

Supplementary information

Peptide–lipid hybrid vesicles with stimuli-responsive phase separation for controlled membrane functions

Avanashiappan Nandakumar,^a Yoshihiro Ito,^{a,b} Motoki Ueda^{a,b}*

^aNano Medical Engineering Laboratory, RIKEN Cluster for Pioneering Research, 2-1 Hirosawa, Wako, Saitama, 351-0198, Japan. ^bEmergent Bioengineering Materials Research Team, RIKEN Center for Emergent Matter Science (CEMS), 2-1 Hirosawa, Wako, Saitama, 351-0198, Japan.

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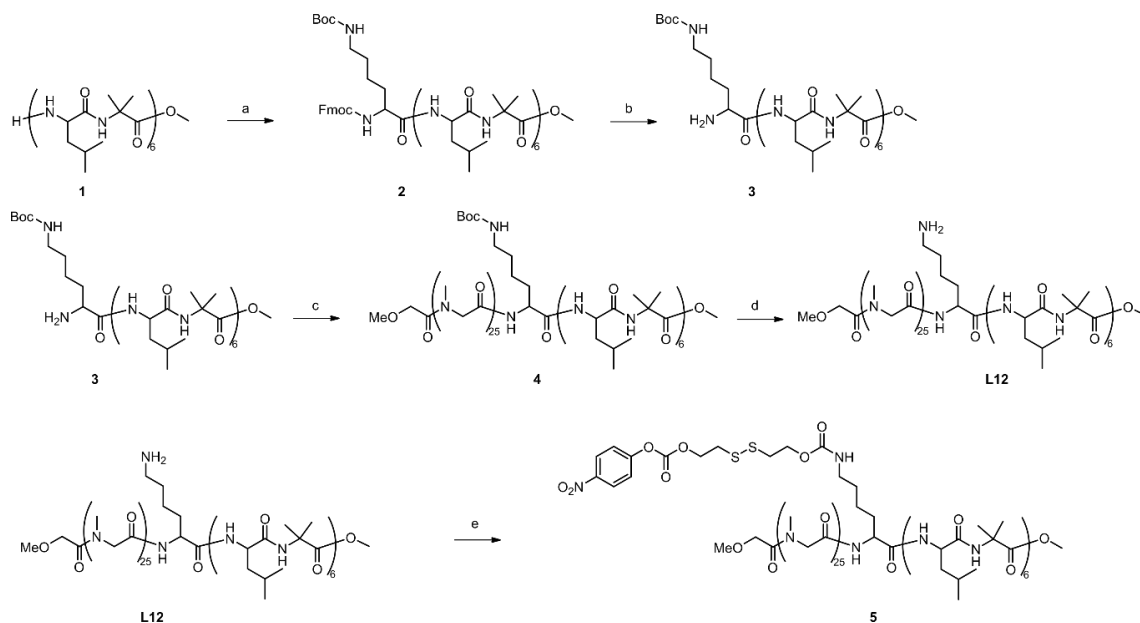
Materials and Methods

Materials

All amino acids and condensation reagents involving *N,N*-diisopropylethylamine (DIPEA, CAS: 7087-68-5), (1-cyano-2-ethoxy-2-oxoethylideneaminoxy)dimethylaminomorpholino-carbenium hexafluorophosphate (COMU, CAS: 1075198-30-9) and ethyl cyano(hydroxyimino)acetate (OxymaPure, CAS: 3849-21-6) were purchased from Watanabe Chemical Ind., Ltd., Hiroshima, Japan. 1,6-Diphenyl-1,3,5-hexatriene (DPH) (CAS:1720-32-7) was purchased from Cayman Chemical Company, Ann Arbor, MI, USA. Super-dehydrated ethanol, (+/-)-Dithiothreitol (DTT, CAS: 3483-12-3) and doxorubicin hydrochloride (DOX, CAS: 25316-40-9) were purchased from FUJIFILM Wako Pure Chemical Co., Osaka, Japan. Saline (product ID: 991-4987035132509) was obtained from Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan. 1-[Bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluorophosphate (HATU, CAS: 148893-10-1) was purchased from GenScript corporation. 4-Nitrophenyl chloroformate (CSA: 7693-46-1) and 2,2'-dithiodiethanol (CAS: 1892-29-1) were purchased from Sigma-Aldrich.

Synthesis of peptide-lipid duo amphiphile L12C16

Synthesis of amphiphilic polypeptide disulfide conjugate



Scheme S1. Synthesis of peptide(L12)-disulfide conjugate. a) Fmoc-Lys(Boc)-OH (1.8 equiv.), HATU (3 equiv.), DIEA (1.8 equiv.), DMF, rt, 24 h. b) 2 M *N,N*-dimethylamine in MeOH, 1:2 ratio of MeOH:CH₂Cl₂, rt, 4 h. c) i) SarNCA (25 equiv.), DMF, Ar, rt, overnight; ii) CH₃OCH₂COOH (1.5 equiv.), COMU (1.5 equiv.), DIPEA (2.4 equiv.), rt, 12 h. d) 4N HCl-dioxane, 30 minutes, rt. e) i) 4-nitrophenyl-OCO-OCH₂CH₂-S-S-CH₂CH₂-OH (1.2 equiv.), DIPEA (5 equiv.), DMF, rt, 3 h; ii) 4-nitrophenyl chloroformate (5 equiv.), DIPEA (4 equiv.), DCM, rt, 5 h.

The 12 mer hydrophobic unit of (L-Leu-Aib)₆ was prepared by previously reported protocol.¹ To the solution of Fmoc-Lys(Boc)-OH (1.8 equiv.) and HATU (3 equiv.) in DMF, DMF solution of **1** (1 equiv.) and DIPEA (1.8 equiv.) was added under stirring condition at 0 °C. After 30 min at 0 °C, the reaction mixture was warmed up at room

temperature (r.t.) and stirred for 24 h. the reaction progress was monitored by TLC and then, purified by silica gel column using 10% MeOH/CHCl₃ as an eluent. The second purification was carried out through a Sephadex LH-20 gel filtration using MeOH as an eluent to get the compound **2** as a white solid. ¹H NMR (400 MHz, CDCl₃) δ ppm 0.88–0.96 (m, 36H, Leu-CH₃), 1.39–1.83 (m, 67H, Aib-CH₃, Leu-CH₂, Leu-CH, Lys-CH₂), 2.19 (s, 2H, Lys-CH₂), 3.17 (brs, 2H, Lys-CH₂), 3.67 (s, 3H, OCH₃), 3.91–4.04 (m, 5H, Leu-CH or Lys-CH), 4.19–4.29 (m, 3H, Fmoc-CH₂, Fmoc-CH), 4.44 (t, *J* = 8.4 Hz, 1H, Leu-CH and Lys-CH), 5.03 (brs, 1H, Leu-CH and Lys-CH), 7.16–7.22 (m, 2H, NH), 7.31–7.44 (m, 5H, Ar-H, NH), 7.58–7.86 (m, 13H, , Ar-H, NH). MALDI-TOF MS for C₈₇H₁₄₂N₁₄O₁₈; calcd. [M+Na]⁺: 1694.05, found [M+Na]⁺: 1693.97.

