15 (m, 16H), 0.87 – 0.82 (m, 6H). ¹³C NMR (125 MHz, DMSO-*d*₆): δ 198.6, 171.8, 171.7, 52.0, 43.4, 35.4, 31.3, 31.2, 28.6, 28.5, 28.3, 25.4, 25.2, 22.2, 22.1, 14.1, 14.0. HRMS (ESI-TOF) calculated for C₁₉H₃₅NO₄S [M+H]⁺: 374.2360, found 374.2358.



N,S-di-hexanoyl-*L*-cysteine (C₆-Cys)

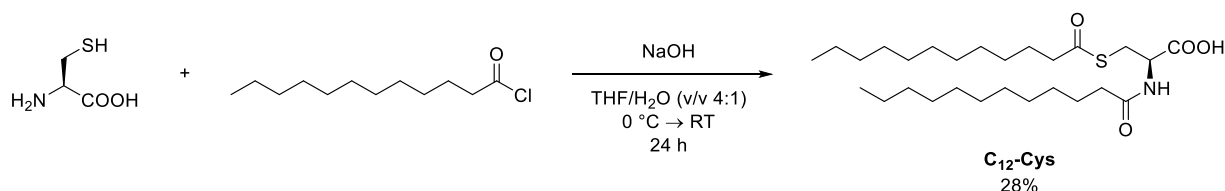
A procedure was adapted from the previous literature⁴ as follows: To a stirred solution of Cys (242 mg, 2.00 mmol, 1.00 eq.) and NaOH (160 mg, 4.00 mmol, 2.00 eq.) in water (1.00 mL), THF (4.00 mL) was added, then the reaction mixture was stirred at 0 °C. Hexanoyl chloride (0.634 mL, 4.40 mmol, 2.20 eq.) was added dropwise, then the reaction mixture was further stirred for 19 h. After the reaction, THF was removed under reduced pressure, then the mixture was acidified by addition of aqueous 6 M HCl solution. The mixture was extracted with EtOAc 3 times, dried over anhydrous MgSO₄, filtered, then concentrated under reduced pressure. Diacylcysteine C₆-Cys was

afforded (305 mg, 48%) as a white powder after purification by flash column chromatography (SiO₂, hexane/EtOAc 70:30 → hexane/EtOAc 30:70). ¹H NMR (500 MHz, CDCl₃): δ 9.14 (brs, 1H), 6.66 (d, *J* = 7.0 Hz, 1H), 4.72 (td, *J* = 7.1, 4.1 Hz, 1H), 3.48 – 3.26 (m, 2H), 2.57 (t, *J* = 7.6 Hz, 2H), 2.31 – 2.14 (m, 2H), 1.75 – 1.49 (m, 4H), 1.38 – 1.18 (m, 8H), 0.88 (t, *J* = 6.8 Hz, 6H). ¹³C NMR (125 MHz, CDCl₃): δ 200.7, 175.1, 172.6, 53.2, 44.0, 36.4, 31.4, 31.1, 30.0, 25.4, 25.2, 22.4, 22.4, 14.1, 14.0. HRMS (ESI-TOF) calculated for C₁₅H₂₇NO₄S [M+H]⁺: 318.1734, found 318.1734.



***N,S*-diheptanoyl-*L*-cysteine (C₇-Cys)**

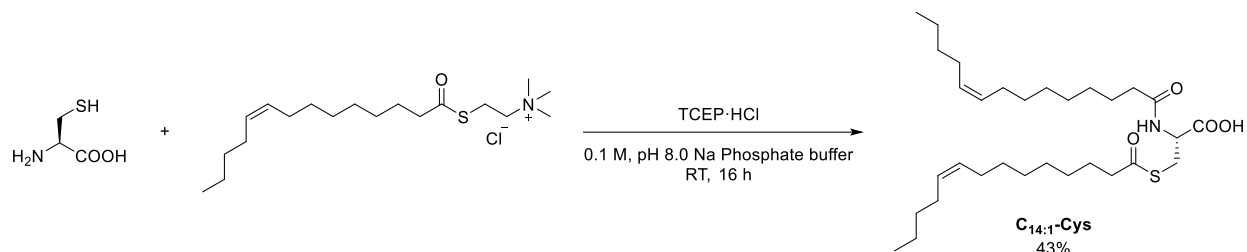
A procedure was adapted from the previous literature⁴ as follows: To a stirred solution of Cys (242 mg, 2.00 mmol, 1.00 eq.) and NaOH (160 mg, 4.00 mmol, 2.00 eq.) in water (1.00 mL), THF (4.00 mL) was added, then the reaction mixture was stirred at 0 °C. Heptanoyl chloride (0.688 mL, 4.40 mmol, 2.20 eq.) was added dropwise, then the reaction mixture was further stirred for 14 h. After the reaction, THF was removed under reduced pressure, then the mixture was acidified by addition of aqueous 6 M HCl solution. The mixture was extracted with EtOAc 3 times, dried over anhydrous MgSO₄, filtered, then concentrated under reduced pressure. Diacylcysteine C₇-Cys was afforded (183 mg, 27%) as a white powder after purification by flash column chromatography (SiO₂, hexane/EtOAc 90:10 → hexane/EtOAc 50:50). ¹H NMR (500 MHz, CDCl₃): δ 7.56 (brs, 1H), 6.76 (d, *J* = 5.6 Hz, 1H), 4.69 (td, *J* = 6.8, 4.5 Hz, 1H), 3.48 – 3.29 (m, 2H), 2.59 (t, *J* = 7.6 Hz, 2H), 2.30 – 2.19 (m, 2H), 1.70 – 1.55 (m, 4H), 1.36 – 1.22 (m, 12H), 0.95 – 0.80 (m, 6H). ¹³C NMR (125 MHz, CDCl₃): δ 201.2, 175.6, 172.3, 53.6, 53.6, 44.1, 36.3, 31.6, 31.5, 29.8, 28.9, 28.7, 25.7, 25.5, 22.6, 22.6, 14.2, 14.2. HRMS (ESI-TOF) calculated for C₁₇H₃₁NO₄S [M-H]⁻: 344.1901, found 344.1900.



***N,S*-didodecanoyl-*L*-cysteine (C₁₂-Cys)**

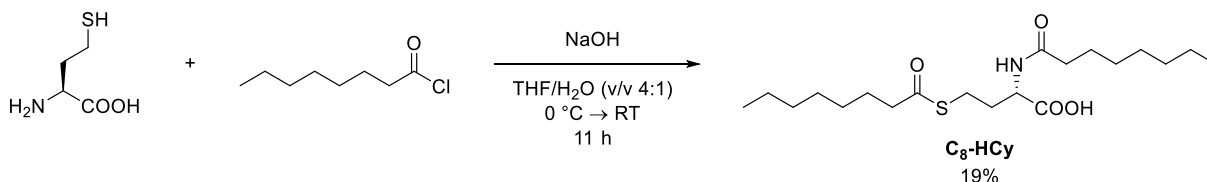
A procedure was adapted from the previous literature⁴ as follows: To a stirred solution of Cys (242 mg, 2.00 mmol, 1.00 eq.) and NaOH (160 mg, 4.00 mmol, 2.00 eq.) in water (1.00 mL), THF (4.00 mL) was added, then the reaction mixture was stirred at 0 °C. Lauroyl chloride (1.06 mL, 4.40 mmol, 2.20 eq.) was added dropwise, then the reaction mixture was further stirred for 24 h. After the reaction, THF was removed under reduced pressure, then the mixture was acidified by addition of aqueous 6 M HCl solution. The mixture was extracted with EtOAc 3 times, dried over anhydrous MgSO₄, filtered, then concentrated under reduced pressure. Diacylcysteine C₁₂-Cys was afforded (269 mg, 28%) as a white powder after purification by flash column chromatography (SiO₂, hexane/EtOAc 90:10 → hexane/EtOAc 70:30). ¹H NMR (500 MHz, CDCl₃): δ 6.70 (d, *J* =

6.1 Hz, 1H), 4.65 (d, $J = 5.9$ Hz, 1H), 3.55 – 3.14 (m, 2H), 2.59 (t, $J = 7.5$ Hz, 2H), 2.24 (t, $J = 7.7$ Hz, 2H), 1.76 – 1.44 (m, 4H), 1.29 – 1.23 (m, 32H), 0.87 (t, $J = 6.9$ Hz, 6H). ^{13}C NMR (125 MHz, CDCl_3): δ 201.8, 175.8, 171.9, 54.0, 44.1, 36.4, 33.6, 32.1, 32.1, 32.1, 29.9, 29.9, 29.9, 29.8, 29.8, 29.8, 29.7, 29.6, 29.6, 29.6, 29.5, 29.5, 29.5, 29.5, 29.4, 29.3, 29.1, 25.8, 25.5, 23.4, 22.9, 22.8, 18.5, 14.3. HRMS (ESI-TOF) calculated for $\text{C}_{27}\text{H}_{51}\text{NO}_4\text{S}$ $[\text{M}+\text{H}]^+$: 486.3612, found 486.3615.



***N,S*-di((*Z*)-tetradec-9-enoyl)-*L*-cysteine ($\text{C}_{14:1}\text{-Cys}$)**

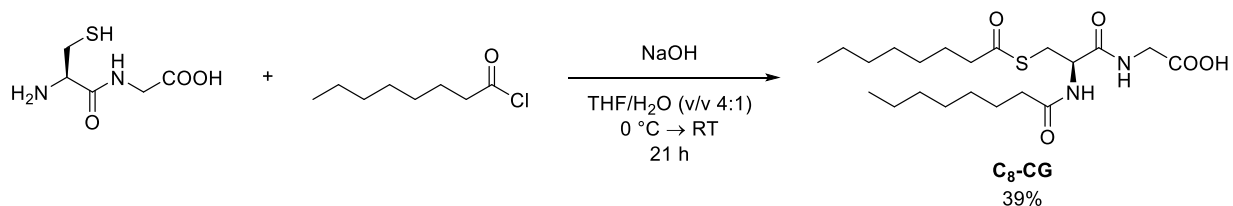
To a stirred solution of $\text{C}_{14:1}\text{-CT}$ (109 mg, 0.300 mmol, 2.00 eq.) and TCEP·HCl (86.0 mg, 0.300 mmol, 2.00 eq.) in 0.1 M, pH 8.0 sodium phosphate buffer, Cys (18.2 mg, 0.150 mmol, 1.00 eq.) was added, then the reaction mixture was stirred at RT for 16 h. After the reaction, the reaction mixture was acidified by addition of aqueous 6 M HCl solution. The mixture was extracted with EtOAc 3 times, dried over anhydrous MgSO_4 , filtered, then concentrated under reduced pressure. Diacylcysteine $\text{C}_{14:1}\text{-Cys}$ was afforded (35.0 mg, 43%) as a colorless gel after purification by flash column chromatography (SiO_2 , hexane/EtOAc 90:10 $\rightarrow$ hexane/EtOAc 50:50). ^1H NMR (500 MHz, CDCl_3): δ 7.38 (brs, 1H), 6.61 (d, $J = 6.7$ Hz, 1H), 5.44 – 5.22 (m, 4H), 4.68 (td, $J = 6.7$, 4.7 Hz, 1H), 3.37 (dd, $J = 6.0$, 2.7 Hz, 2H), 2.58 (t, $J = 7.6$ Hz, 2H), 2.28 – 2.16 (m, 2H), 2.08 – 1.83 (m, 8H), 1.71 – 1.51 (m, 4H), 1.38 – 1.22 (m, 24H), 0.95 – 0.80 (m, 6H). ^{13}C NMR (125 MHz, CDCl_3): δ 201.1, 175.2, 172.4, 130.1, 130.1, 129.9, 129.8, 53.5, 44.1, 36.4, 32.1, 29.9, 29.8, 29.8, 29.4, 29.3, 29.3, 29.2, 29.2, 29.0, 27.3, 27.3, 27.1, 25.8, 25.5, 22.5, 14.2. HRMS (ESI-TOF) calculated for $\text{C}_{31}\text{H}_{55}\text{NO}_4\text{S}$ $[\text{M}-\text{H}]^-$: 536.3779, found 536.3786.



***N,S*-dioctanoyl-*L*-homocysteine ($\text{C}_8\text{-HCy}$)**

A procedure was adapted from the previous literature⁴ as follows: To a stirred solution of *L*-homocysteine (41.4 mg, 0.300 mmol, 1.00 eq.) and NaOH (24.0 mg, 0.600 mmol, 2.00 eq.) in water (0.500 mL), THF (2.00 mL) was added, then the reaction mixture was stirred at 0 °C. Octanoyl chloride (0.114 mL, 0.660 mmol, 2.20 eq.) was added dropwise, then the reaction mixture was further stirred for 11 h. After the reaction, THF was removed under reduced pressure, then the mixture was acidified by addition of aqueous 6 M HCl solution. The mixture was extracted with EtOAc 3 times, dried over anhydrous MgSO_4 , filtered, then concentrated under reduced pressure. Diacylcysteine $\text{C}_8\text{-HCy}$ was afforded (22.5 mg, 19%) as a white powder after purification by flash column chromatography (SiO_2 , hexane/EtOAc 90:10 $\rightarrow$ hexane/EtOAc 60:40). ^1H NMR (500 MHz, CDCl_3): δ 8.00 (brs, 1H), 6.62 (d, $J = 7.3$ Hz, 1H), 4.57 (td, $J = 7.6$, 4.5 Hz, 1H), 3.03 – 2.88 (m, 1H), 2.88 – 2.76 (m, 1H), 2.54 (t, $J = 7.6$ Hz, 2H), 2.30 (t, $J = 7.7$ Hz, 2H), 2.20 – 2.08 (m, 1H), 2.09 – 1.94 (m, 1H), 1.73 – 1.56 (m, 4H), 1.40 – 1.15 (m, 16H), 1.02 – 0.68 (m, 6H). ^{13}C

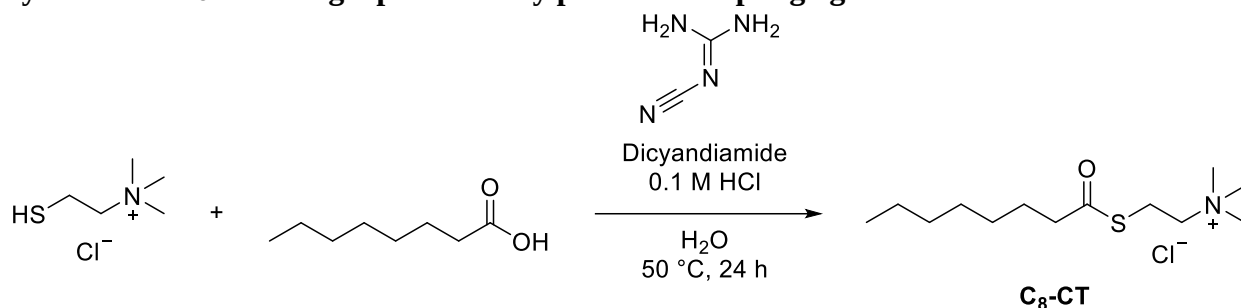
NMR (125 MHz, CDCl₃): δ 200.6, 174.9, 174.5, 51.9, 51.9, 44.2, 36.5, 32.3, 31.8, 31.7, 29.3, 29.1, 29.0, 29.0, 25.7, 25.7, 24.8, 22.7, 22.7, 14.2, 14.2. HRMS (ESI-TOF) calculated for C₂₀H₃₇NO₄S [M+H]⁺: 388.2516 found 388.2519.



***N,S*-di-octanoyl-*L*-cysteinylglycine (C₈-CG)**

A procedure was adapted from the previous literature⁴ as follows: To a stirred solution of CG (77.4 mg, 0.369 mmol, 1.00 eq.) and NaOH (29.6 mg, 0.739 mmol, 2.00 eq.) in water (0.185 mL), THF (0.739 mL) was added, then the reaction mixture was stirred at 0 °C. Octanoyl chloride (0.141 mL, 0.813 mmol, 2.20 eq.) was added dropwise, then the reaction mixture was further stirred for 21 h. After the reaction, THF was removed under reduced pressure, then the mixture was acidified by addition of aqueous 6 M HCl solution. The mixture was extracted with EtOAc 3 times, dried over anhydrous MgSO₄, filtered, then concentrated under reduced pressure. Diacylated peptide C₈-CG was afforded (62.2 mg, 39%) as a white powder after purification by flash column chromatography (SiO₂, hexane/EtOAc 90:10 → hexane/EtOAc 50:50). ¹H NMR (500 MHz, CDCl₃): δ 7.54 (brs, 1H), 7.07 (d, J = 8.0 Hz, 1H), 4.86 – 4.67 (m, 1H), 4.16 – 3.92 (m, 2H), 3.28 (d, J = 6.6 Hz, 2H), 2.56 (t, J = 7.6 Hz, 2H), 2.31 – 2.12 (m, 2H), 1.69 – 1.49 (m, 4H), 1.31 – 1.15 (m, 16H), 0.92 – 0.77 (m, 6H). ¹³C NMR (125 MHz, CDCl₃): δ 200.9, 175.4, 172.1, 170.5, 53.3, 44.2, 41.6, 36.4, 31.8, 31.7, 30.6, 29.3, 29.1, 29.0, 25.8, 25.7, 22.8, 22.7, 14.2, 14.2. HRMS (ESI-TOF) calculated for C₂₁H₃₈N₂O₅S [M-H]⁻: 429.2429, found 429.2432.

Synthesis of C₈-CT using a prebiotically plausible coupling agent



To a stirred solution of thiocholine chloride (38.9 mg, 0.250 mmol, 2.00 eq.) and dicyandiamide (52.8 mg, 0.625 mmol, 5.00 eq.) in 0.1 M aqueous HCl solution (0.50 mL), octanoic acid (20.2 μL , 0.125 mmol, 1.00 eq.) was added, then the reaction mixture was stirred at 50 $^\circ\text{C}$. After 24 hours of reaction, 10 μL of the reaction mixture was diluted with 990 μL H₂O/MeOH (v/v 1:1), then filtered through 0.45 μm PTFE filter. C₈-CT was detected by LCMS analysis using the TIC signal.

LCMS analysis of the reaction mixtures of Cys and C₇- or C₆-CT

Stock solution A: 5 mM Cys in 0.1 M, pH 8.0 sodium phosphate buffer containing 10 mM TCEP·HCl

Stock solution B: 10 mM C₇-CT (or C₆-CT) in 0.1 M, pH 8.0 sodium phosphate buffer containing 10 mM TCEP·HCl

Stock solution A and B were prepared right before the experiment. To 500 μL of the stock solution A in the glass vial, 500 μL of the stock solution B was added, then the reaction mixture was tumbled at RT (final concentration of Cys = 2.5 mM, C₇-CT (or C₆-CT) = 5 mM). After 2 hours of reaction, 80 μL of the reaction mixture was diluted with 240 μL MeOH, then filtered through 0.45 μm PTFE filter. The diacylated product was detected by LCMS analysis using the ELSD signal.

Cryogenic electron microscopy (cryo-EM) of C₈-Cys vesicles

Cryo-TEM grids (Quantifoil 200 mesh coated with thin carbon film, Ted Pella) were glow-discharged using a PELCO easiGlowTM for 30 s. 2.5 μL of a 1 mM C₈-Cys dispersion of sample was applied to the grids, which were blotted for 2 s with filter paper and plunged into liquid ethane using a manual plunger. The images were acquired on a FEI Tecnai TF20 high resolution Transmission Electron Microscope at an accelerating voltage of 200 kV, equipped with a K2 Direct Detection Camera, and collected with a total dose of 20 e/ $\text{\AA}^2$ at 2.36 $\text{\AA}/\text{pixel}$ and a 4 μm nominal defocus.

Encapsulation of fluorescent dyes

Encapsulation of HPTS and PI

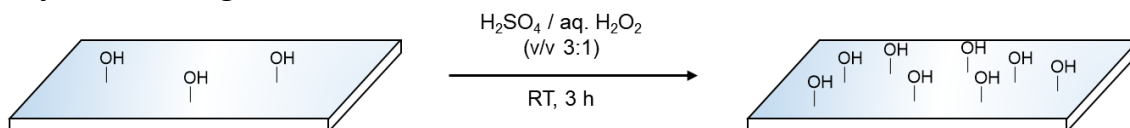
A thin film of diacylcysteine C₈-Cys was prepared by dissolving 4 mg of C₈-Cys in a minimal amount of chloroform, followed by drying by Ar blowing. The films were further dried in air at RT for several hours. Subsequently, a 25 mM C₈-Cys solution was prepared by film hydration in 0.1 M, pH 8.0 sodium phosphate buffer containing 4 mM TCEP·HCl and 1 mM HPTS or PI. After incubation at RT for overnight, 5 µL of the C₈-Cys solution was diluted with 245 µL of 0.1 M, pH 8.0 sodium phosphate buffer containing 4 mM TCEP·HCl (final concentration of C₈-Cys = 0.5 mM). The resulting samples were analyzed by bright field and fluorescence microscopy.