The compound **2** was dissolved in 1:2 ratio of MeOH:CH₂Cl₂ mixture and then 10 mL of 2M MeOH solution of dimethylamine was added at 0 °C. The reaction mixture was brought to r.t. and stirred for 4 h. the reaction was monitored by TLC. After the completion of reaction, the reaction mixture was evaporated to dryness and purified by a Sephadex LH-20 gel column using MeOH as an eluent to get a compound **3** as a white solid. ¹H NMR (400 MHz, CDCl₃) δ ppm 0.84–0.99 (m, 36H, Leu-CH₃), 1.40–2.03 (m, 69H, Aib-CH₃, Leu-CH₂, Leu-CH, Lys-CH₂), 3.12 (brs, 2H, Lys-CH₂), 3.68 (s, 3H, OCH₃), 3.89–4.02 (m, 5H, Leu-CH and Lys-CH), 4.24 (brs, 1H, Leu-CH and Lys-CH), 4.96 (brs, 1H, Leu-CH and Lys-CH), 7.36–7.84 (m, 12H, NH), 8.41 (brs, 1H, NH). MALDI-TOF MS for C₇₂H₁₃₂N₁₄O₁₆; calcd. [M+Na]⁺: 1471.98, found [M+Na]⁺: 1471.95.

Sarcosine *N*-carboxyanhydride (SarNCA) was synthesized by using previously reported protocol.¹ The polymerization reaction was carried out under dry condition in order to control the sarcosine length. SarNCA (25 equiv.) was dissolved in minimum amount of

anhydrous DMF. The solution of **3** (1 equiv.) in dry DMF was added to the solution of SarNCA at r.t. under Argon atmosphere. The reaction mixture was stirred for 12 h at r.t. 2-Methoxyacetic acid (1.5 equiv.), COMU (1.5 equiv.) and DIPEA (2.5 equiv.) were added to the reaction solution to cap the end of polymerized sarcosine chain. The resulting compounds were concentrated and purified using a Sephadex LH-20 gel column with 1:1 ratio of MeOH:CHCl₃ mixture as an eluent. The compound Ac-(PSar)₂₅-*b*-Lys(Boc)-(L-Leu-Aib)₆ (**4**) was obtained as a white solid. ¹H NMR (400 MHz, CDCl₃) δ ppm 0.83–1.00 (m, 36H, Leu-CH₃), 1.43–1.84 (m, 67H, Aib-CH₃, Leu-CH₂, Leu-CH, Lys-CH₂), 2.22 (brs, 2H, Lys-CH₂), 2.95–3.24 (br, 77H, Sar-CH₃, Lys-CH₂), 3.37–3.48 (m, 3H, OCH₃), 3.67 (s, 3H, OCH₃), 3.92–4.38 (m, 58H, Sar-CH₂, Leu-CH and Lys-CH), 5.08 (brs, 1H, Leu-CH and Lys-CH), 7.00–7.24 (m, 2H, NH), 7.31–7.98 (m, 11H, NH). MALDI-TOF MS for C₁₅₀H₂₆₁N₃₉O₄₃; calcd. [M+Na]⁺: 3319.93, found [M+Na]⁺: 3319.94.

To a solution of compound **4** in 2:1 ratio of MeOH:CHCl₃ mixture, 3 mL of 4N HCl in dioxane was added. The resulting solution was stirred for 30 min at r.t. Then, the solvent was evaporated to dryness. The compound was washed twice with diethyl ether and dried under vacuum to provide a product L12 as a white solid. ¹H NMR (400 MHz, CDCl₃) δ ppm 0.87–0.98 (m, 36H, Leu-CH₃), 1.49–2.24 (m, 60H, Aib-CH₃, Leu-CH₂, Leu-CH, Lys-CH₂), 2.95–3.05 (m, 77H, Sar-CH₃, Lys-CH₂), 3.37–3.44 (m, 3H, OCH₃), 3.71 (s, 3H, OCH₃), 3.78–4.26 (m, 59H, Sar-CH₂, Leu-CH and Lys-CH), 7.30–7.93 (m, 13H, NH). MALDI-TOF MS for C₁₄₅H₂₅₃N₃₉O₄₁; calcd. [M+Na]⁺: 3219.88, found [M+Na]⁺: 3219.82.

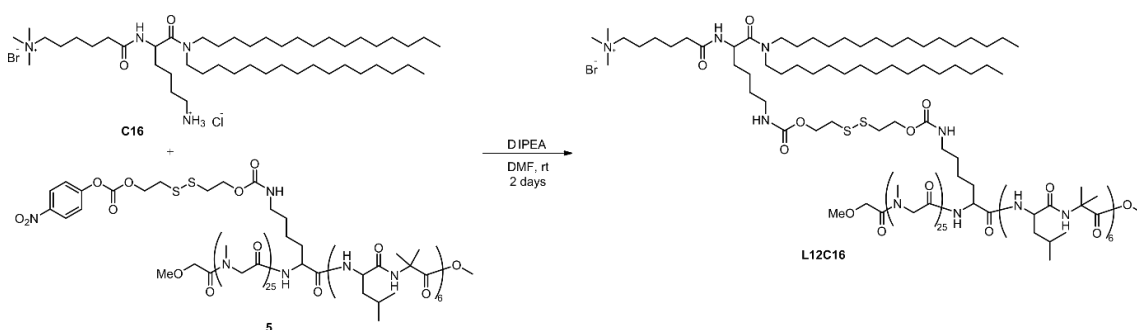
The compound 4-nitrophenyl-OCO-OCH₂CH₂-S-S-CH₂CH₂-OH was synthesized using previously reported protocol.² ¹H NMR (400 MHz, CDCl₃) δ ppm 2.92 (t, *J* = 6 Hz, 2H, S-CH₂), 3.05 (t, *J* = 6.4 Hz, 2H, S-CH₂), 3.92 (t, *J* = 6 Hz, 2H, O-CH₂), 4.57 (t, *J* = 6.4 Hz, 2H, O-CH₂), 7.40 (d, *J* = 9.2 Hz, 2H, Ar-*H*), 8.29 (d, *J* = 9.2 Hz, 2H, Ar-*H*).

To a solution of L12 (1 equiv.) in DMF, the compound 4-nitrophenyl-OCO-OCH₂CH₂-S-S-CH₂CH₂-OH (1.2 equiv.) and DIPEA (5 equiv.) were added at 0 °C. The solution was brought to r.t. and stirred for 3h at r.t. The reaction progress was monitored by using TLC. After the reaction, the solvent was evaporated to dryness and the product was purified by using a Sephadex LH-20 gel column with MeOH as an eluent. Hydroxyethyl disulfide conjugated L12 was obtained as a white solid. ¹H NMR (400 MHz, CDCl₃) δ ppm 0.77–1.05 (m, 36H, Leu-CH₃), 1.35–2.02 (m, 60H, Aib-CH₃, Leu-CH₂, Leu-CH, Lys-CH₂), 2.83–3.44 (m, 81H, Sar-CH₃, Lys-CH₂, S-CH₂), 3.37–3.44 (m, 3H, OCH₃), 3.67 (s, 3H, OCH₃), 3.84–4.14 (m, 63H, Sar-CH₂, Leu-CH and Lys-CH, OCH₂), 7.30–7.94 (m, 13H, NH). MALDI-TOF MS for C₁₅₀H₂₆₁N₃₉O₄₄S₂; calcd. [M+Na]⁺: 3399.87, found [M+Na]⁺: 3399.67.

To a solution of hydroxyethyl disulfide conjugated L12 in CH₂Cl₂, the solution of 4-nitrophenyl chloroformate (5 equiv.) in CH₂Cl₂ was added at 0 °C. The reaction mixture was warmed to r.t. and the stirring was continued for 3h. After the completion of reaction, the solvent was evaporated to dryness and the product **5** was purified by filtering through a Sephadex LH-20 column to get a white solid. ¹H NMR (400 MHz, CDCl₃) δ ppm 0.84–1.00 (m, 36H, Leu-CH₃), 1.44–1.87 (m, 60H, Aib-CH₃, Leu-CH₂, Leu-CH, Lys-CH₂), 2.91–3.26 (m, 81H, Sar-CH₃, Lys-CH₂, S-CH₂), 3.37–3.45 (m, 3H, OCH₃), 3.67 (s, 3H,

OCH₃), 3.85–4.36 (m, 61H, Sar-CH₂, Leu-CH and Lys-CH, OCH₂), 4.55 (t, *J* = 6.4 Hz, 2H, O-CH₂), 7.30–7.78 (m, 15H, NH), 8.29 (d, *J* = 9.2 Hz, 2H, Ar-H).

Synthesis of L12C16



Scheme 2. Synthesis of peptide-lipid disulfide conjugate

The compound C16 was synthesised by using previously reported protocol.^{3,4} ¹H NMR (400 MHz, CDCl₃) δ ppm 0.88 (t, *J* = 6.4 Hz, 6H, CH₃), 1.25–1.94 (m, 68H, CH₂), 2.43 (brs, 2H, CH₂), 3.01–3.26 (m, 5H, CH₂), 3.34–3.43 (m, 10H, CH₂, NCH₃), 3.59–3.72 (m, 2H, CH₂), 4.72 (t, *J* = 7.2 Hz, 1H, CH), 8.08 (brs, 1H, NH), 8.57 (brs, 3H, NH); ¹³C NMR (125 MHz, CDCl₃) δ ppm 14.0, 22.3, 22.6, 24.8, 25.0, 26.5, 26.8, 27.0, 27.5, 29.2, 29.6, 31.1, 31.8, 34.7, 38.7, 46.6, 48.1, 49.6, 53.5, 66.4, 171.6, 173.5. MALDI-TOF MS for C₄₇H₉₇N₄O₂⁺; calcd. [M]⁺: 749.76, found [M]⁺: 749.68. To a solution of **5** in DMF, C16 (2 equiv.) and DIPEA (1 equiv.) were added at r.t. The solution was stirred for 2 days at r.t. condition. Then, the solvent was evaporated in vacuum condition and the product was purified by using a Sephadex LH-20 gel filtration column with MeOH as an eluent to get product L12C16 as a white solid. ¹H NMR (400 MHz, CDCl₃) δ ppm 0.78–1.00 (m, 42H, Leu-CH₃, C16-CH₃), 1.26–1.85 (m, 128H, Aib-CH₃, Leu-CH₂, Leu-CH, Lys-CH₂, C16-CH₂), 2.26 (brs, 2H, C16-CH₂), 2.91–3.29 (m, 96H, Sar-CH₃, Lys-CH₂, S-CH₂, C16-CH₂), 3.37–3.44 (m, 3H, OCH₃), 3.68 (s, 3H, OCH₃), 3.86–4.41 (m, 65H, Sar-CH₂, Leu-CH and

Lys-CH, OCH₂, C16-CH₂), 4.80 (s, 1H, C16-CH), 6.77–6.88 (brs, 1H, NH), 7.30–7.98 (m, 13H, NH). MALDI-TOF MS for C₁₉₈H₃₅₆N₄₃O₄₇S₂⁺; calcd. [M]⁺: 4152.62, found [M+Na]⁺: 4152.65.

Preparation of molecular assembly

A stock solution of L12, C16 and L12C16 were prepared using a concentration of 40 mg/800 μL of EtOH. Assembly of L12C16 was achieved by the ethanolic injection of 10 μL of L12C16 stock solution into a 990 μL of saline under stirring condition at r.t. (0.5 mg of L12C16 per 1 mL solution). Then, the dispersion was stirred for 10 min at r.t. and followed by, kept at 90 °C for 1 h to get self-assembled morphology.

Transmission electron microscope (TEM)

TEM images of assembly were taken using JEOL JEM-1230 (JEOL, Tokyo, Japan) at an accelerating voltage of 80 kV. For observations, a drop of dispersion was mounted on a carbon-coated copper grid (Okenshoji Co., Ltd., Tokyo, Japan) and stained negatively with 2% samarium acetate, followed by removal of the excess fluid with a filter paper.

Dynamic light scattering (DLS)

The hydrodynamic diameter of molecular assemblies in saline was analyzed by ELSZ-2PL (Photal Otsuka Electronics, Co. Ltd., Osaka, Japan) using a He-Ne laser (633 nm). All measurements were performed at 25 °C.

Circular dichroism (CD)

Circular dichroism (CD) data were acquired with a JASCO J-1500 instrument (JEOL, Japan), using a cell with an optical path length of 1 cm. Data were recorded at 25 °C based on the self-assembly of 0.12 mM L12C16 dispersions (0.5 mg/mL) in saline. Each dispersion was diluted to final concentration of 0.024 mM before collecting CD data.