Encapsulation of sfGFP

A thin film of diacylcysteine C₈-Cys was prepared by dissolving 0.4 mg of C₈-Cys in a minimal amount of chloroform, followed by drying by Ar blowing. The films were further dried in air at RT for several hours. Subsequently, a 25 mM C₈-Cys solution was prepared by film hydration in 0.1 M, pH 8.0 sodium phosphate buffer containing 4 mM TCEP·HCl and 5 µM sfGFP. After incubation at RT overnight, 2 µL of the C₈-Cys solution was diluted with 98 µL of 0.1 M, pH 8.0 sodium phosphate buffer containing 4 mM TCEP·HCl (final concentration of C₈-Cys = 0.5 mM). The resulting samples were analyzed by bright field and fluorescence microscopy.

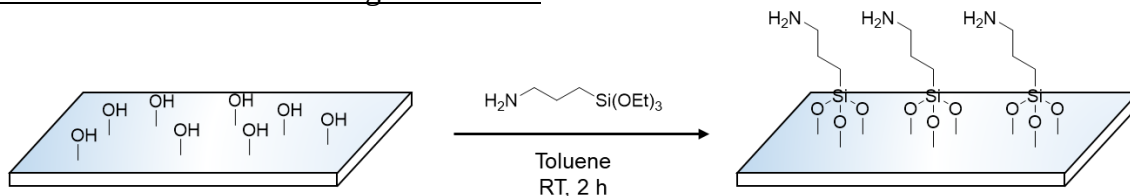
Chemical modification of glass surfaces

Hydroxylation of the glass surface⁵



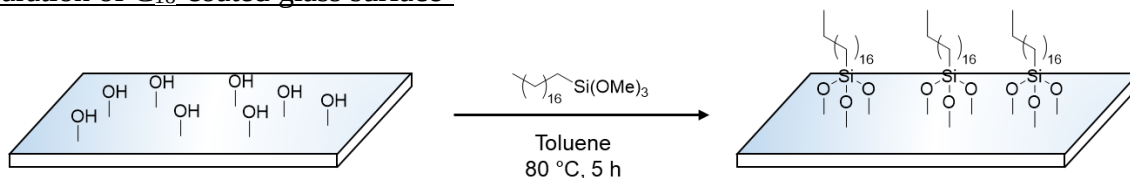
Piranha solution was prepared right before the experiment by mixing H_2SO_4 and ~30% aqueous H_2O_2 (v/v 3:1). Glass slides and coverslips were immersed in the piranha solution and incubated at RT. After 3-h incubation, the glass slides and coverslips were thoroughly washed by DIW and stored in DIW until further modification.

Preparation of APTES-modified glass surface⁵



The piranha-treated glass slides and coverslips were completely dried by Ar blowing. Subsequently, the glass substrates were immersed in 2 wt% APTES solution in toluene, then incubated at RT for 2 h. The slides were rinsed with toluene, MeOH and DIW, then dried *in vacuo*.

Preparation of C_{18} -coated glass surface⁶



The piranha-treated glass slides and coverslips were completely dried by Ar blowing. To a flame-dried round bottom flask (RBF), the glass substrates were added, then the RBF was flushed with Ar. Subsequently, the glass substrates were immersed in 10 wt% ODTMS solution in anhydrous toluene, and incubated at 80 °C for 5 h under Ar atmosphere. The slides were rinsed with toluene, then dried *in vacuo*.

Effect of the precursor ratios on vesicle formation

In situ vesicle formation on the glass surface with various precursor ratios

Stock solution A: Cys at twice the indicated final concentration in 0.1 M, pH 8.0 sodium phosphate buffer containing 4 mM TCEP·HCl

Stock solution B: C₈-CT at twice of the indicated final concentration in 0.1 M, pH 8.0 sodium phosphate buffer containing 4 mM TCEP·HCl

Stock solution A and B were prepared right before the experiment. 2 μ L of stock solution A was carefully pipetted onto a glass slide, followed by addition of 2 μ L of the stock solution B. The solution was gently mixed by pipetting up and down, then covered with a glass coverslip. The resulting mixture was monitored by phase contrast microscopy to analyze the *in situ* formation of vesicles.

Film hydration of C₈-Cys in the presence of additional C₈ precursors

C₈-Cys and C₈-CT (or octanoic acid or octanol) were added to a glass vial in the indicated molar ratios, dissolved in a minimal amount of MeOH, then a dry thin film was prepared by gently blowing with Ar. The films were further dried for several hours, then hydrated by addition of 0.1 M, pH 8.0 sodium phosphate buffer (final concentration of C₈-Cys = 1 mM). The resulting mixture was tumbled at RT for 1 h, then phase contrast microscopy images were obtained.

Addition of Cys to the preformed mixed membrane of C₈-Cys and C₈-CT

C₈-Cys and C₈-CT were added to a glass vial in a molar ratio of 1:4, dissolved in a minimal amount of MeOH, then a thin dry film was prepared by gently blowing with Ar. The film was further dried for several hours, then hydrated by addition of 0.1 M, pH 8.0 sodium phosphate buffer containing 10 mM TCEP·HCl (concentration of C₈-Cys = 2 mM, C₈-CT = 8 mM). After tumbling at RT for 1 h, 40 μ L of the mixture was aliquoted and added to 40 μ L of 8 mM Cys solution in 0.1 M, pH 8.0 sodium phosphate buffer containing 10 mM TCEP·HCl (final concentration of C₈-Cys = 1 mM, C₈-CT = 4 mM, Cys = 4 mM). The resulting mixture was tumbled at RT, then phase contrast microscopy images were obtained after the indicated time points.

Effect of the ionic strength on vesicle formation

In situ vesicle formation on the glass surface in the presence of NaCl

Stock solution A: 2 mM Cys in 0.1 M (or 0.01 M), pH 8.0 sodium phosphate buffer containing 4 mM TCEP·HCl

Stock solution B: 4 mM C₈-CT and NaCl at twice of the indicated final concentration in 0.1 M (or 0.01 M), pH 8.0 sodium phosphate buffer containing 4 mM TCEP·HCl

Stock solution A and B were prepared right before the experiment. 2 µL of the stock solution A was carefully pipetted onto a glass slide, followed by addition of 2 µL of the stock solution B. The solution was gently mixed by pipetting up and down, then covered by a glass coverslip. The resulting mixture was monitored by phase contrast microscopy to analyze the *in situ* formation of vesicles and glass adhered membranes (final concentration of Cys = 1 mM, C₈-CT = 2 mM).

Evaluation of Laurdan generalized polarization (GP) during *in situ* vesicle formation on the glass surface

Stock solution A: 2 mM Cys in 0.1 M, pH 8.0 sodium phosphate buffer containing 4 mM TCEP·HCl and 5 µM Laurdan

Stock solution B: 4 mM C₈-CT and NaCl at twice of the indicated final concentration in 0.1 M, pH 8.0 sodium phosphate buffer containing 4 mM TCEP·HCl

Stock solution A and B were prepared right before the experiment. To the 100 µL of stock solution A in a 1.5 mL Eppendorf tube, 100 µL of stock solution B was added, then the reaction mixture was gently vortexed for a few seconds. Subsequently, 80 µL of the mixture was transferred to a glass-bottomed 96-well plate and analyzed on a Tecan Infinite plate reader (final concentration of Cys = 1 mM, C₈-CT = 2 mM). The samples were excited at 364 nm and emission spectra were acquired over 400 – 600 nm every 5 minutes for a period of 2 hours. Generalized polarization (GP) was calculated as follows:

$$GP = \frac{I_{440} - I_{490}}{I_{440} + I_{490}}$$

Vesicle formation from lipopeptides

Stock solution A: 5 mM peptide in 0.1 M, pH 8.0 sodium phosphate buffer containing 10 mM TCEP·HCl and 1 µM Nile Red.

Stock solution B: 10 mM choline thioester in 0.1 M, pH 8.0 sodium phosphate buffer containing 10 mM TCEP·HCl and 1 µM Nile Red.

Stock solution A and B were prepared right before the experiment. To 100 µL of stock solution A in the glass vial, 100 µL of the stock solution B was added, then the reaction mixture was tumbled at RT. After the indicated time points, 2 µL of the reaction mixture was dispensed on the glass slide, covered by a glass coverslip, then phase contrast, bright field and fluorescence microscopy images were obtained (final concentration of peptide = 2.5 mM, choline thioester = 5 mM).

Stability of C₈-Cys vesicles toward divalent cations

A 5 mM solution of C₈-Cys in MeOH was added to a glass vial, and then a dry thin film was prepared by gently blowing with Ar. The film was further dried under Ar atmosphere for at least 4 h, then hydrated by addition of 0.1 M, pH 8.0 sodium phosphate buffer containing CaCl₂ or MgCl₂ of indicated concentration (final concentration of C₈-Cys = 2.5 mM). The resulting mixture was tumbled at RT for overnight, and the presence of vesicles was evaluated by phase contrast microscopy.

***In situ* C₈-Cys vesicle formation in aminoacylation ribozyme buffer**

Stock solution A: 40 mM Cys in aminoacylation ribozyme buffer (0.1 M HEPES, 0.1 M NaCl, 0.1 M KCl, 5 mM MgCl₂, pH 7.0) with CaCl₂ (5, 2 or 0 mM).

Stock solution B: 80 mM C₈-CT in aminoacylation ribozyme buffer (0.1 M HEPES, 0.1 M NaCl, 0.1 M KCl, 5 mM MgCl₂, pH 7.0) with CaCl₂ (5, 2 or 0 mM).

Stock solution A and B were prepared right before the experiment. To 100 µL of stock solution A in a glass vial, 100 µL of stock solution B was added, then the reaction mixture was tumbled at 37 °C (final concentration of Cys = 20 mM, C₈-CT = 40 mM). After overnight incubation, vesicle formation was confirmed by phase contrast microscopy.

Stability of the mixed vesicles of C₈-Cys and monoacylglycerols in aminoacylation ribozyme buffer

50 µL of a solution containing 80 mM C₈-Cys and monoacylglycerols in various molar ratios in MeOH/chloroform (v/v 2:1) was added to a glass vial, then dry thin films were prepared by gentle Ar blowing. The films were further dried under an Ar atmosphere overnight, then hydrated by addition of 200 µL of aminoacylation ribozyme buffer (0.1 M HEPES, 0.1 M NaCl, 0.1 M KCl, 5 mM MgCl₂, pH 7.0) (final concentration of C₈-Cys = 20 mM). The resulting mixtures were tumbled at 37 °C overnight, and the stability of the vesicles was evaluated by phase contrast microscopy.

Stability of the mixed vesicles of C₈-Cys and monoacylglycerols toward mechanical force

Spin filtration of vesicles³

Mixed vesicles of 80 mM C₈-Cys and monoacylglycerols (in various molar ratios) in aminoacylation buffer (0.1 M HEPES, 0.1 M NaCl, 0.1 M KCl, 5 mM MgCl₂, pH 7.0) were prepared by film hydration following the previously discussed procedure. Afterward, 50 μ L of the resulting solution was further diluted with an additional 150 μ L of aminoacylation buffer and transferred to a 100 kDa molecular weight cut-off (MWCO) centrifugal membrane filter (Millipore Sigma) and centrifuged at 800 g for 30 min. Then, the solution was diluted and centrifuged with 150 μ L of aminoacylation buffer for additional 4 times. Stability of the vesicles was evaluated by phase contrast microscopy.

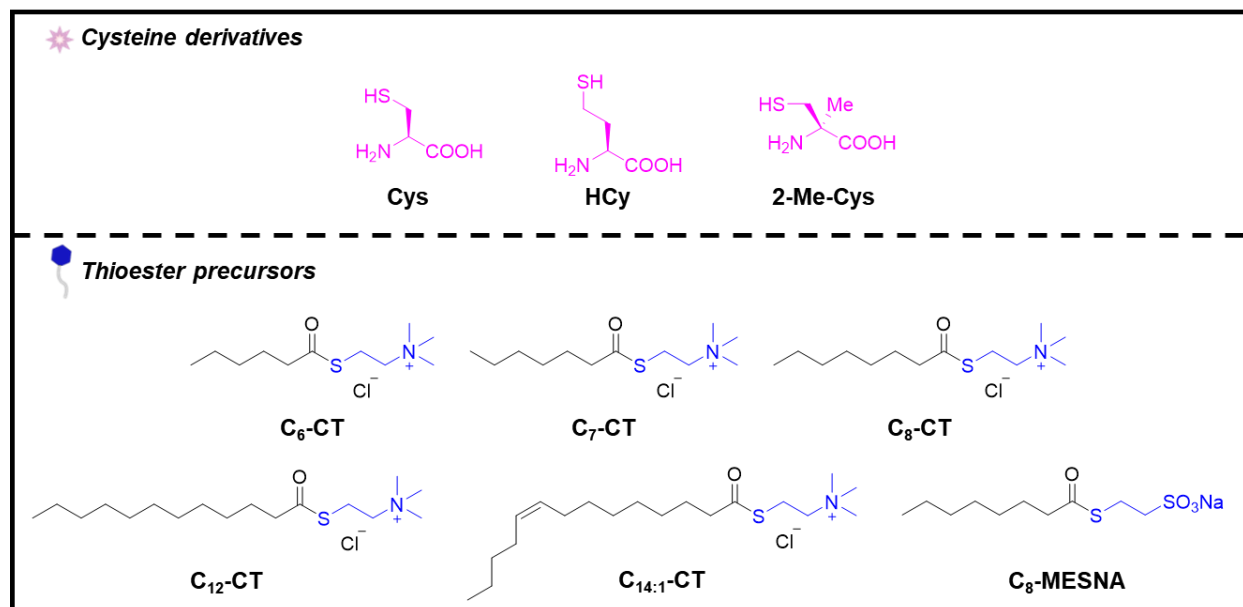
Size exclusion column

Mixed vesicles of 80 mM C₈-Cys and monoacylglycerols (in various molar ratios) in aminoacylation buffer (0.1 M HEPES, 0.1 M NaCl, 0.1 M KCl, 5 mM MgCl₂, pH 7.0) with or without 5 mM CaCl₂ were prepared by film hydration following the previously discussed procedure. Afterward, 50 μ L of the resulting solution was further diluted with an additional 150 μ L of aminoacylation buffer and transferred to a Sephadex G-25 size exclusion column and centrifuged at 800 g for 2 min. Then, the collected solution was transferred to another column and centrifuged and the process was repeated an additional 3 times. Stability of the vesicles was evaluated by phase contrast and fluorescence microscopy. For the fluorescence microscopy experiment, 20 μ M Nile Red was added to the mixture.

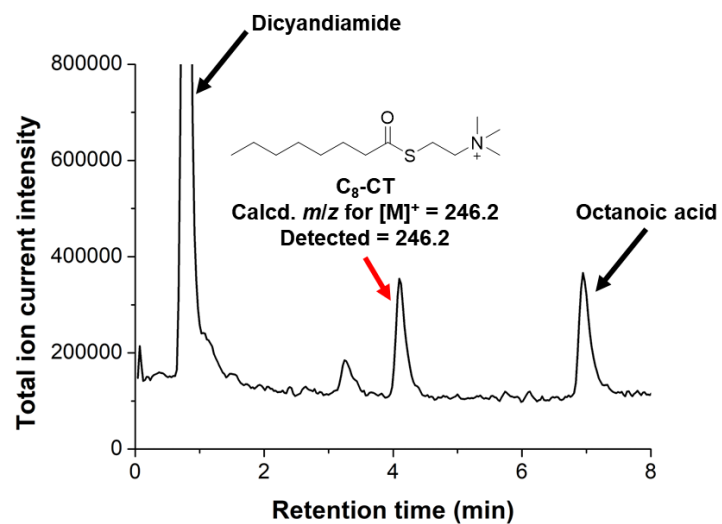
Encapsulation of fluorescent dyes into the mixed vesicles of C₈-Cys and C₁₂-*rac*-glycerol

The mixed vesicles of 20 mM C₈-Cys and C₁₂-*rac*-glycerol in 1:0.5 molar ratio in aminoacylation buffer (0.1 M HEPES, 0.1 M NaCl, 0.1 M KCl, 5 mM MgCl₂, pH 7.0) containing 0.1 mM HPTS or PI were prepared by film hydration following the previously discussed procedure. To remove unencapsulated dye, 200 μ L of the vesicle solution was transferred to a Sephadex G-25 size exclusion column and centrifuged at 800 g for 2 min. Then, the resulting solution was collected and transferred to another column and centrifuged and this process was repeated an additional 2 times. The resulting samples were analyzed by phase contrast and fluorescence microscopy.

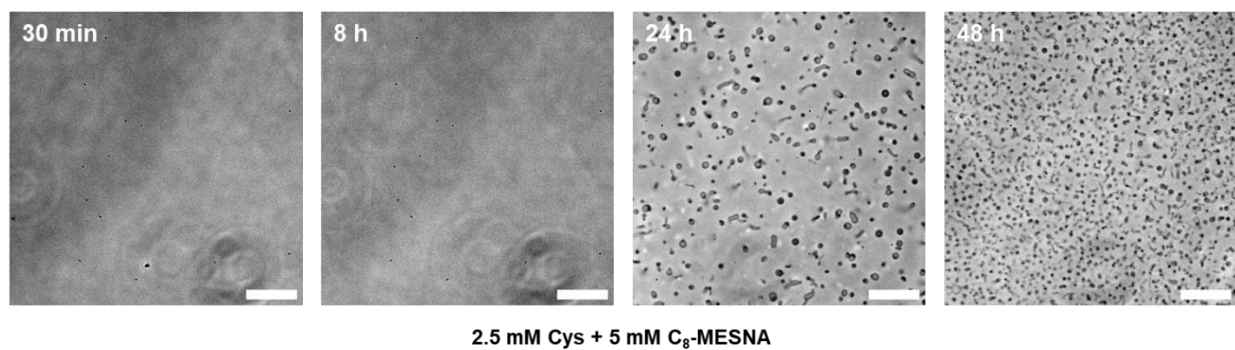
Supplementary Figures



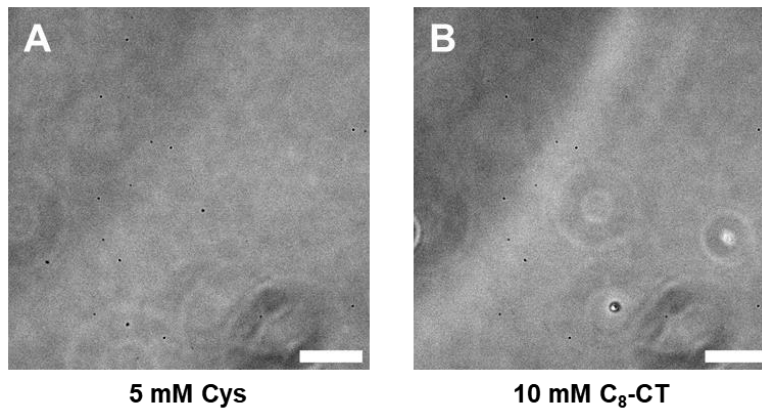
Supplementary Fig. 1. Chemical structures of the cysteine derivatives and thioesters used in this study.