Förster resonance energy transfer (FRET) analysis

FRET experiments in vesicles were carried out using a JASCO FP-6500 fluorometer (JASCO, Tokyo, Japan). For the NBD-PE/Rhodamine-PE donor/acceptor pair, the excitation wavelength was set at 460 nm and emission spectra were collected from 480 to 650 nm. FRET was measured in liposomes containing known concentrations of donor- and acceptor-labeled vesicles. L12C16 vesicles were labeled with 1.2 mol % of both NBD-PE and Rhod-PE, respectively. In brief, the stock ethanol solutions of the L12C16, NBD-PE and Rhod-PE were mixed with the desired ratio (L12C16, NBD-PE, Rhod-PE; 120, 1.4, 1.4 μ mol) and then injected into saline (1 mL) at one time. The mixture solution was then stirred at room temperature for 15 min and heated in the same manner described in the preparation of molecular assembly section. In addition, respective donor-labeled (NBD-PE only) L12C16 vesicles were also prepared to calculate FRET efficiency E in the absence and presence of the acceptor according to $E (\%) = (I_D - I_{DA}) / (I_D) \times 100$, where I_D and I_{DA} are the donor intensities of samples containing only donor-labeled vesicles and samples with both donor- and acceptor-labeled vesicles, respectively.

Membrane fluidity evaluation using DPH

The membrane fluidity of the L12C16 vesicle before and after the addition of DTT was determined using a fluorescence spectrophotometer FP-6500 (JASCO, Tokyo, Japan), with DPH which inserts into the hydrophobic layer of assembly as a fluorescent polarization probe. Each sample (1 mL) and 100 μ M DPH in ethanol (10 μ L) was incubated for 60 min in the dark. The samples were excited with vertically polarized light (360 nm), and emission light (430 nm) was recorded in both the perpendicular, I_{\parallel} (0° , 0°), and parallel, I_{\perp} (0° , 90°), directions. The polarization (P) value of DPH was calculated using $P = (I_{\parallel} - GI_{\perp}) / (I_{\parallel} + GI_{\perp})$, where G is the correction factor. The following formula was also used: $G = i_{\perp} / i_{\parallel}$, where i_{\perp} and i_{\parallel} are the emission intensities perpendicular (90° , 0°) and parallel (90° , 90°) to the horizontally polarized light, respectively. The membrane fluidity of different samples was measured from 15 $^{\circ}$ C to 50 $^{\circ}$ C.

Cargo release study

Before studying release kinetics of cargo from L12C16 vesicle, the encapsulation of DOX was performed using L12C16 vesicle. Briefly, the stock solution of L12C16 (10 μ L) was injected into DOX solution (990 μ L; DOX concentration used for assembly process is 1 mg/mL) and the mixture was stirred at room temperature for 15 min. Next, the mixture was heated at 90 $^{\circ}$ C for 1 h and purified through Amicon filtration as well as PD-10 column to remove unencapsulated DOX. A loading amount of DOX by L12C16 dispersion was calculated by using UV-Vis calibration curve. DOX releasing kinetics were carried out as follows: 90 μ L DOX loaded L12C16 vesicle dispersion and 10 μ L of

DTT (0.1 M stock solution) was dialyzed against 10 mM DTT in 1800 μ L of saline at 20 °C and 37 °C. The control experiment was carried out in the absence of DTT at both 20 °C and 37 °C. 600 μ L of aliquots were removed from dialyzed medium at different time interval to measure the release amount of DOX. The released DOX concentration was measured by using UV-Vis calibration curve.

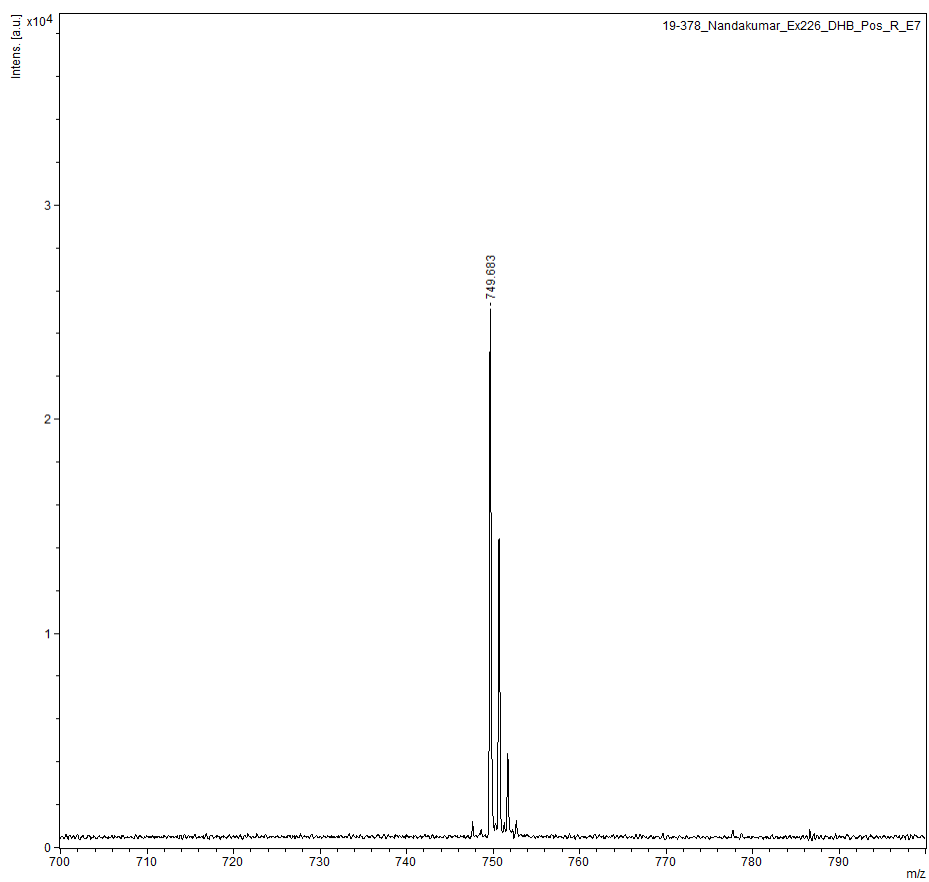
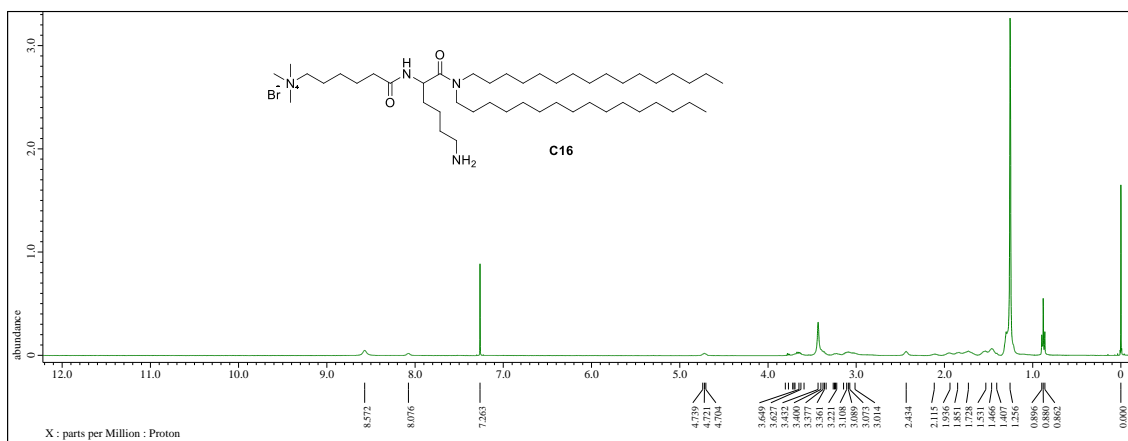


Fig. S1. NMR and MALDI-TOF MS of C16

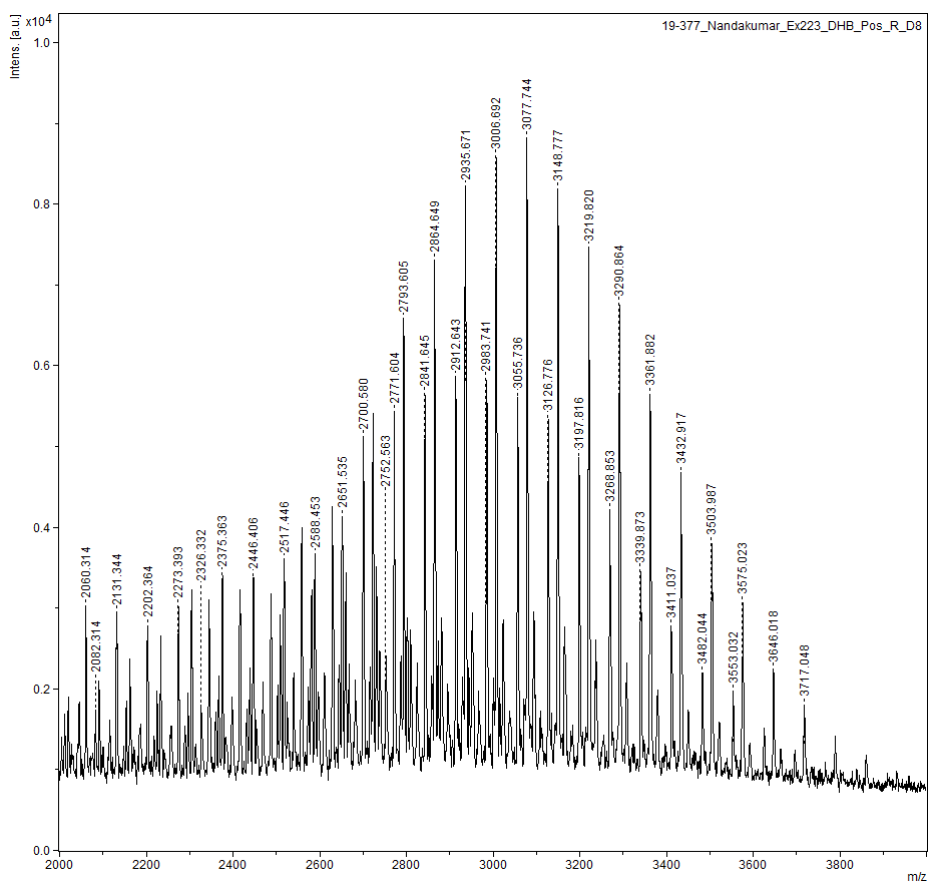
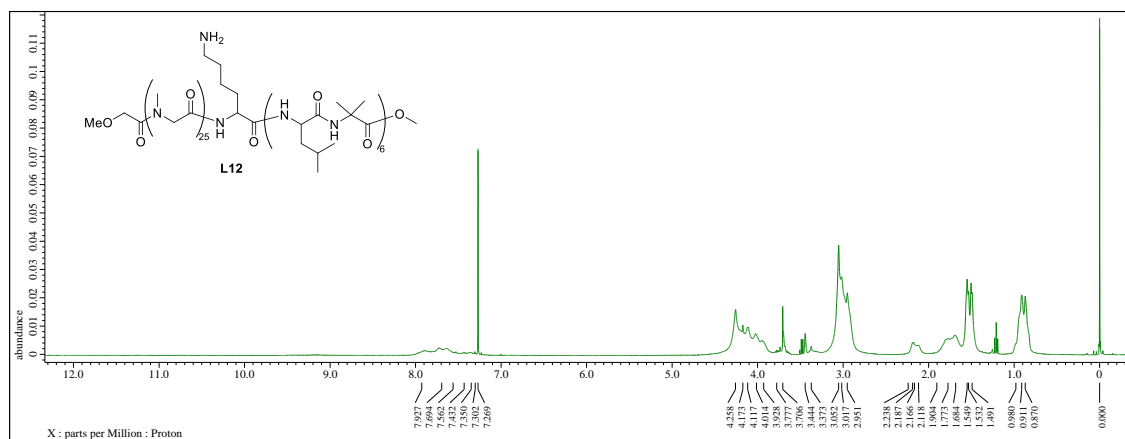