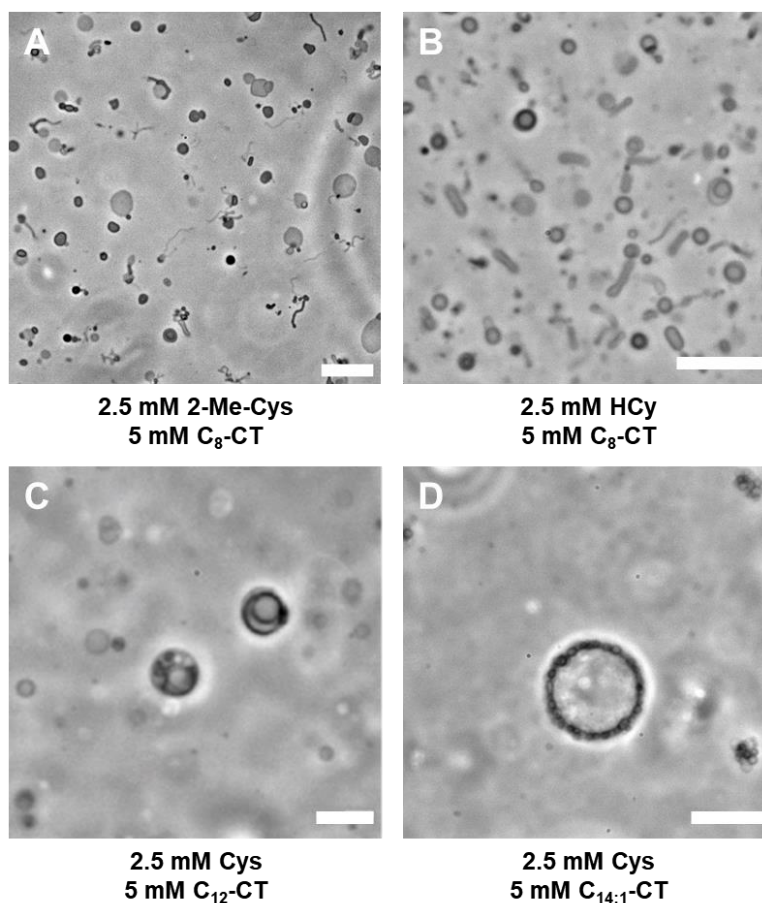
Supplementary Fig. 2. LCMS chromatogram of the reaction mixture of 0.25 M octanoic acid, 0.5 M thiocholine chloride and 1.25 M dicyandiamide.



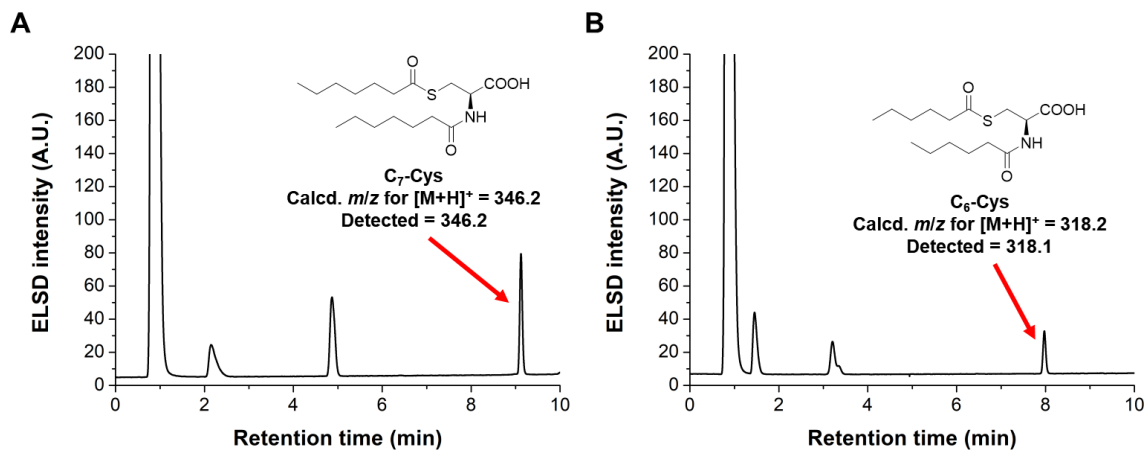
Supplementary Fig. 3. Phase contrast microscopy images of the reaction mixtures of 2.5 mM Cys and 5 mM C₈-MESNA in 0.1 M, pH 8.0 sodium phosphate buffer. Scale bars: 25 μ m.



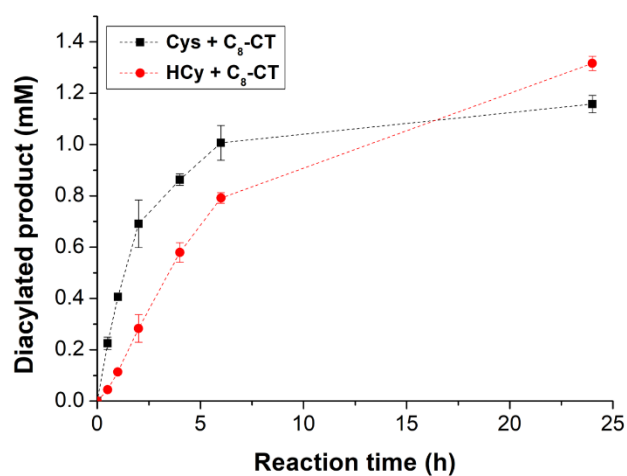
Supplementary Fig. 4. Phase contrast microscopy images of solutions of **(a)** 5 mM Cys and **(b)** 10 mM C₈-CT in 0.1 M, pH 8.0 sodium phosphate buffer after 2-h incubation. Scale bars: 25 μ m.



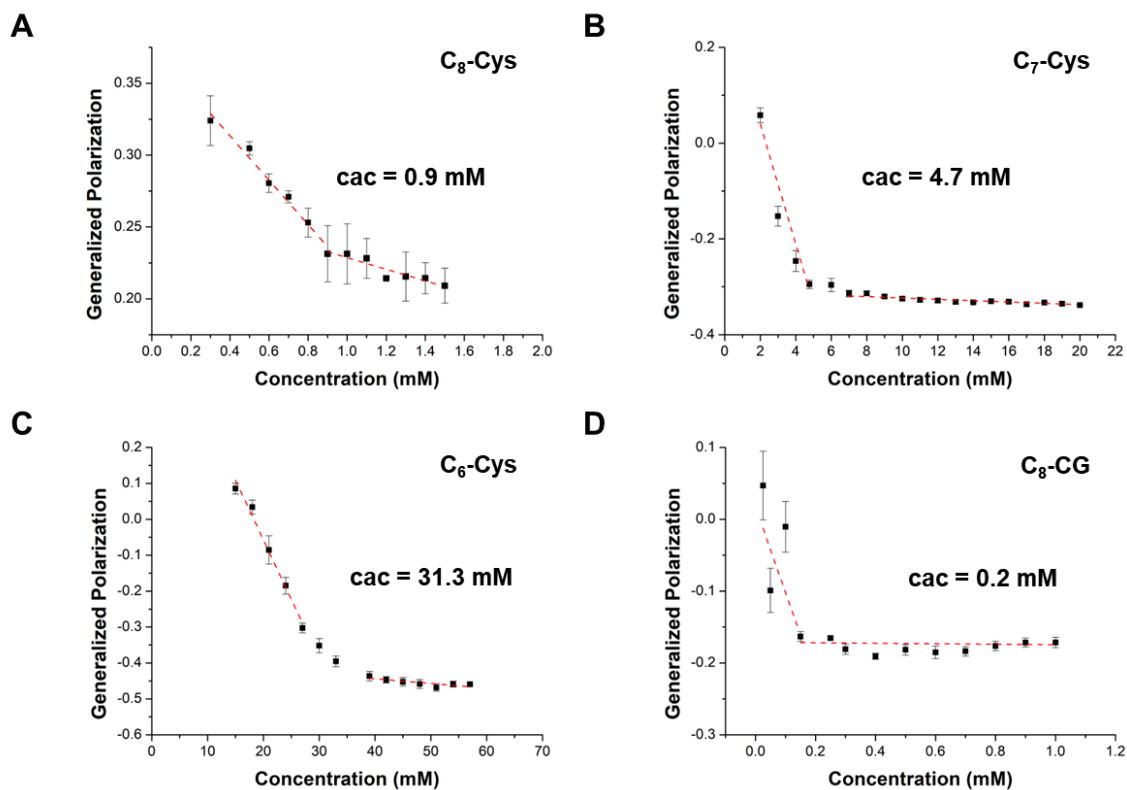
Supplementary Fig. 5. Phase contrast microscopy images of the reaction mixtures of (a) 2.5 mM 2-Me-Cys and 5 mM C₈-CT, (b) 2.5 mM HCy + 5 mM C₈-CT after 2-h incubation and (c) 2.5 mM Cys and 5 mM C₁₂-CT, (d) 2.5 mM Cys and 5 mM C_{14:1}-CT after overnight incubation in 0.1 M, pH 8.0 sodium phosphate buffer. Scale bars: 20 μm.



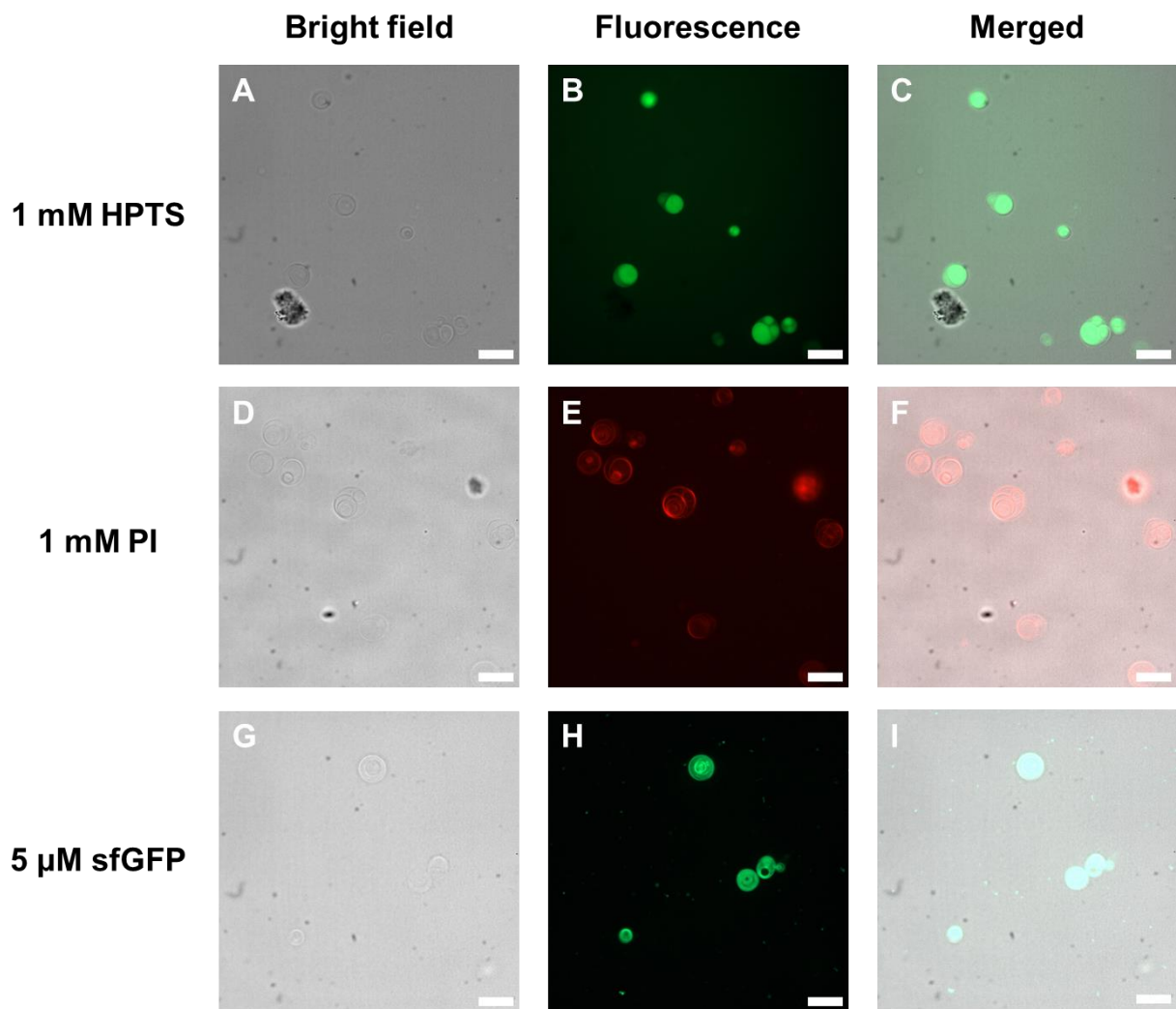
Supplementary Fig. 6. LCMS chromatogram of the reaction mixture of 2.5 mM Cys and (a) 5 mM C₇-CT or (b) 5 mM C₆-CT.



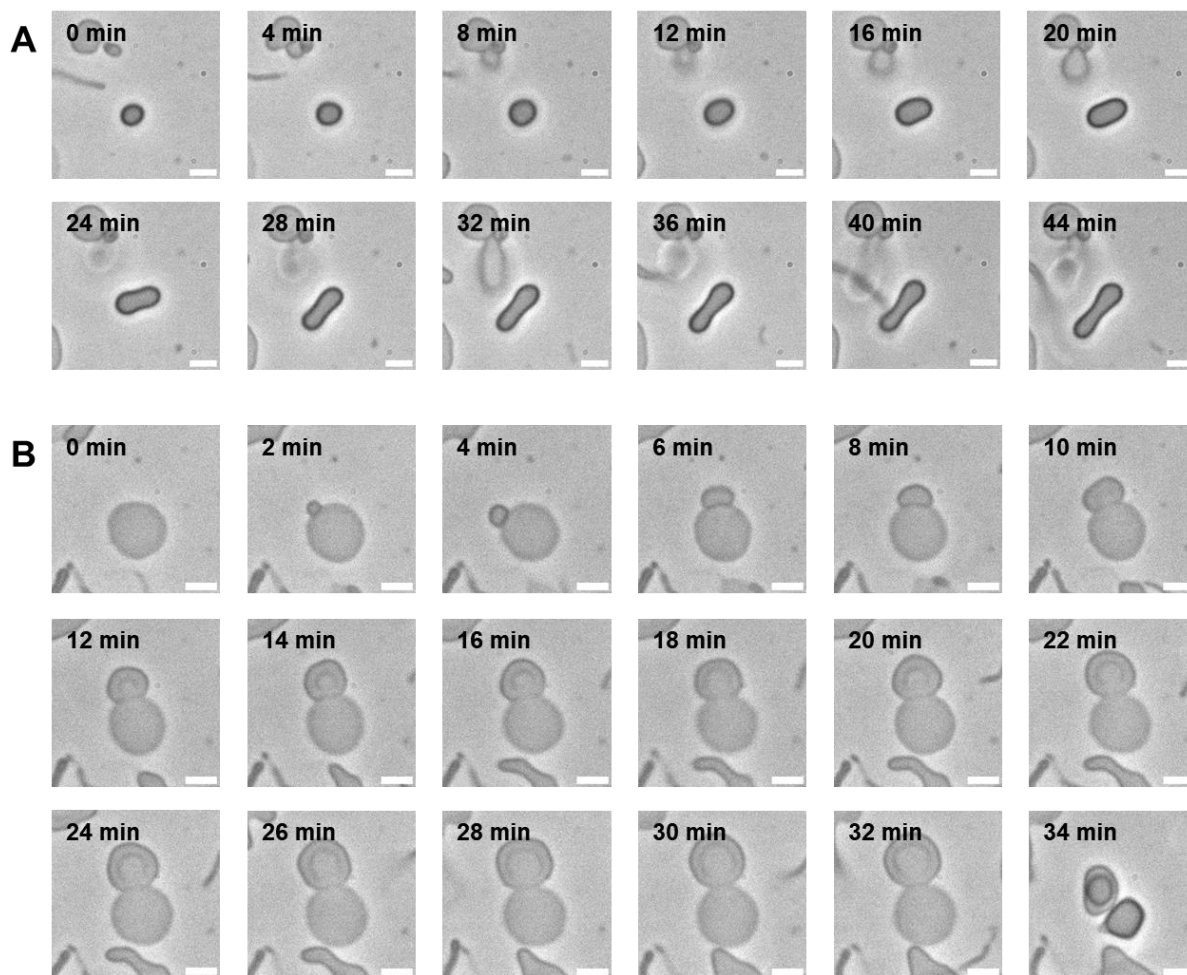
Supplementary Fig. 7. Comparison of the diacylation reaction kinetics of Cys and HCy. The reactions were carried out using 2.5 mM Cys or HCy and 5 mM C₈-CT in 0.1 M, pH 8.0 sodium phosphate buffer at RT. The products were quantified by LCMS using 230 nm absorbance. The data points are presented as means $\pm$ standard deviation ($n = 3$ technically independent samples).



Supplementary Fig. 8. Critical aggregation concentrations (cac) of (a) C₈-Cys, (b) C₇-Cys, (c) C₆-Cys and (d) C₈-CG. The data points are presented as means $\pm$ standard deviation ($n = 3$ technically independent samples).

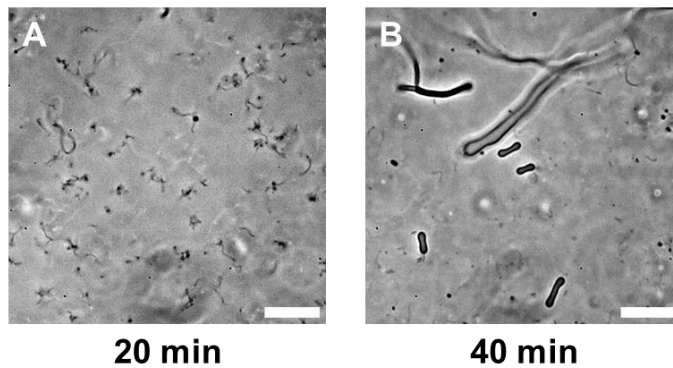


Supplementary Fig. 9. Bright field and fluorescence microscopy images of C₈-Cys vesicles containing (a–c) 1 mM HPTS, (d–f) 1 mM PI and (g–i) 5 μ M sfGFP. Scale bars: 25 μ m.



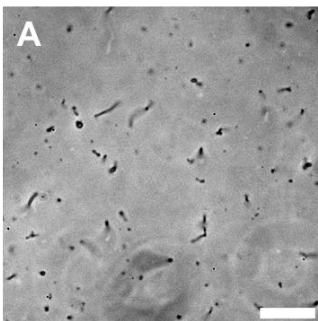
Supplementary Fig. 10. Representative phase contrast microscopy images showing (a) growth, (b) budding and fission of a C₈-Cys vesicle during the reaction of 1 mM Cys and 2 mM C₈-CT in 0.1 M, pH 8.0 sodium phosphate buffer. Scale bars: 5 μ m.

**1 mM CG
2 mM C₈-CT**

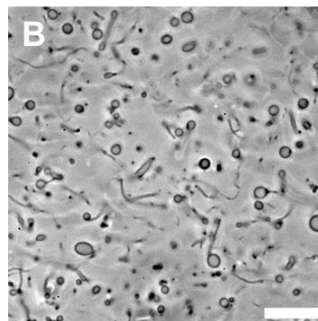


Supplementary Fig. 11. *In situ* C₈-CG membrane formation from 1 mM CG and 2 mM C₈-CT at RT on a glass surface. Phase contrast microscopy images were obtained after (a) 20-min and (b) 40-min incubation. Scale bars: 25 μ m.