Fig. S2. NMR and MALDI-TOF MS of **L12**

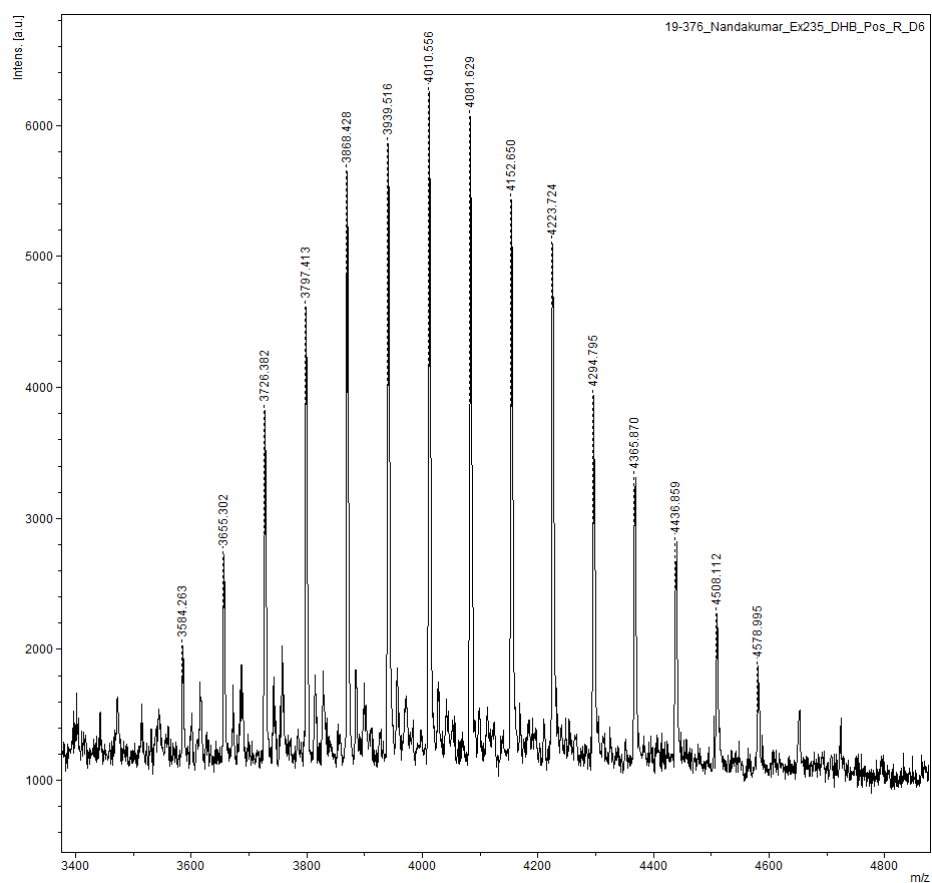
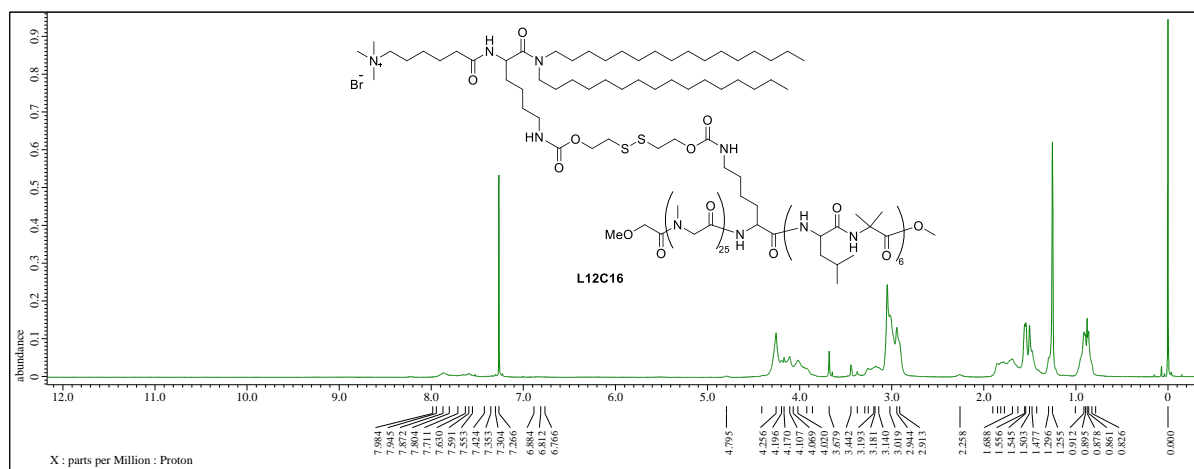


Fig. S3. NMR and MALDI-TOF MS of L12C16

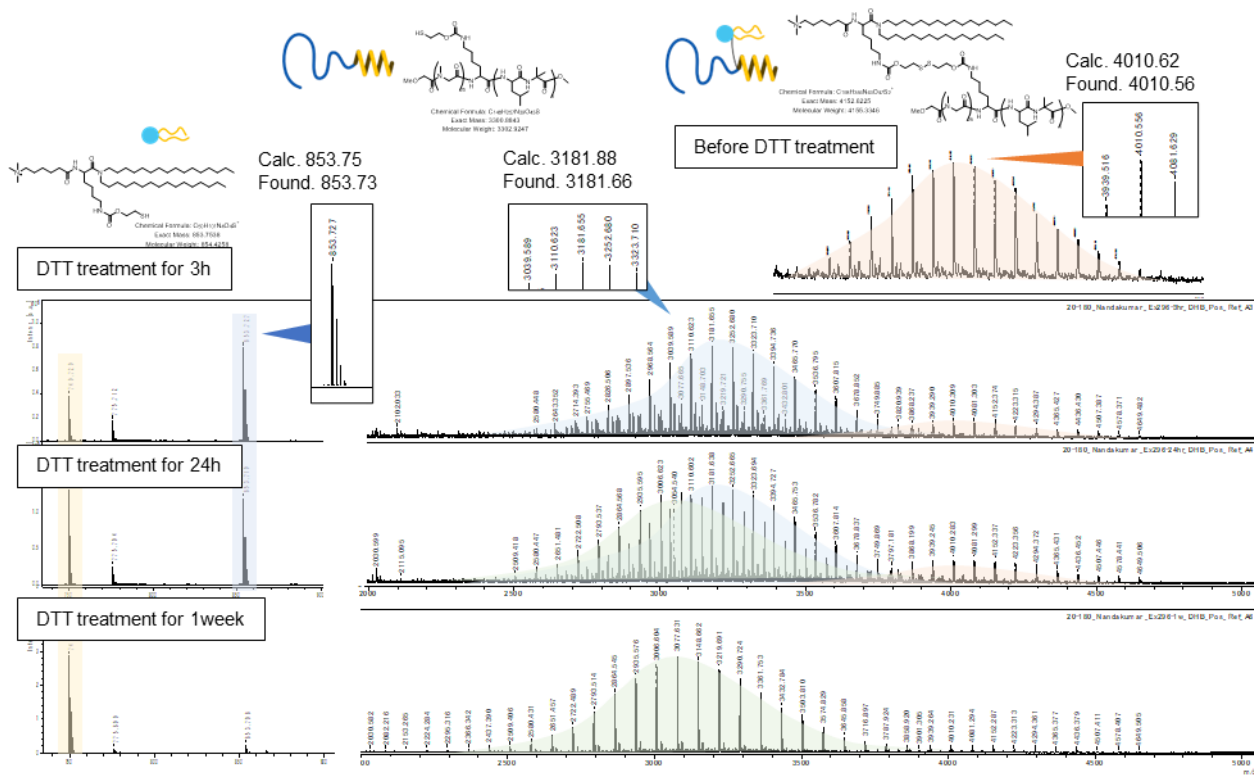


Fig. S4 MALDI-TOF MS spectra of L12C16 before and after treatment with DTT for 3h, 24h and 1 week.