**1 mM CGGG
2 mM C₈-CT**

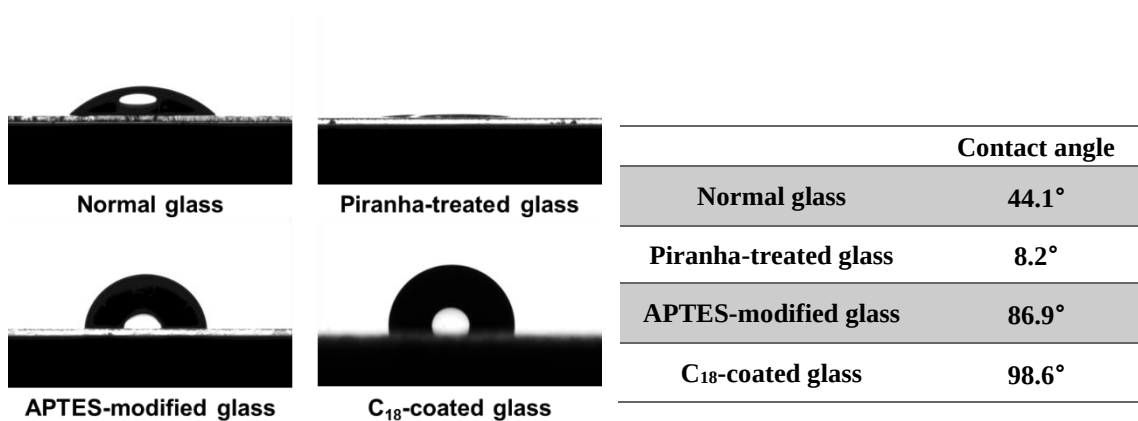


40 min

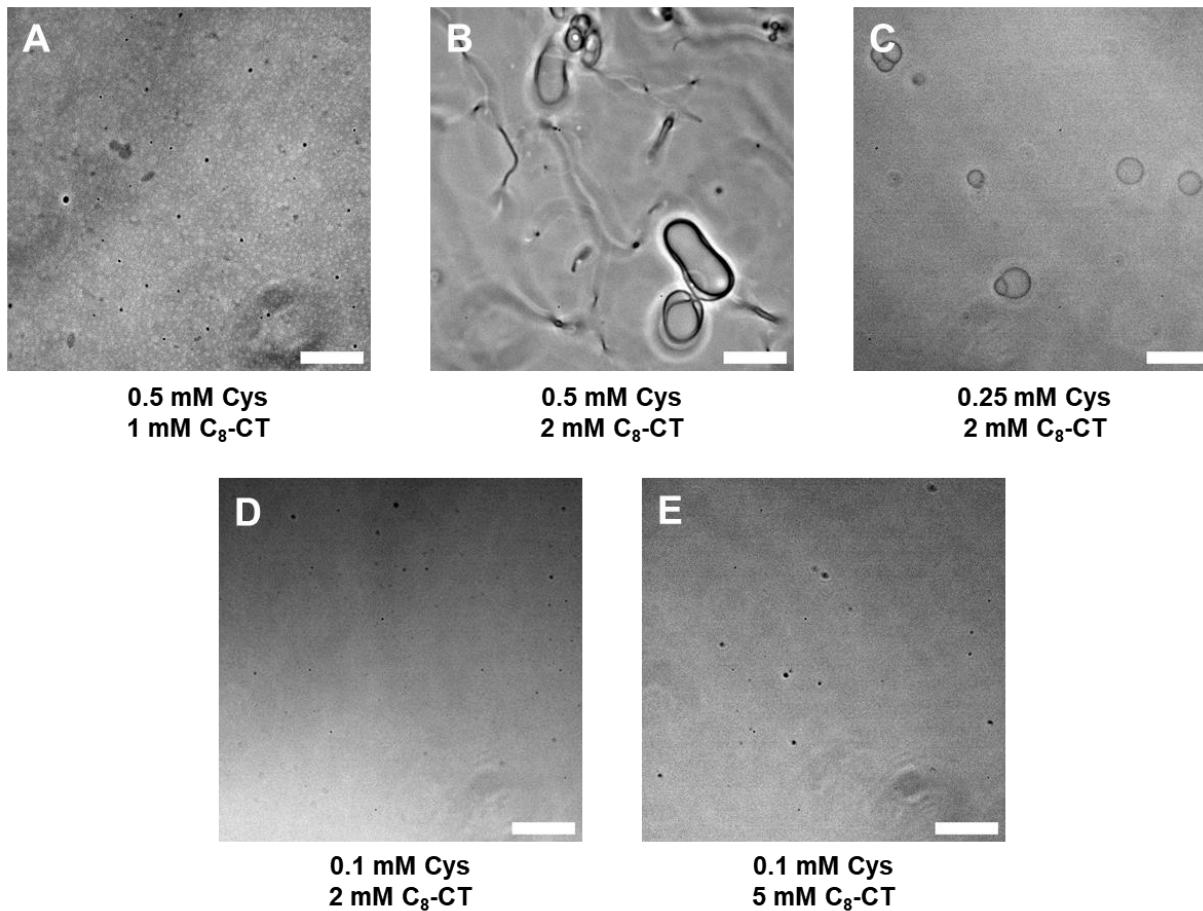


120 min

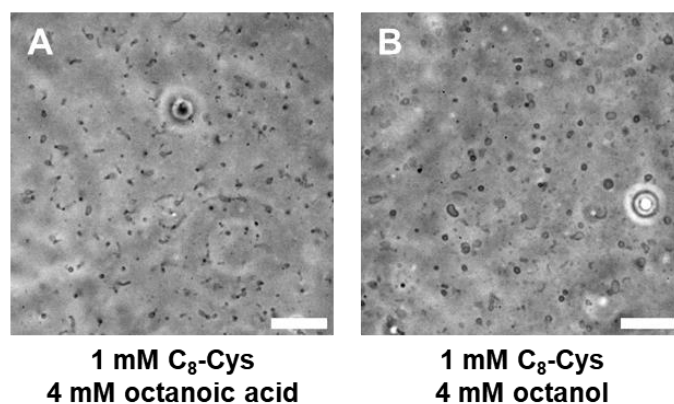
Supplementary Fig. 12. *In situ* C₈-CGGG membrane formation from 1 mM CGGG and 2 mM C₈-CT at RT on a glass surface. Phase contrast microscopy images were obtained after (a) 40-min and (b) 2-h incubation. Scale bars: 25 μ m.



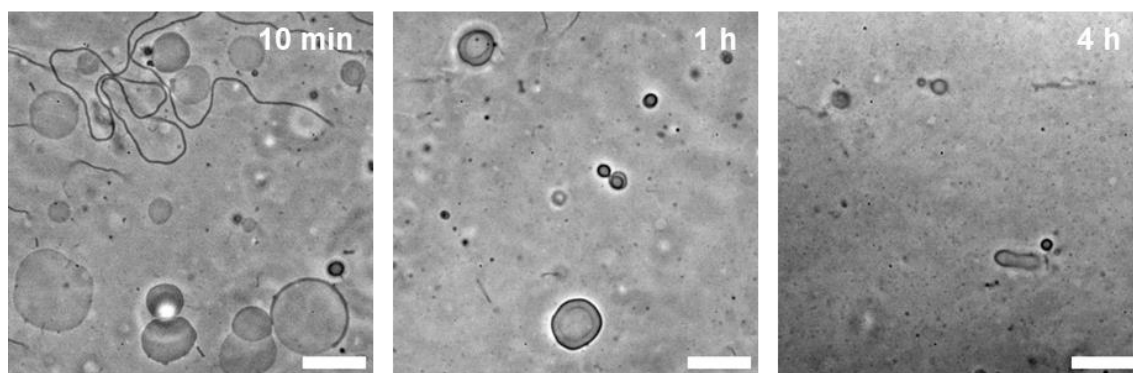
Supplementary Fig. 13. Water contact angles on the glass surfaces before and after chemical modification.



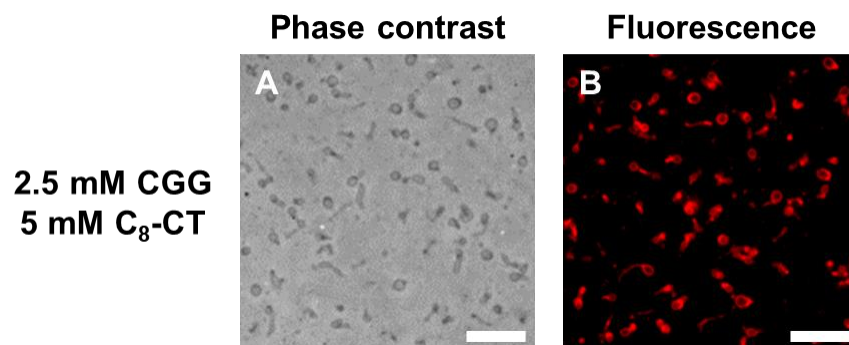
Supplementary Fig. 14. *In situ* C₈-Cys membrane formation using submillimolar concentrations of Cys and various concentrations of C₈-CT at RT on a glass surface. Phase contrast microscopy images were obtained after the indicated time points. **(a)** 0.5 mM Cys + 1 mM C₈-CT after 48 h. **(b)** 0.5 mM Cys + 2 mM C₈-CT after 2 h. **(c)** 0.25 mM Cys + 2 mM C₈-CT after 24 h. **(d)** 0.1 mM Cys + 2 mM C₈-CT after 24 h. **(e)** 0.1 mM Cys + 5 mM C₈-CT after 24 h. Scale bars: 25 μ m.



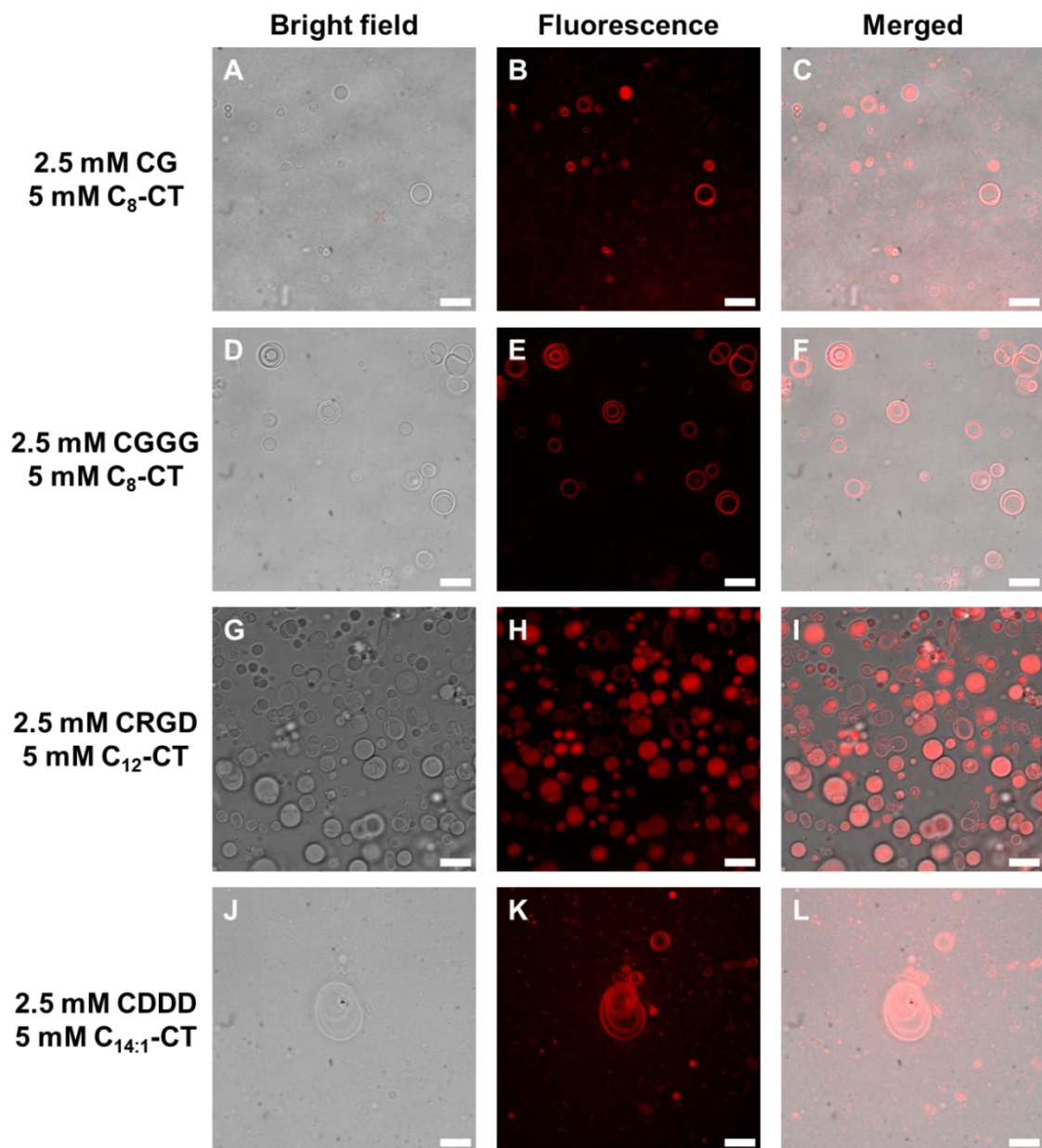
Supplementary Fig. 15. Film hydration of 1 mM C₈-Cys in 0.1 M, pH 8.0 sodium phosphate buffer at RT with (a) 4 mM octanoic acid or (b) 4 mM octanol. Phase contrast microscopy images were obtained after 1-h incubation. Scale bars: 25 μ m.



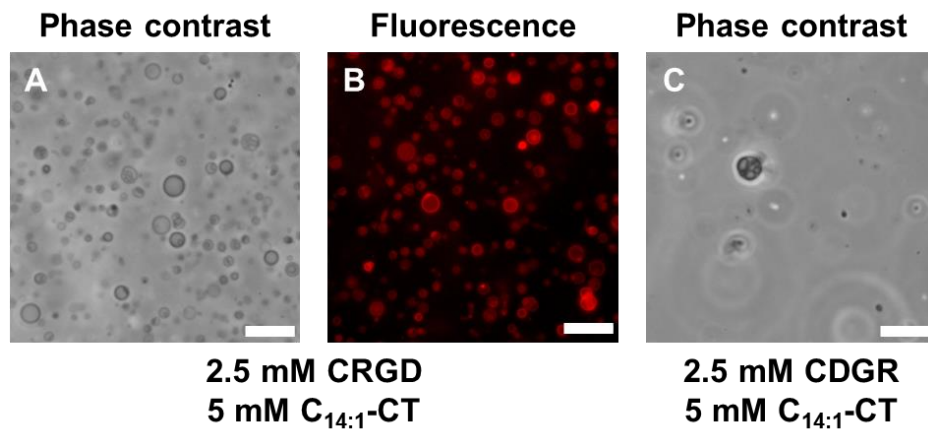
Supplementary Fig. 16. Disappearance of the flattened lipid structures on the glass surface as the reaction proceeds. Flattened membranes were produced by film hydration of 1 mM C₈-Cys in the presence of 4 mM C₈-CT. Subsequently 4 mM Cys was added to the mixture. The phase contrast microscopy images were obtained after the indicated time points. Scale bars: 25 μ m.



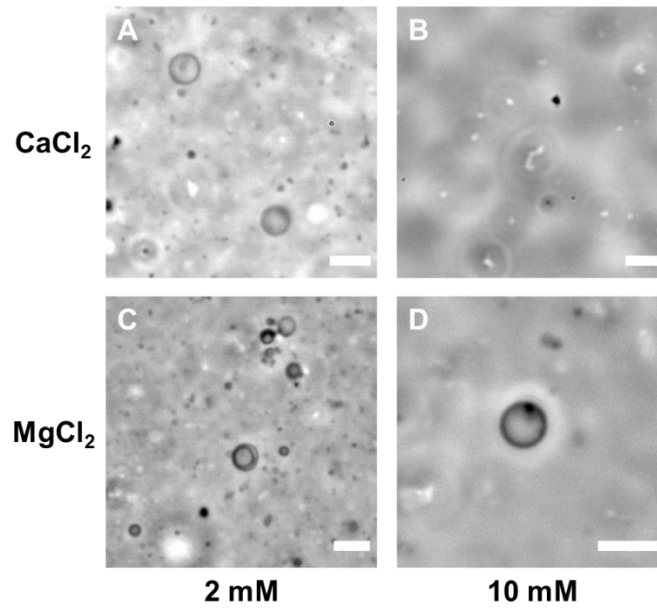
Supplementary Fig. 17. Phase contrast and fluorescence microscopy images of the reaction mixtures of 2.5 mM CGG and 5 mM C₈-CT after overnight incubation in 0.1 M, pH 8.0 sodium phosphate buffer containing 1 μ M Nile Red at RT. Scale bars: 20 μ m.



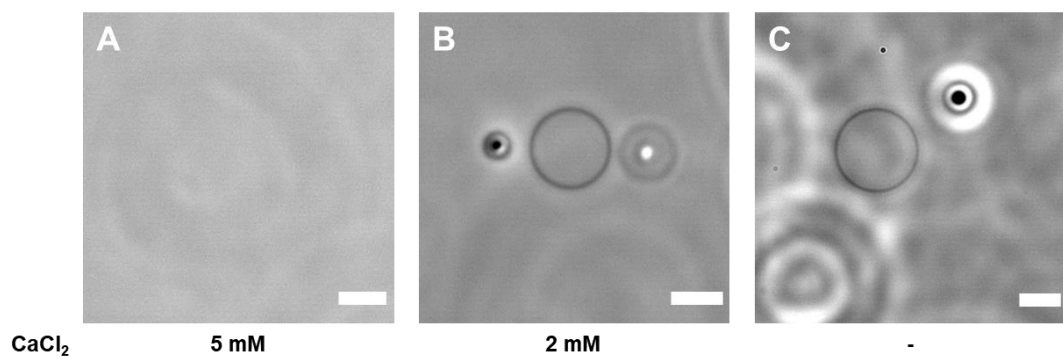
Supplementary Fig. 18. Bright field and fluorescence microscopy images of the diacylated peptide vesicles produced by in situ reactions in 0.1 M, pH 8.0 sodium phosphate buffer containing 1 μ M Nile Red at RT. (a–c) 2.5 mM CG + 5 mM C₈-CT after 5 h, (d–f) 2.5 mM CGGG + 5 mM C₈-CT after 5 h, (g–i) 2.5 mM CRGD + 5 mM C₁₂-CT after 2 h, (j–l) 2.5 mM CDDD + 5 mM C_{14:1}-CT after 48 h. Scale bars: 25 μ m.



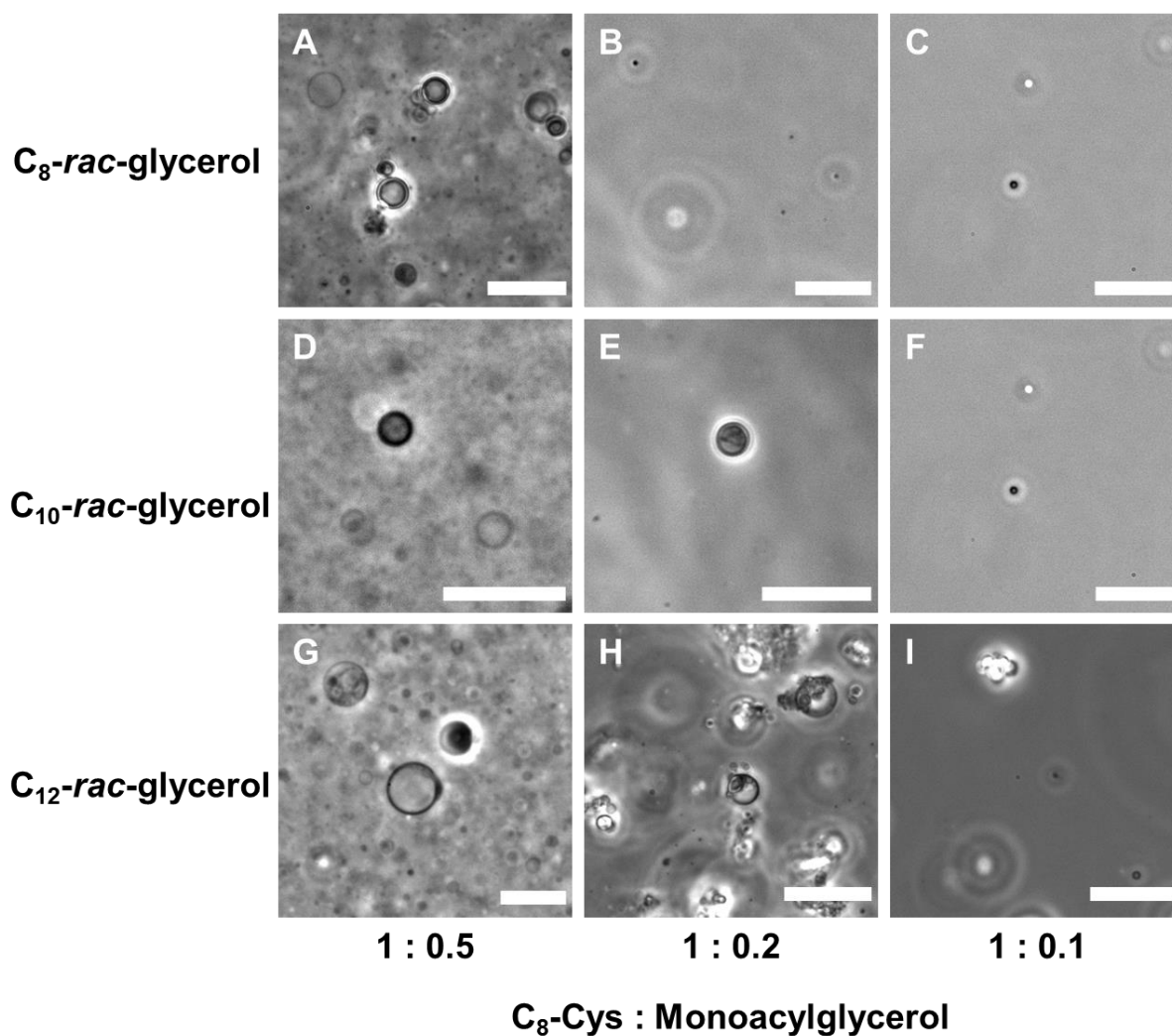
Supplementary Fig. 19. (a) Phase contrast and (b) fluorescence microscopy images of the reaction mixture of 2.5 mM CRGD and 5 mM C_{14:1}-CT and (c) phase contrast microscopy image of the reaction mixture of 2.5 mM CDGR and 5 mM C_{14:1}-CT after overnight incubation in 0.1 M, pH 8.0 sodium phosphate buffer containing 1 μM Nile Red at RT. Scale bars: 20 μm.