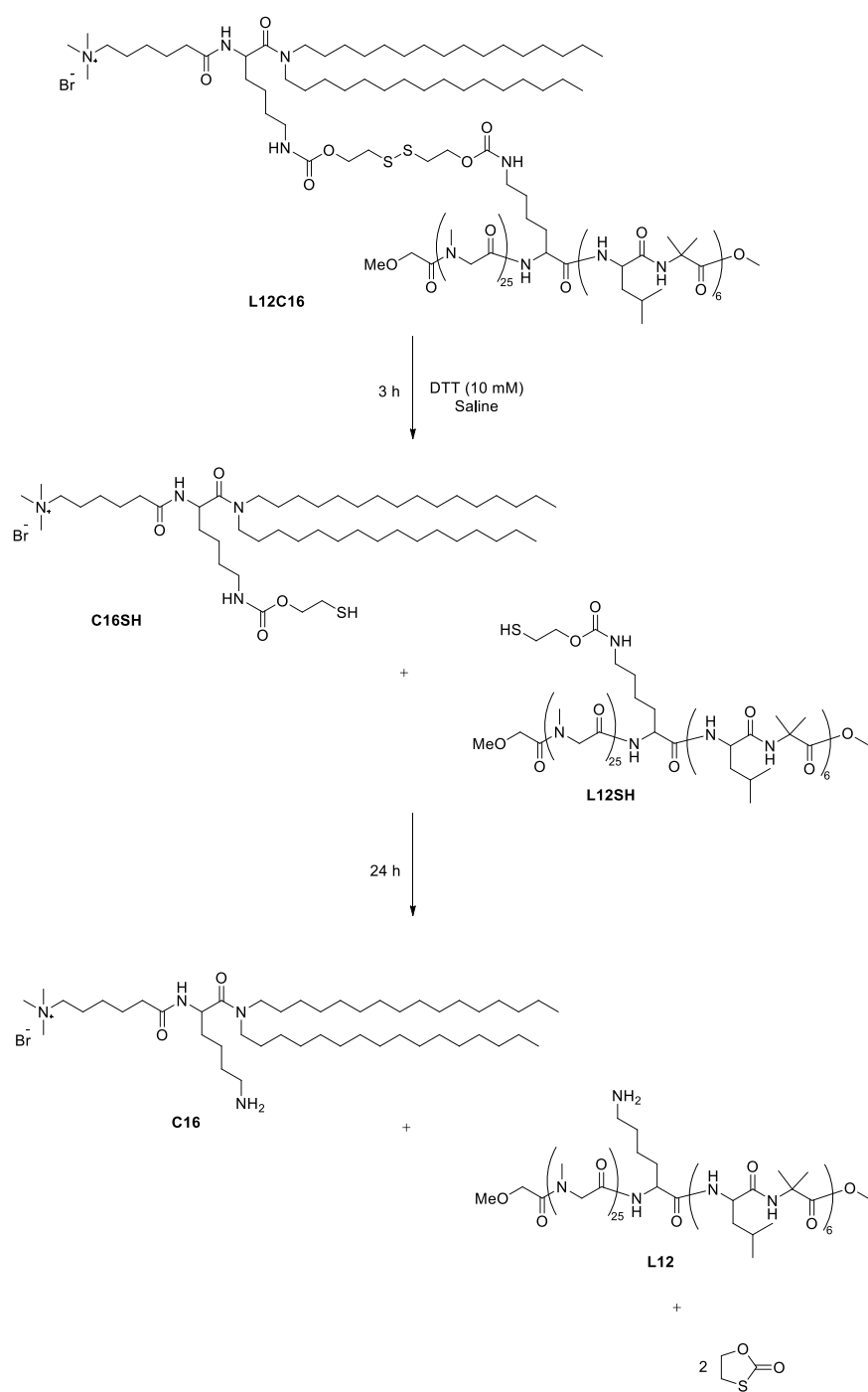


Fig. S5. Schematic representation of chemical transformation of **L12C16** in the presence of DTT over 24 h period