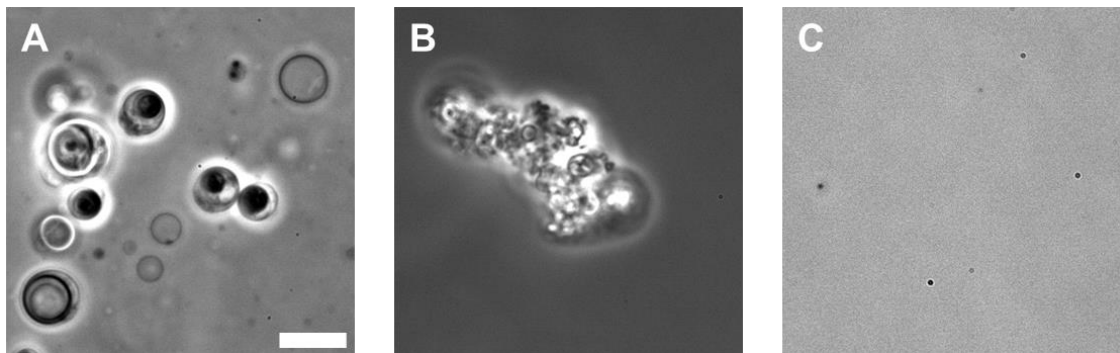
Supplementary Fig. 20. Phase contrast microscopy images of 2.5 mM C₈-Cys vesicles in 0.1 M, pH 8.0 sodium phosphate buffer containing (a) 2 mM CaCl₂, (b) 10 mM CaCl₂, (c) 2 mM MgCl₂ and (d) 10 mM MgCl₂. Scale bars: 10 μm.



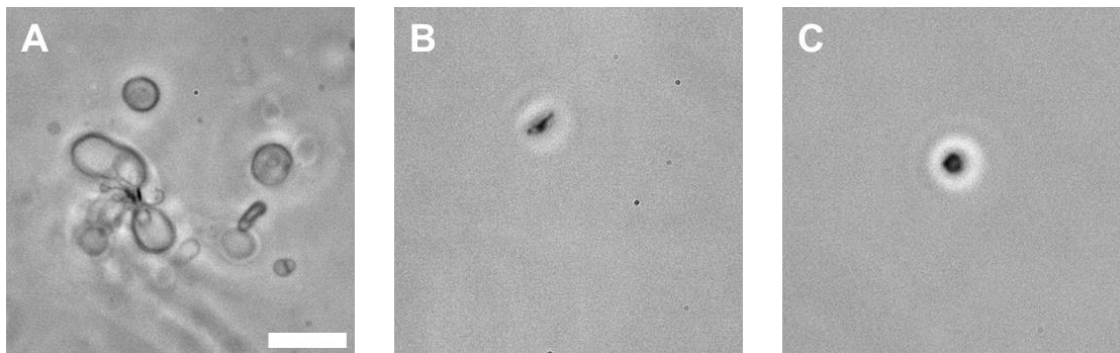
Supplementary Fig. 21. Phase contrast microscopy images of the reaction mixture of 20 mM Cys and 40 mM C₈-CT in aminoacylation ribozyme buffer containing (a) 5 mM, (b) 2 mM and (c) no CaCl₂. Scale bars: 10 μ m.



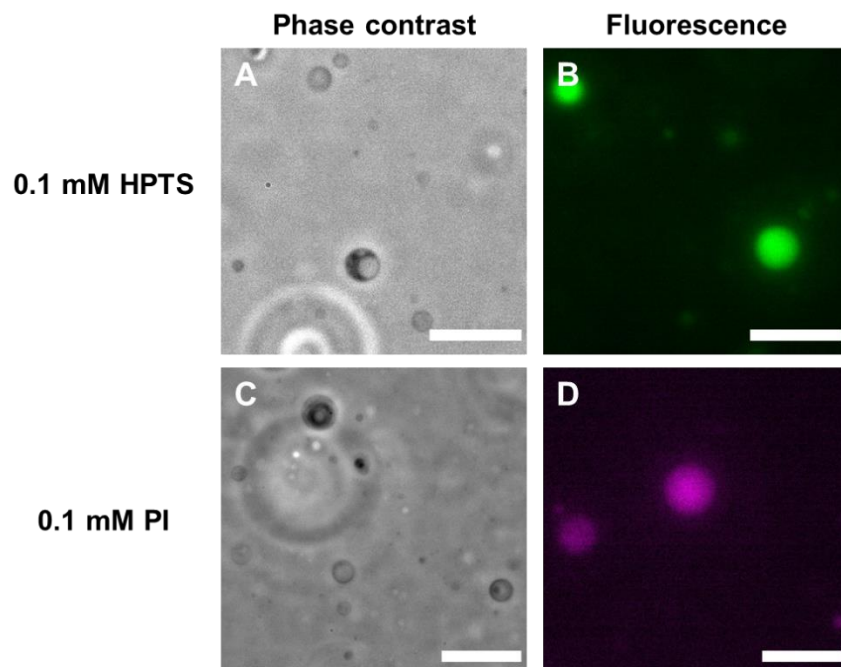
Supplementary Fig. 22. Phase contrast microscopy images of mixed vesicles formed from 20 mM C₈-Cys and monoacylglycerols in aminoacylation ribozyme buffer. The mixed vesicles consisted of C₈-Cys and (a–c) C₈-*rac*-glycerol in (a) 1:0.5, (b) 1:0.2 and (c) 1:0.1 molar ratios, (d–f) C₁₀-*rac*-glycerol in (d) 1:0.5, (e) 1:0.2 and (f) 1:0.1 molar ratios, (g–i) C₁₂-*rac*-glycerol with (g) 1:0.5, (h) 1:0.2 and (i) 1:0.1 molar ratios. Scale bars: 20 μ m.



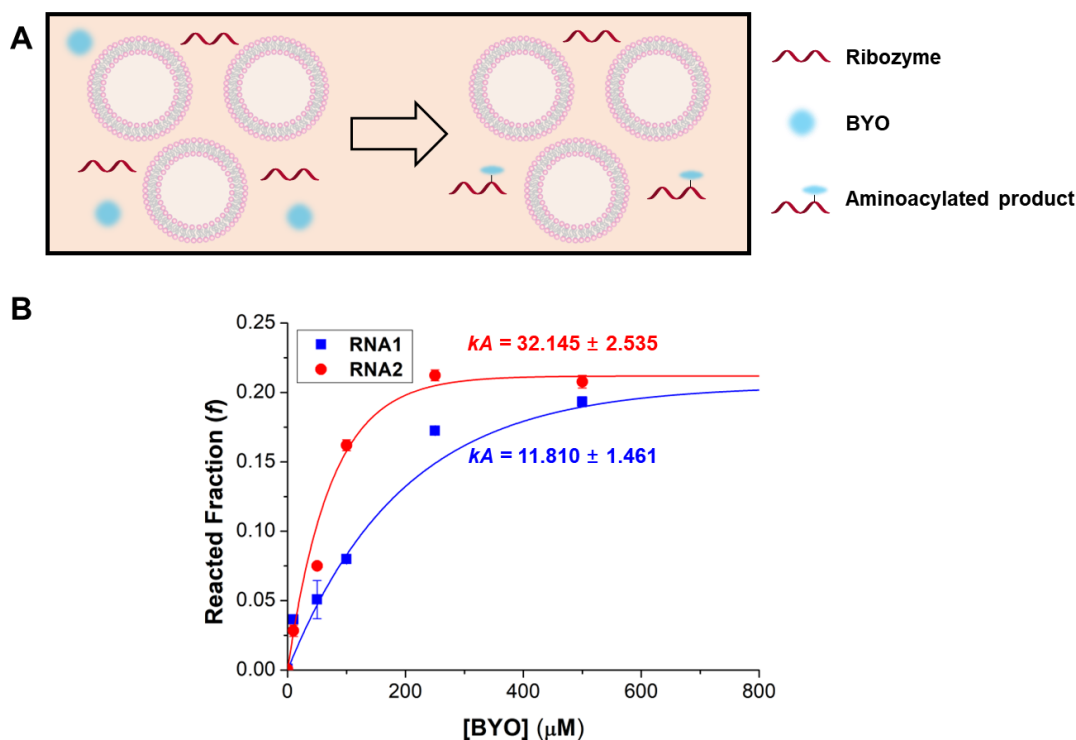
Supplementary Fig. 23. Phase contrast microscopy images of the mixed vesicles of 20 mM C₈-Cys and monoacylglycerols in aminoacylation ribozyme buffer after spin filtration. Mixed vesicles of C₈-Cys and (a, b) C₁₂-*rac*-glycerol in 1:0.5 and 1:0.2 molar ratio, respectively, and (c) C₁₀-*rac*-glycerol in 1:0.5 molar ratio. Scale bar: 20 μ m.



Supplementary Fig. 24. Phase contrast microscopy images of the mixed vesicles of 20 mM C₈-Cys and monoacylglycerols in aminoacylation ribozyme buffer after centrifugation through size exclusion column. The mixed vesicles of C₈-Cys and **(a, b)** C₁₂-*rac*-glycerol in 1:0.5 and 1:0.2 molar ratio, respectively, and **(c)** C₁₀-*rac*-glycerol in 1:0.5 molar ratio. Scale bar: 20 μ m.



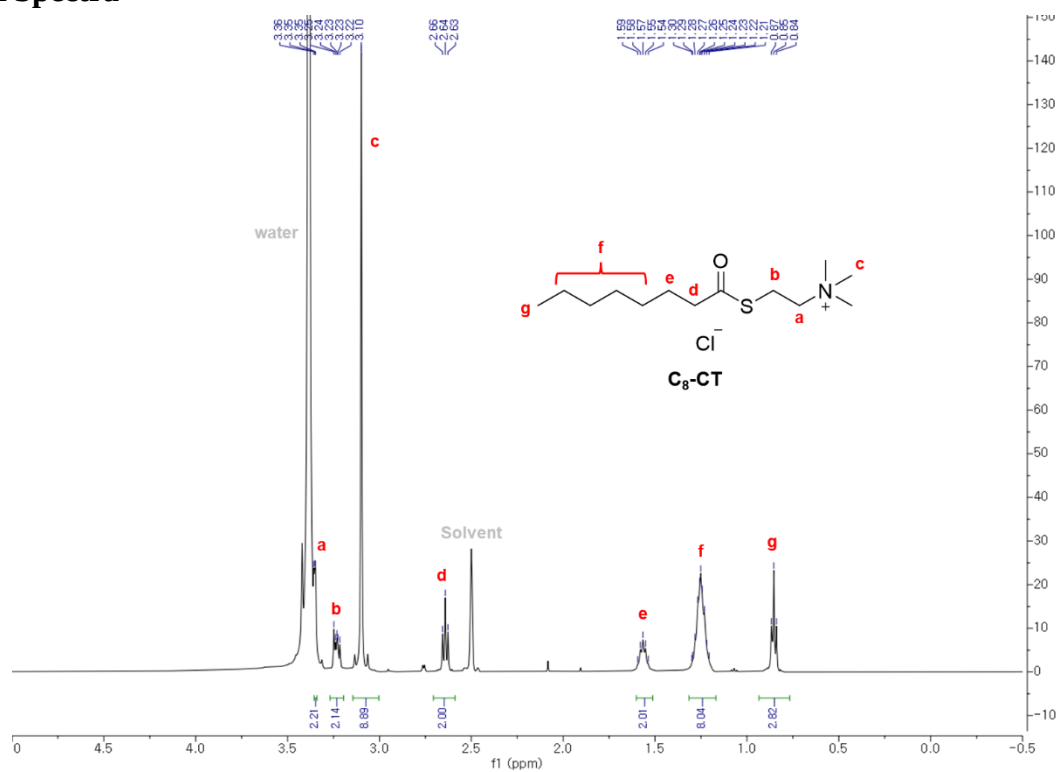
Supplementary Fig. 25. Encapsulation of fluorescent dyes in mixed vesicles of 20 mM C₈-Cys and C₁₂-*rac*-glycerol in 1:0.5 molar ratio. (a) Phase contrast and (b) fluorescence microscopy images of the vesicles encapsulating 0.1 mM HPTS. (c) Phase contrast and (d) fluorescence microscopy images of the vesicles encapsulating 0.1 mM PI (pseudo-color change from red to magenta). Scale bars: 20 μ m.



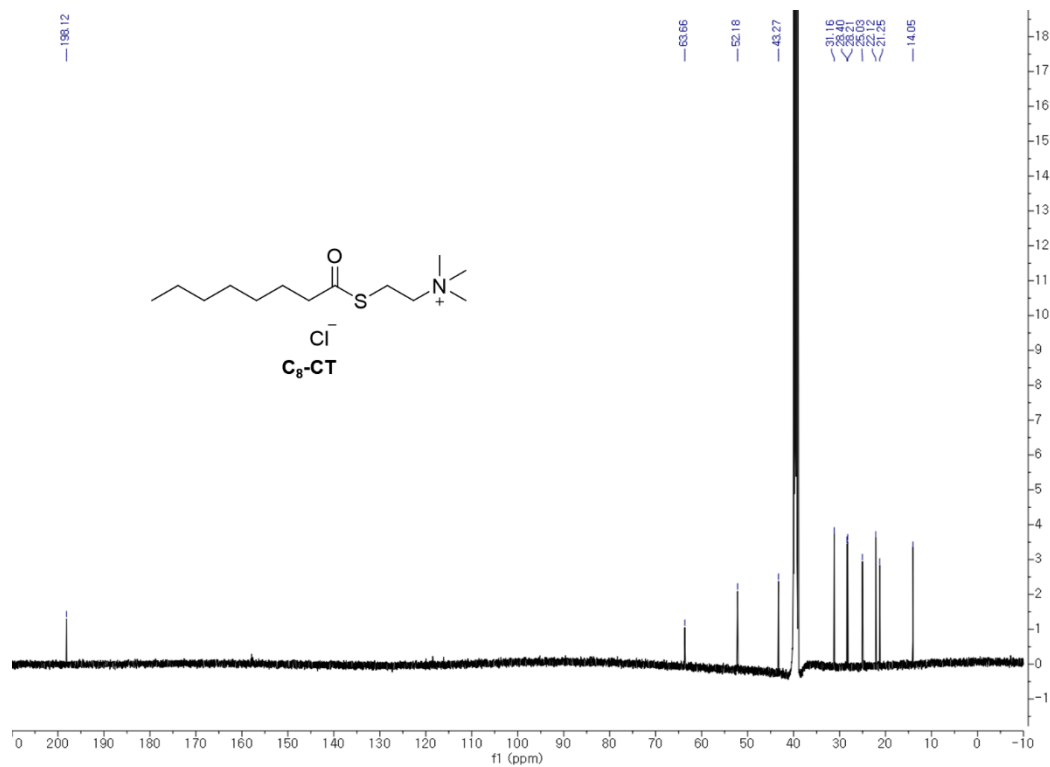
Supplementary Fig. 26. (a) Schematic diagram of aminoacylation of the model ribozymes outside the vesicles. **(b)** The activities of the ribozyme RNA1 (blue) and RNA2 (red) outside the mixed vesicles composed of C₈-Cys/C₁₂-*rac*-glycerol (1:0.5 molar ratio), measured by RT-qPCR. The ribozyme-containing vesicles were extruded through polycarbonate membranes with 100 nm pore size before addition of BYO. The reaction mixture of 50 mM C₈-Cys, 25 mM C₁₂-*rac*-glycerol, 0.5 μM ribozyme and the indicated concentration of BYO were incubated in the aminoacylation ribozyme buffer solution (0.1 M HEPES, 0.1 M KCl, 5 mM MgCl₂, pH 7.0) for 90 min. Ribozyme activities (k_A) were determined by fitting data to a pseudo-first-order rate equation and are expressed in $\text{min}^{-1} \cdot \text{M}^{-1}$ units. The data points are presented as means $\pm$ standard deviation ($n = 3$ technically independent samples).

Note, due to the instability of BYO in aqueous solution (half-life of 36.5 min)⁷, it is technically difficult to encapsulate BYO in the vesicles for a delayed-start reaction, which would result in a significant decrease in BYO concentration during the purification process. Although the reason for the low ribozyme activity (k_A) inside the vesicles is unclear at this point, the large differences between the k_A inside and outside the vesicles suggest that the interactions between the membrane and ribozyme are not the only contributor. Also, when compared to the k_A inside fatty acid/phospholipid vesicles⁸, the lower k_A inside the diacylcysteine vesicles suggests that the permeation rate of BYO through the diacylcysteine membranes may be an important factor.

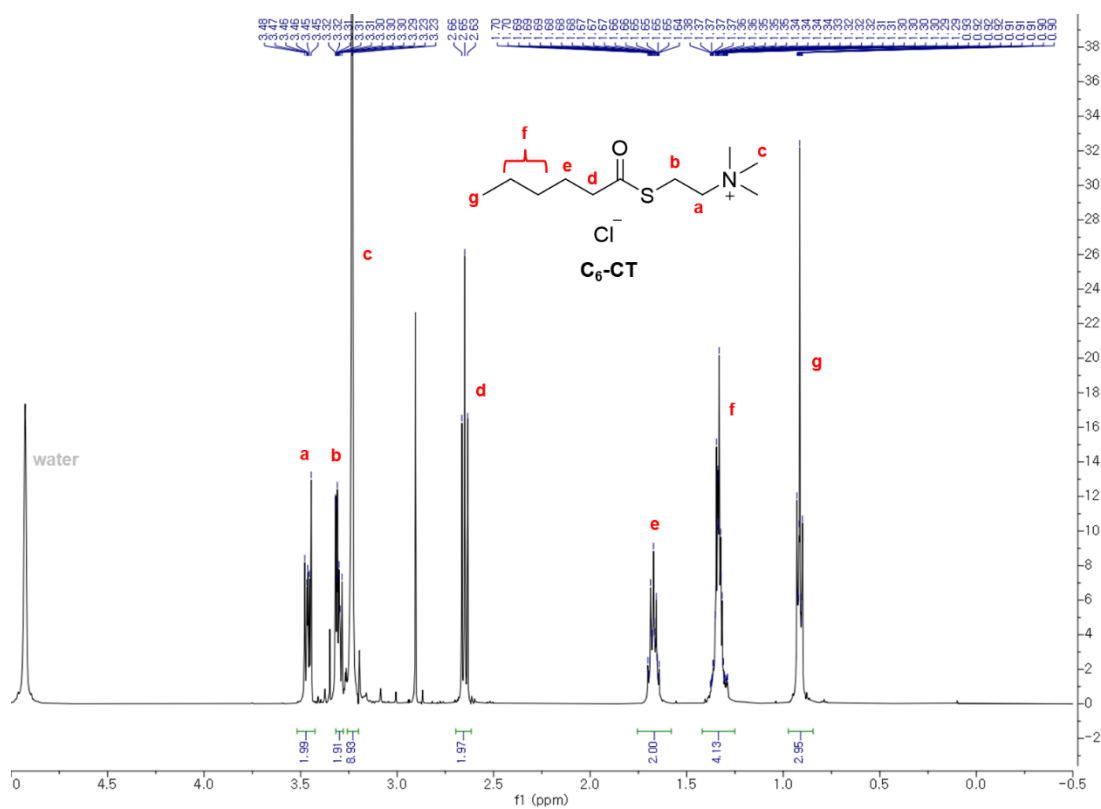
NMR Spectra



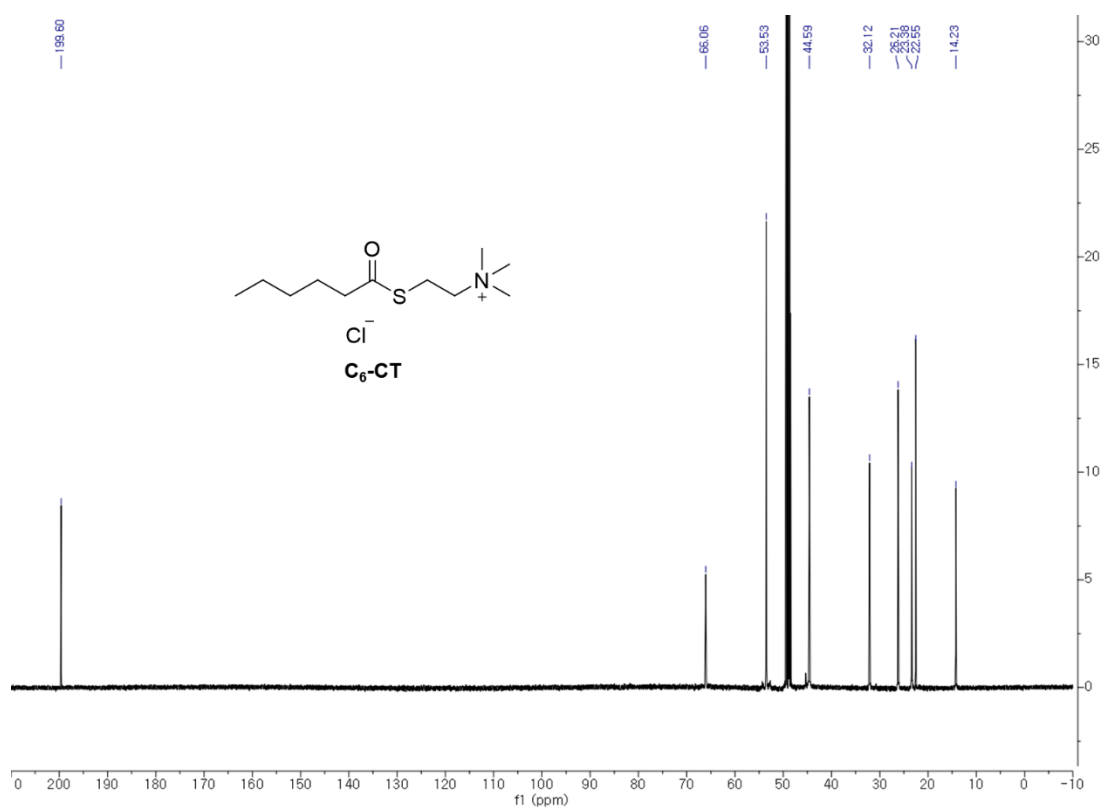
¹H NMR spectrum of C₈-CT in DMSO-*d*₆.



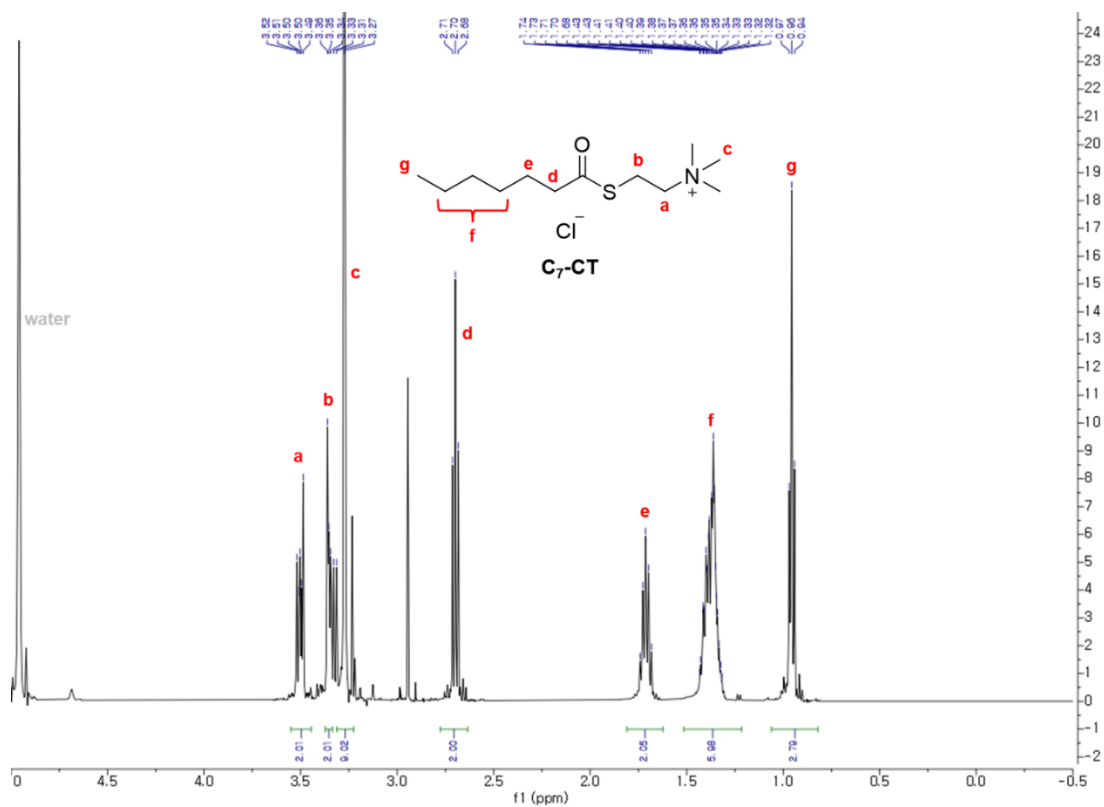
¹³C NMR spectrum of C₈-CT in DMSO-*d*₆.



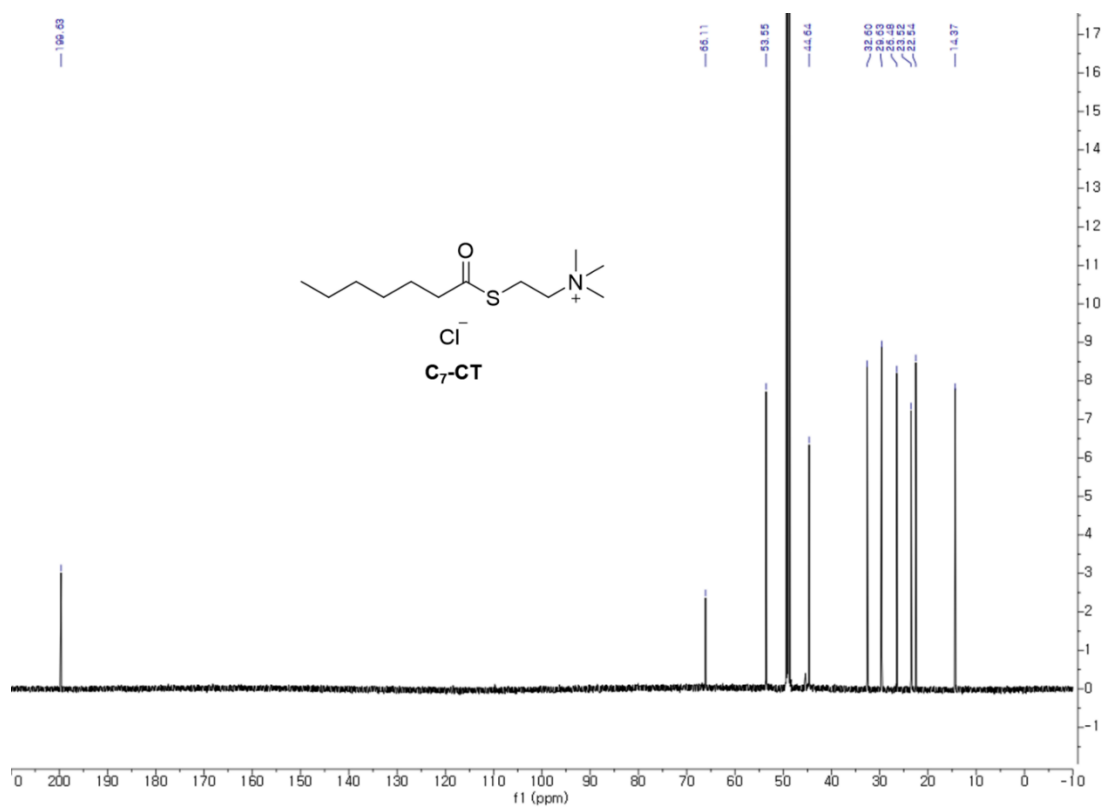
¹H NMR spectrum of C₆-CT in CD₃OD.



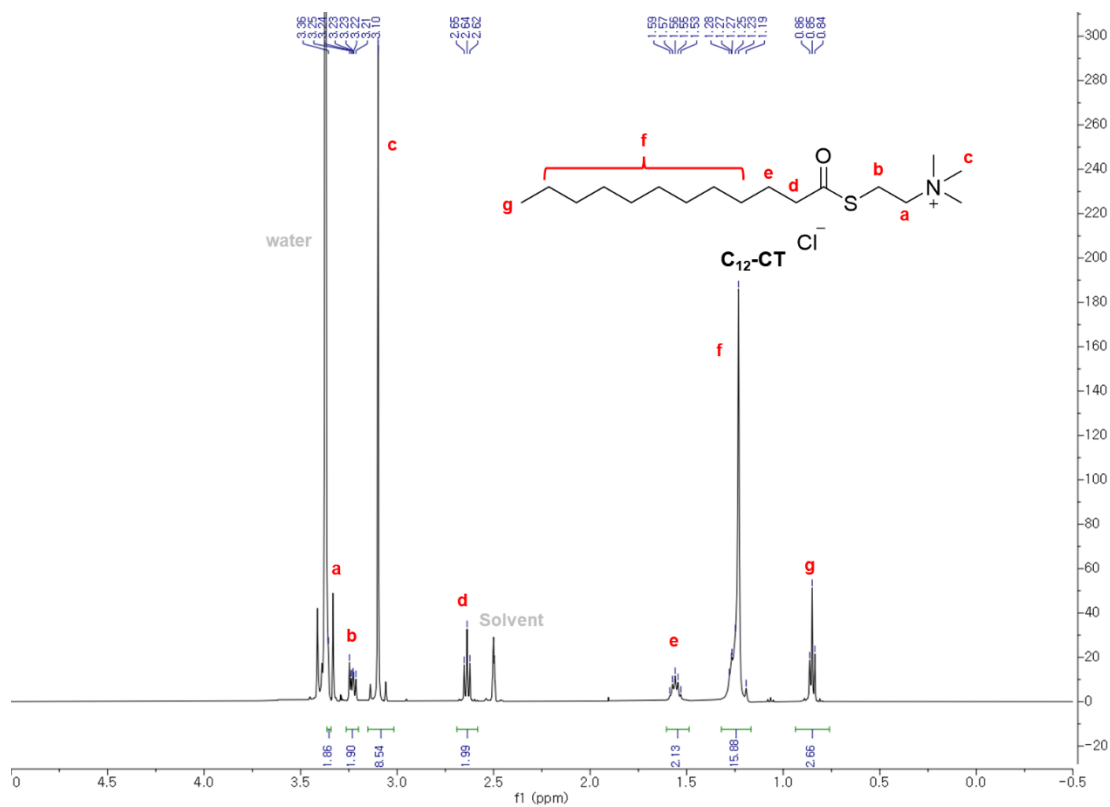
¹³C NMR spectrum of C₆-CT in CD₃OD.



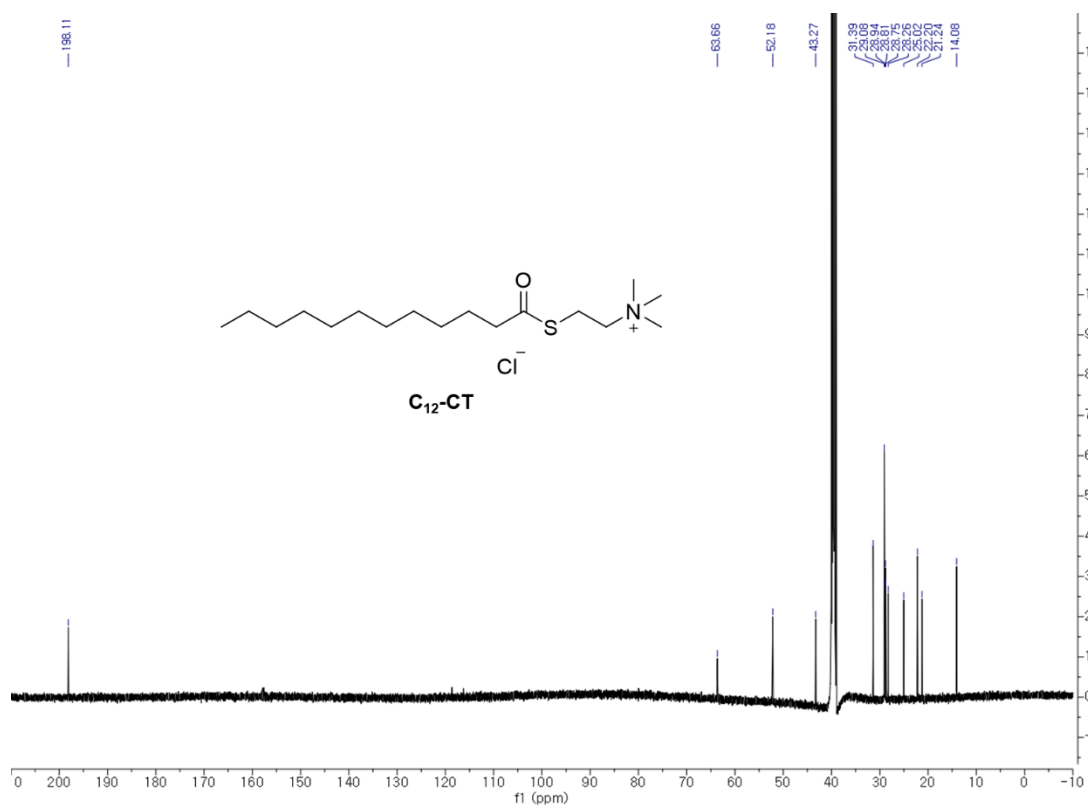
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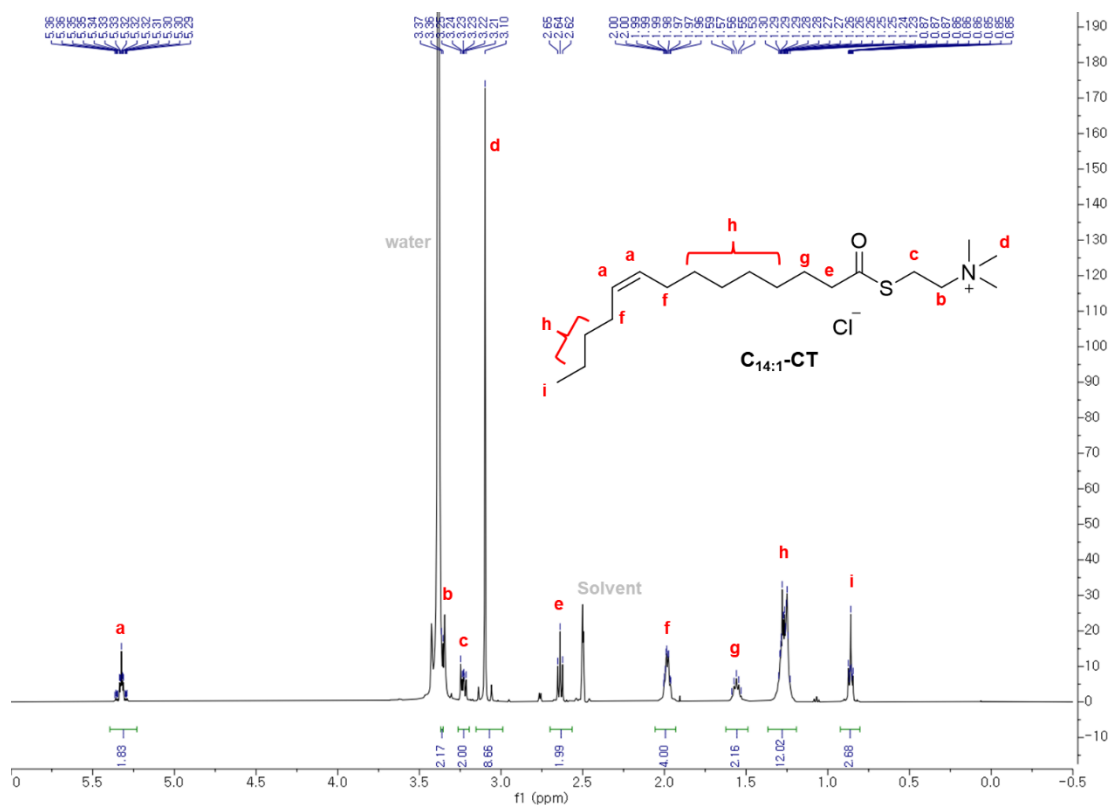


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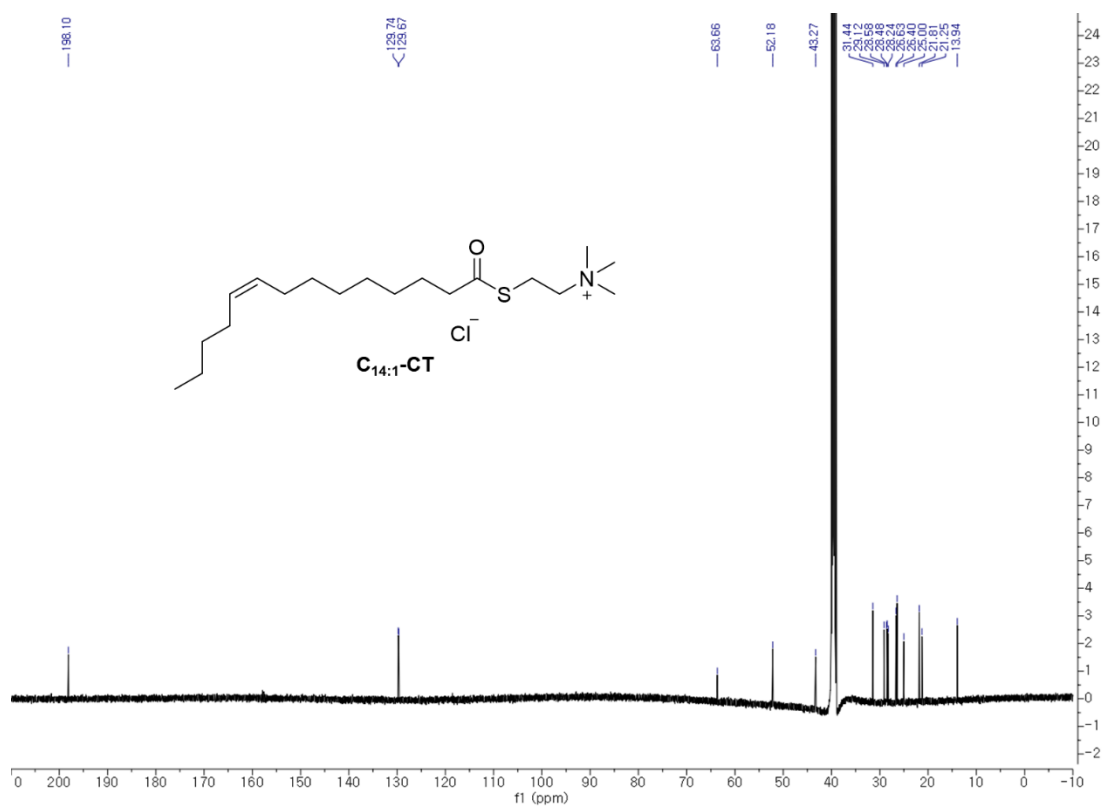


¹H NMR spectrum of C₁₂-CT in DMSO-*d*₆.