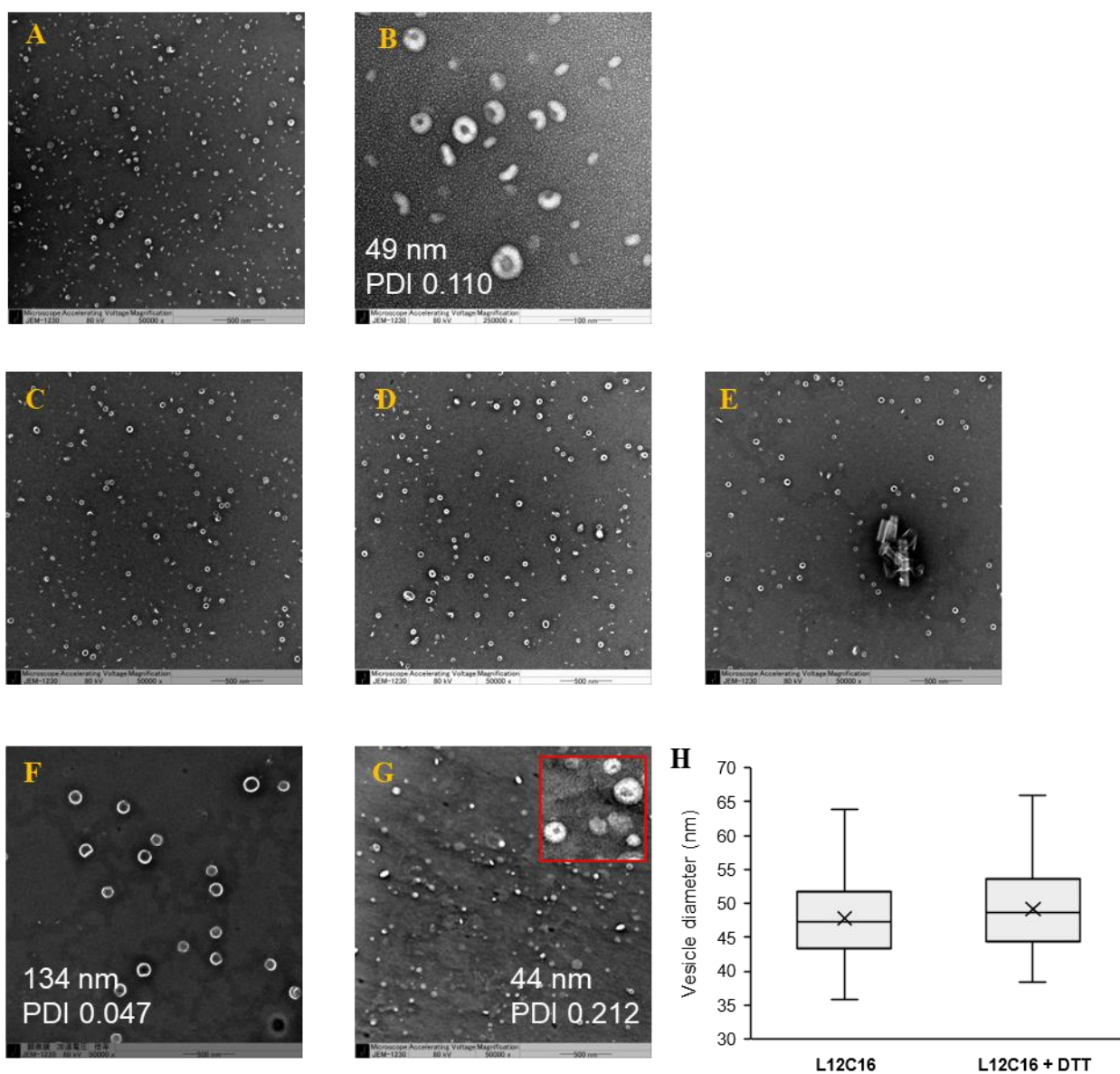


Fig. S6. TEM negative staining images of L12C16 before (A and B) and after DTT treatment for 1 h (C), 3 h (D) and 24 h ageing (E). TEM negative staining image of L12 (F) and C16 (G). Vesicle diameter of L12C16 and L12C16 after DTT treatment for 24 h from TEM images (H).

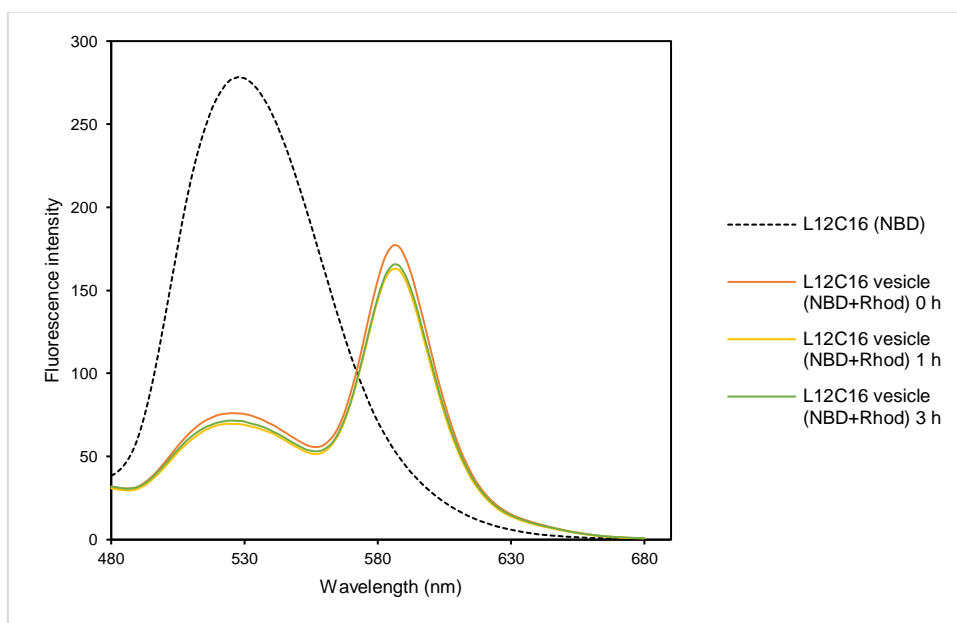


Fig. S7. Fluorescence spectra of L12C16 in the absence of DTT, containing NBD-PE (1.4 μmol) with and without Rhod-PE (1.4 μmol), in saline at 25 $^{\circ}\text{C}$ at an excitation wavelength of 460 nm

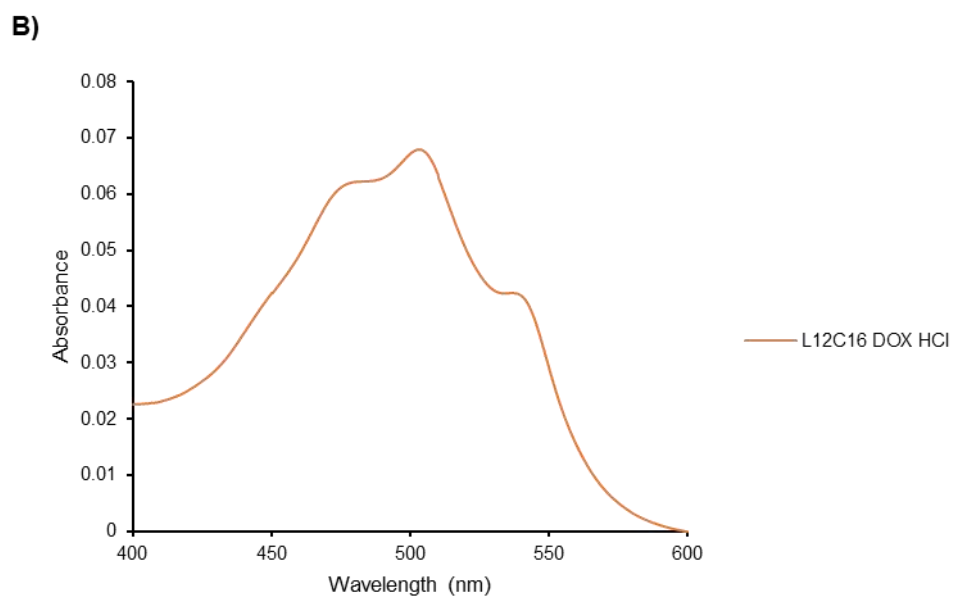
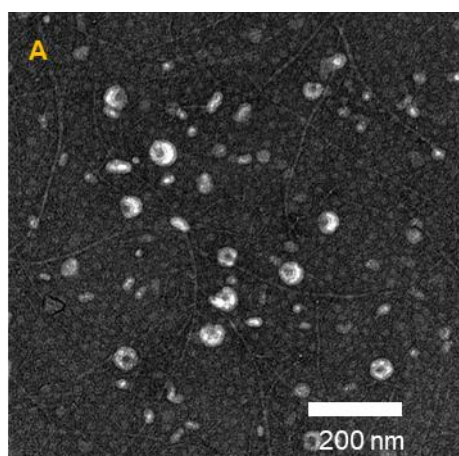


Fig. S8. TEM negative staining image of L12C16 after incorporation of DOX.HCl (A). Absorption spectra of L12C16 with DOX.HCl after treating with triton X100 (B) to calculate the encapsulation efficiency (EE = 4 %).

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