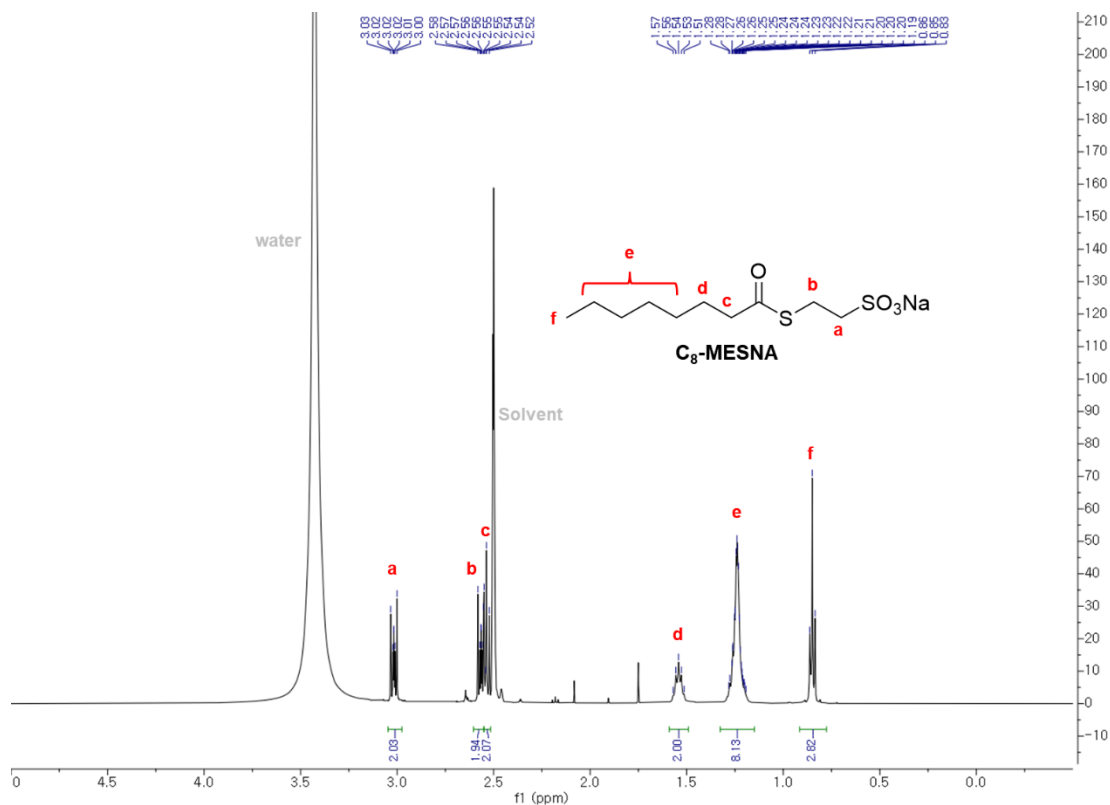




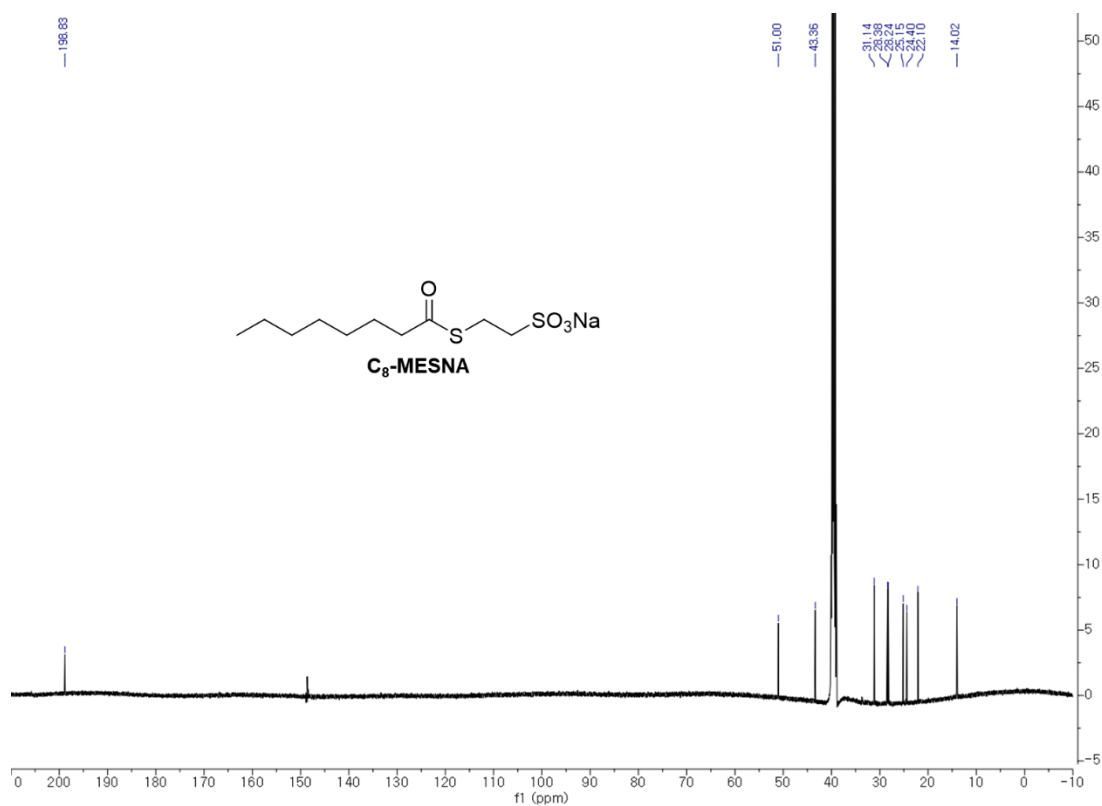
¹H NMR spectrum of C_{14:1}-CT in DMSO-*d*₆.



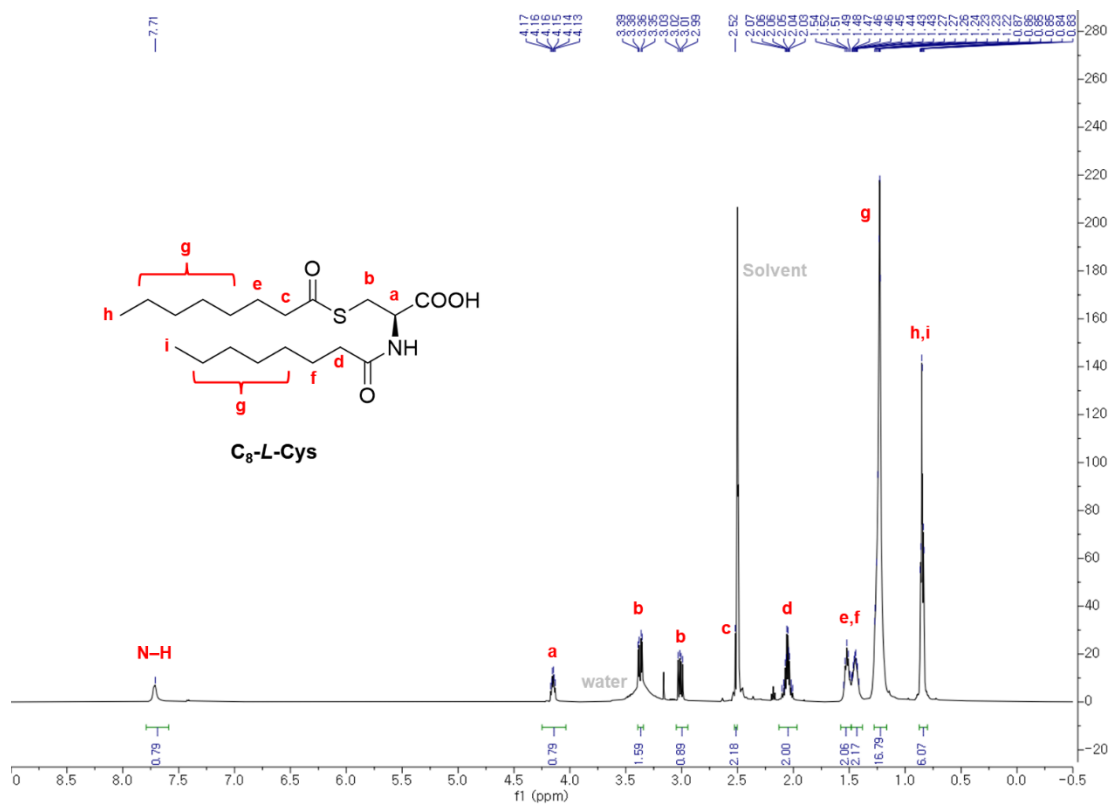
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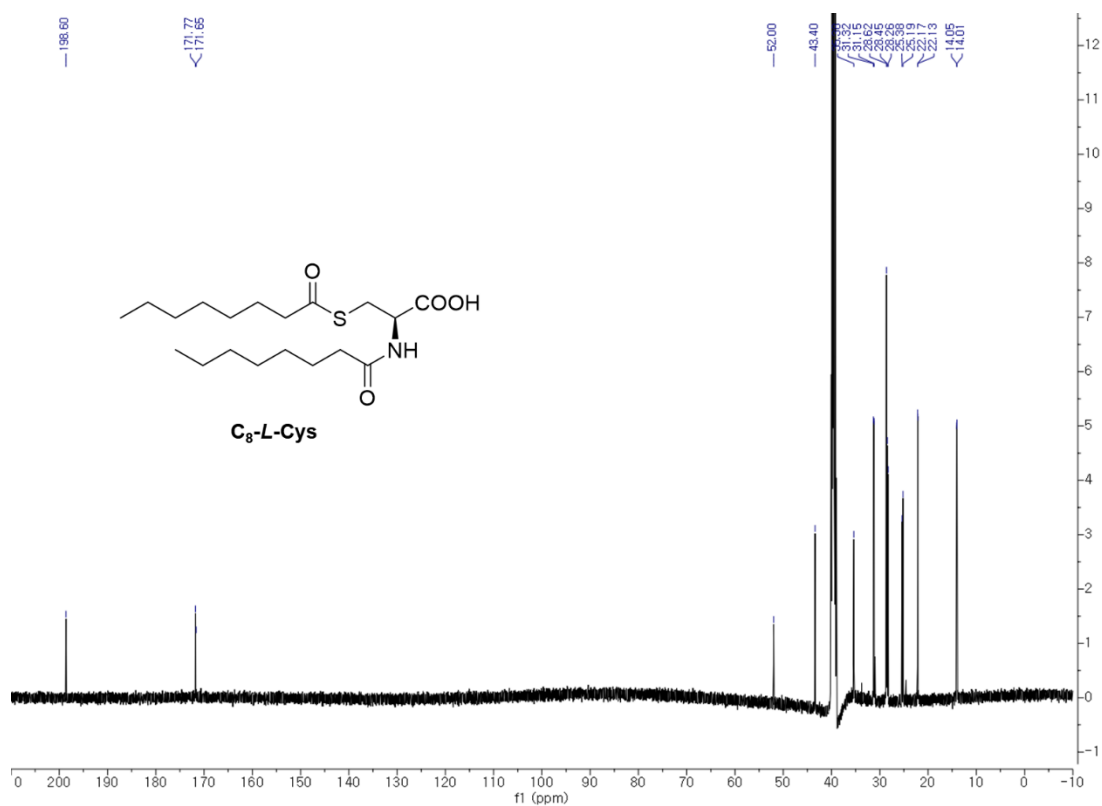
¹H NMR spectrum of C₈-MESNA in DMSO-*d*₆.



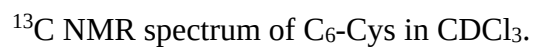
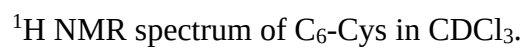
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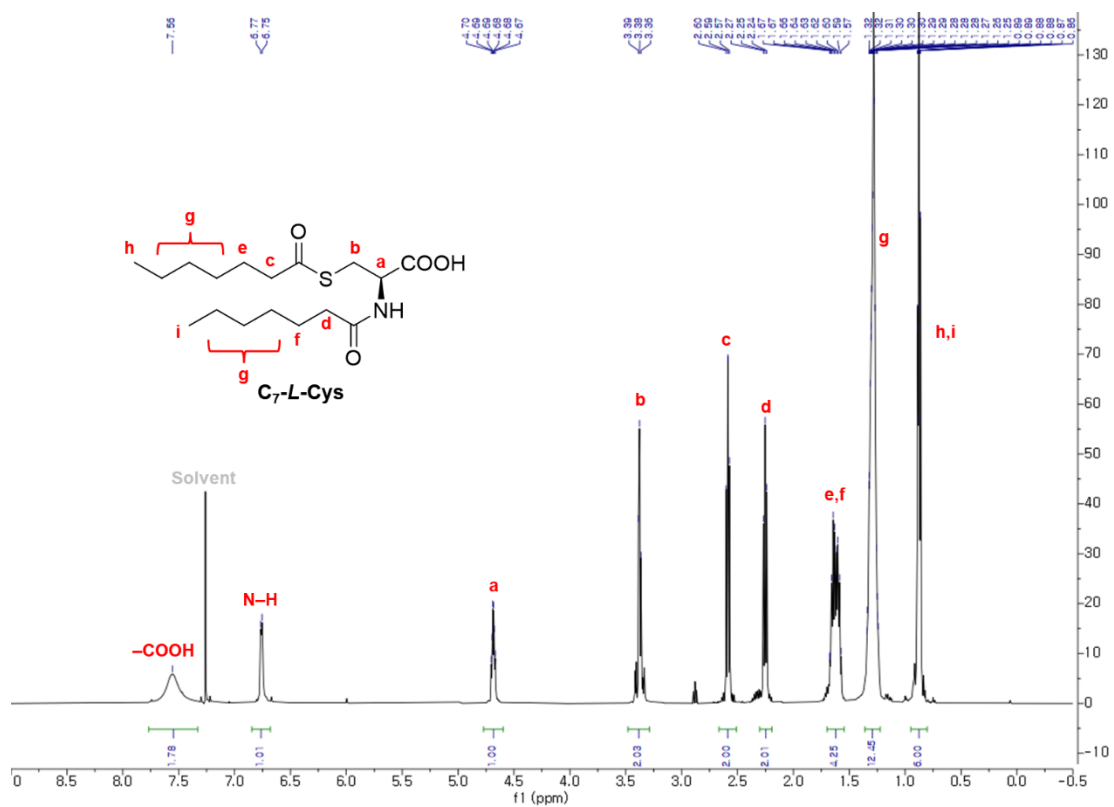


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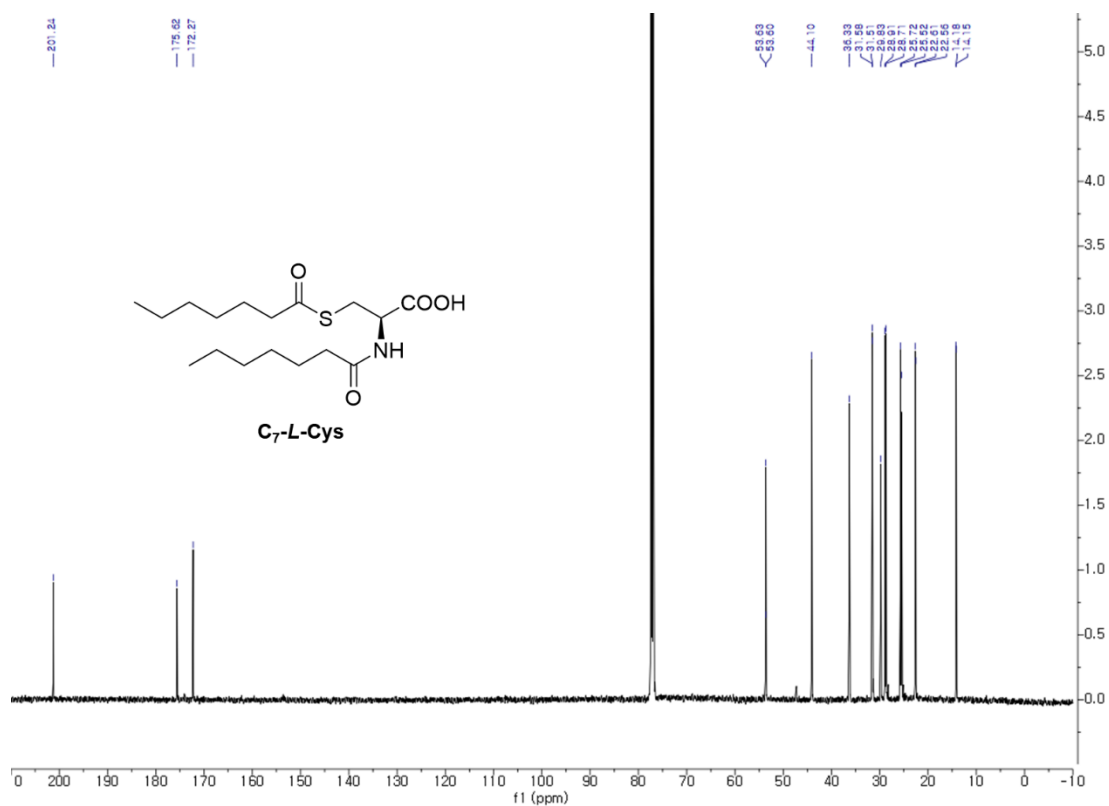


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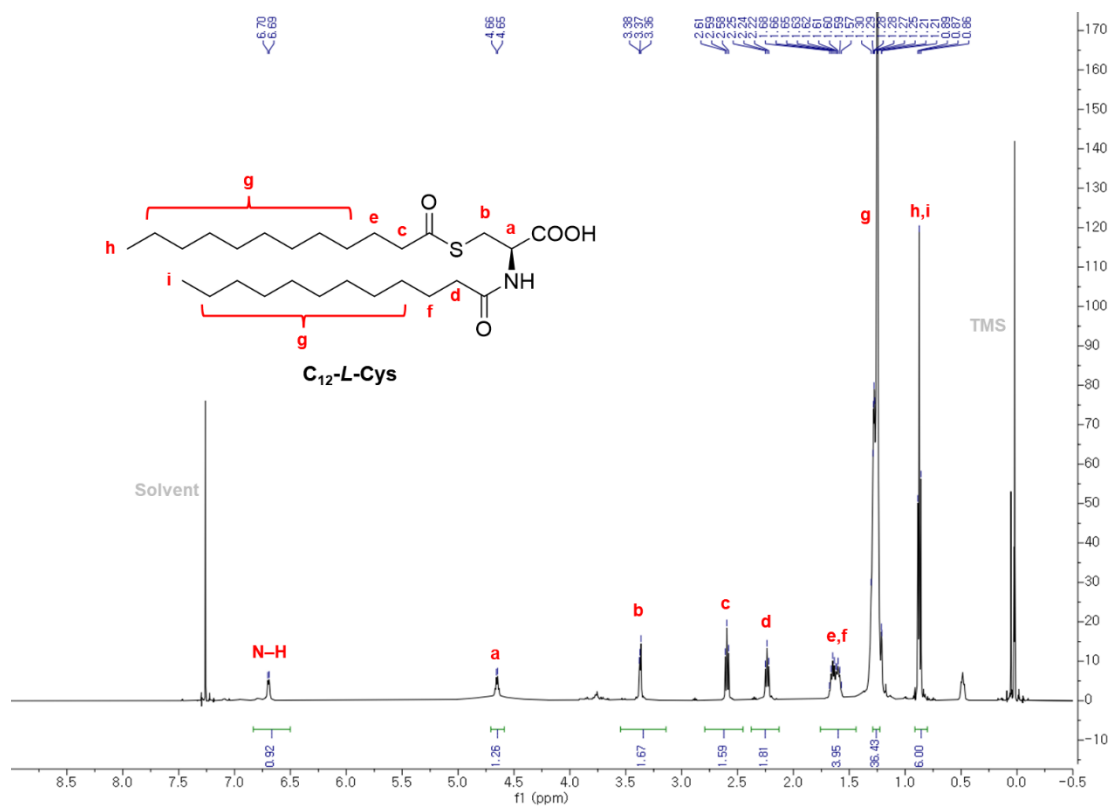




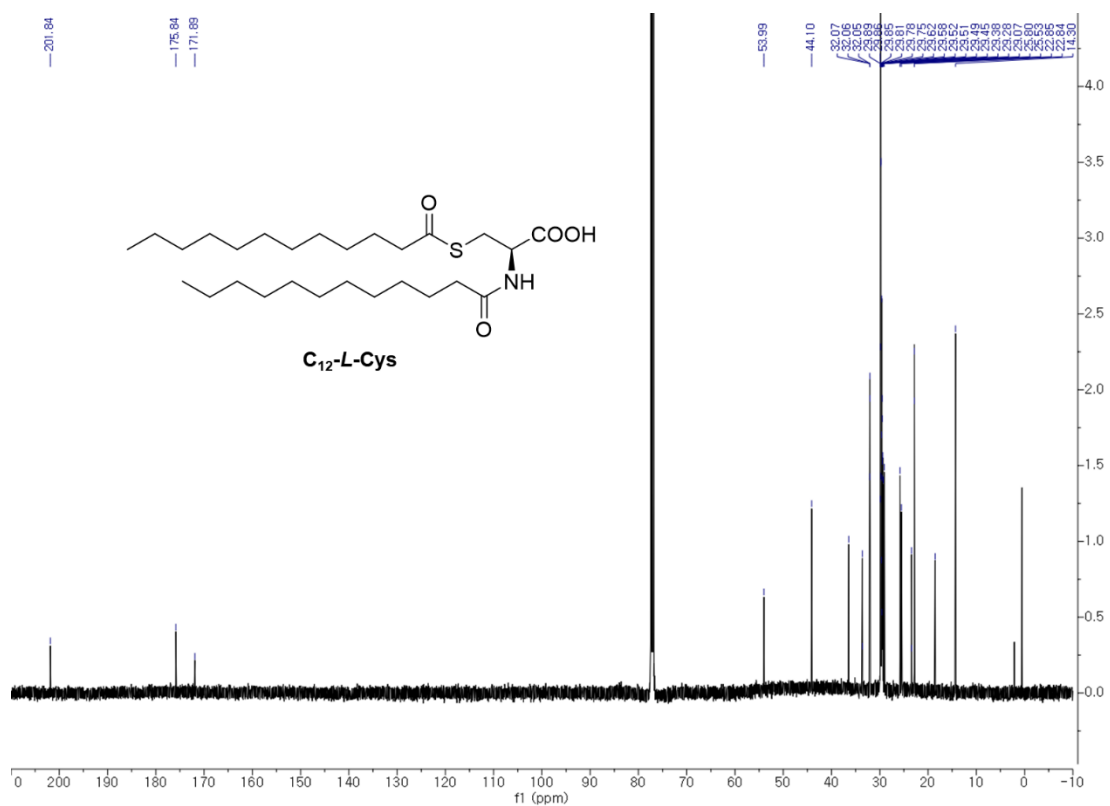
¹H NMR spectrum of C₇-Cys in CDCl₃.



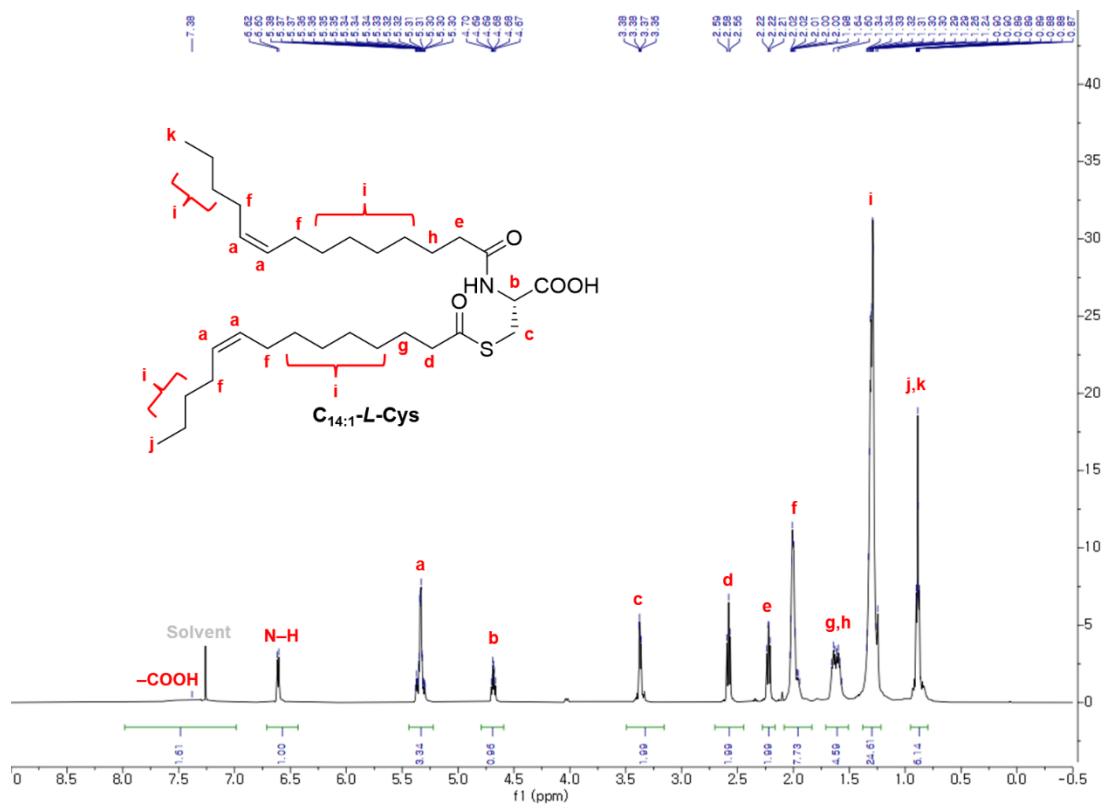
¹³C NMR spectrum of C₇-Cys in CDCl₃.



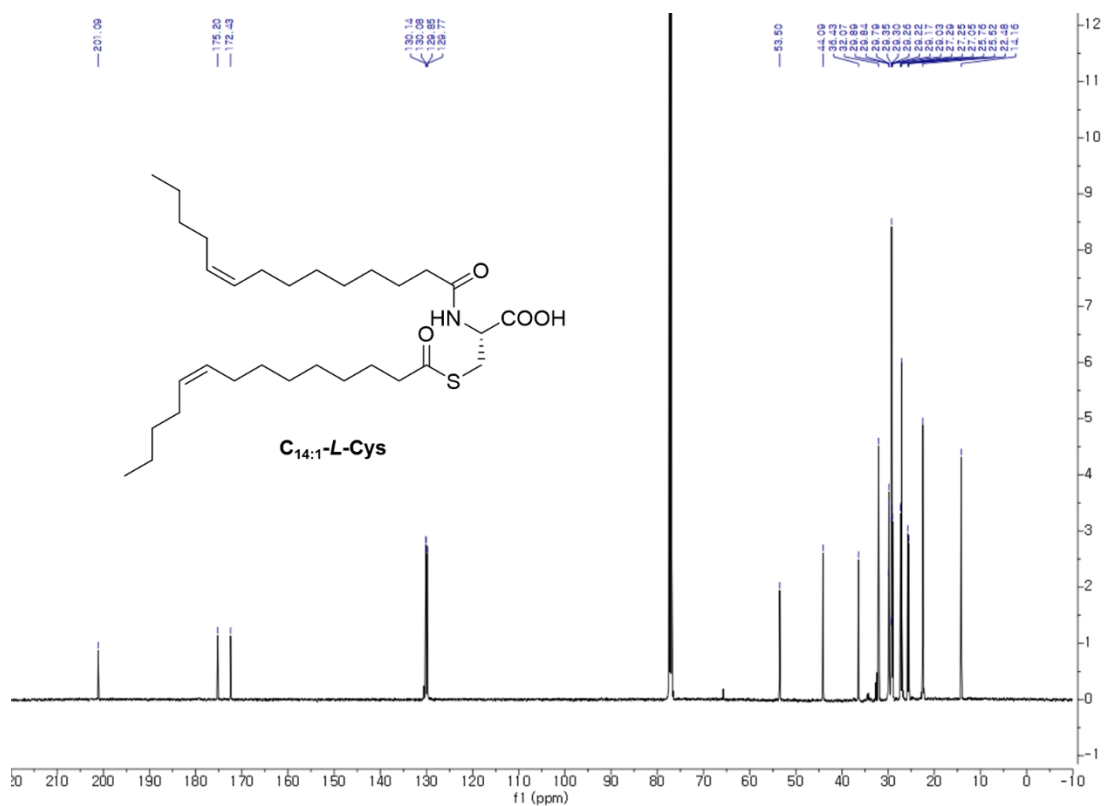
1H NMR spectrum of C_{12} -Cys in $CDCl_3$.



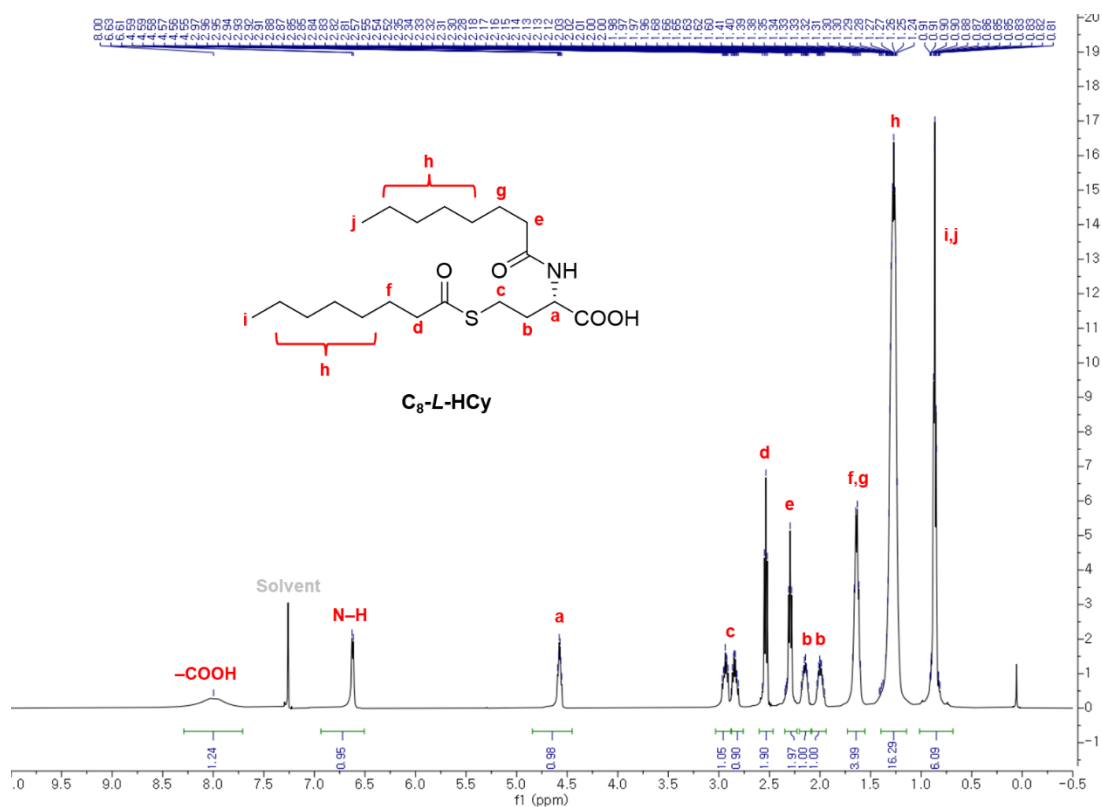
^{13}C NMR spectrum of C_{12} -Cys in $CDCl_3$.



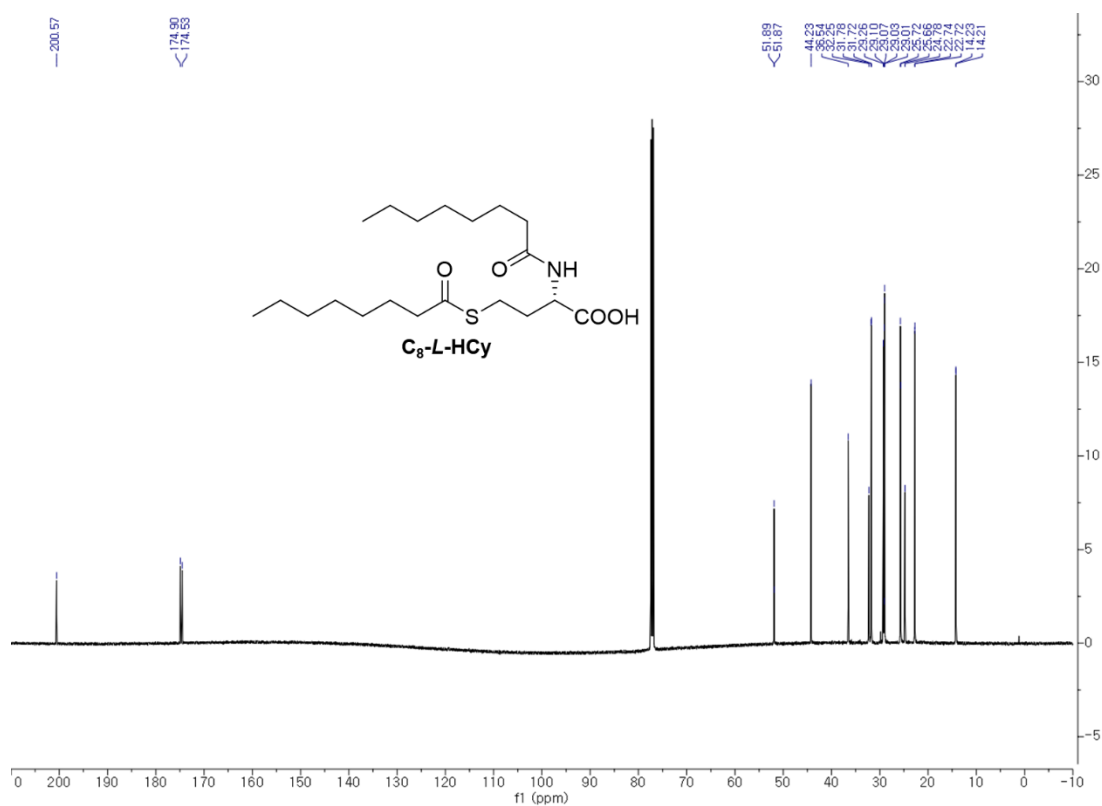
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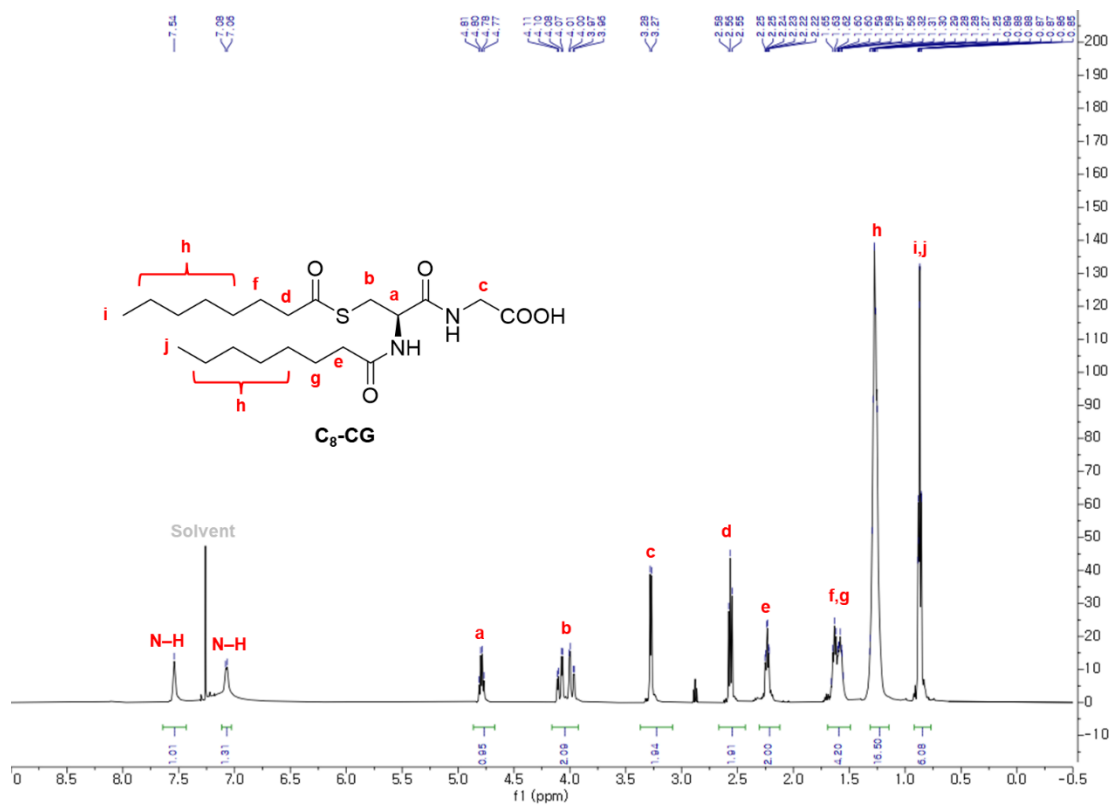
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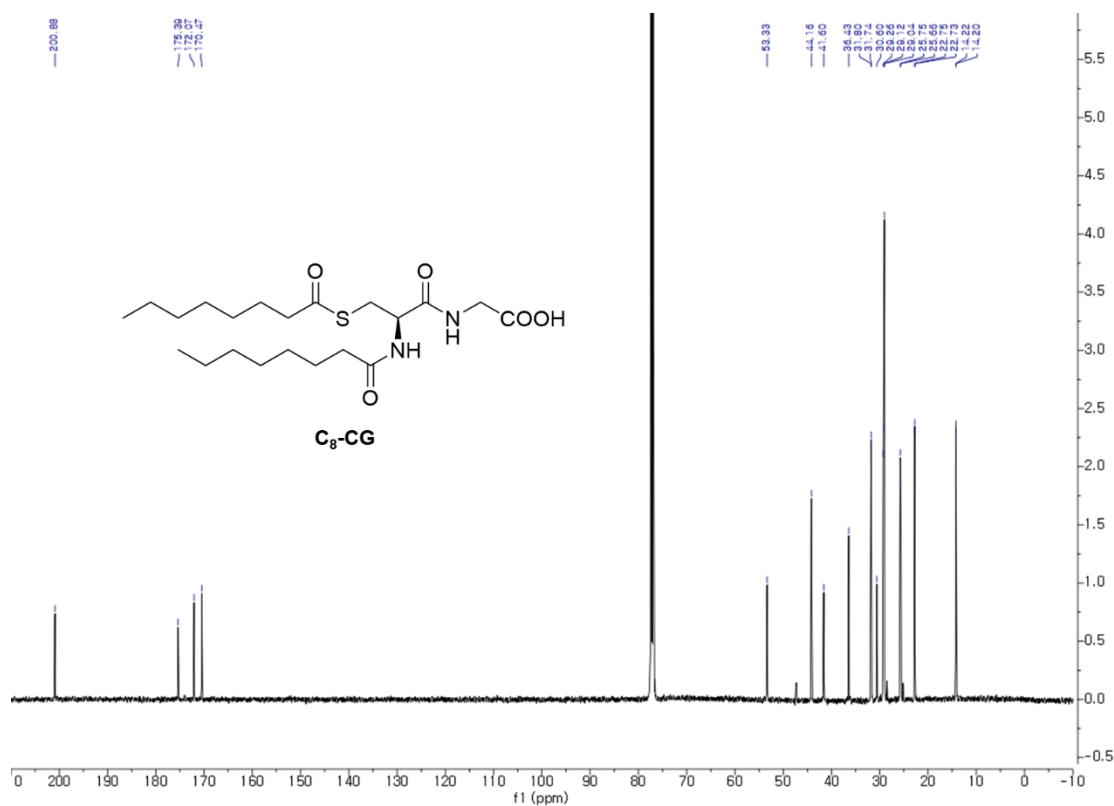
¹H NMR spectrum of C₈-HCy in CDCl₃.



¹³C NMR spectrum of C₈-HCy in CDCl₃.



¹H NMR spectrum of C₈-CG in CDCl₃.



¹³C NMR spectrum of C₈-CG in CDCl₃.

Supplementary References

1. Bernardes, G. J. L., Chalker, J. M., Errey, J. C. & Davis, B. G. Facile Conversion of Cysteine and Alkyl Cysteines to Dehydroalanine on Protein Surfaces: Versatile and Switchable Access to Functionalized Proteins. *J. Am. Chem. Soc.* **130**, 5052–5053 (2008).
2. Liu, L., Zou, Y., Battacharya, A., Zhang, D., Lang, S. Q., Houk, K. N. & Devaraj, N. K. Enzyme-free synthesis of natural phospholipids in water. *Nat. Chem.* **12**, 1029–1034 (2020).
3. Brea, R. J., Cole, C. M. & Devaraj, N. K. In Situ Vesicle Formation by Native Chemical Ligation. *Angew. Chem. Int. Ed.* **53**, 14102–14105 (2014).
4. Vicente-Garcia, C. & Colomer, I. New antimicrobial self-assembling short lipopeptides. *Org. Biomol. Chem.* **19**, 6797–6803 (2021).
5. Xiang, S., Xing, G., Xue, W., Lu, C. & Lin, J.-M. Comparison of two different deposition methods of 3-aminopropyltriethoxysilane on glass slides and their application in the ThinPrep cytologic test. *Analyst* **137**, 1669–1673 (2012).
6. Brambilla, R., Pinto, C. F., Miranda, M. S. L. & dos Santos, J. H. Z. Octadecylsilane-modified silicas in the adsorption of toluene. *Anal. Bioanal. Chem.* **391**, 2673–2681 (2008).
7. Pressman, A. D., Liu, Z., Janzen, E., Blanco, C., Müller, U. F., Joyce, G. F., Pascal, R. & Chen, I. A. Mapping a Systematic Ribozyme Fitness Landscape Reveals a Frustrated Evolutionary Network for Self-Aminoacylating RNA. *J. Am. Chem. Soc.* **141**, 6213–6223 (2019).
8. Lai, Y.-C., Liu, Z. & Chen, I. A. Encapsulation of Ribozymes inside Model Protocells Leads to Faster Evolutionary Adaptation. *Proc. Natl. Acad. Sci. USA* **118**, e2025054118 (2021).