Supporting Information

Creation of polymersomes with changes in the membrane structure via Disulfide crosslinking under reductive conditions for controlled gradual cargo release

*Tomoya Kojima, Kouichi Asakura, and Taisuke Banno**

Department of Applied Chemistry, Keio University, 3-14-1 Hiyoshi, Kohoku-ku, Yokohama, Kanagawa, 223-8522, Japan

*E-mail: banno@applc.keio.ac.jp

Table of Contents

1. Materials and methods

1.1. Materials

Chloroform, dichloromethane, 1,4-dioxane, ethyl acetate, hexane, hydrogen chloride, methanol, pyridine, and tetrahydrofuran were purchased from Kanto Chemical Co. (Tokyo, Japan). 2-Bromoisobutyryl bromide, calcein, ethanethiol, glutathione (reduced form), iodine, 2-mercaptoethanol, methacryloyl chloride, Nile Red, polyethylene glycol monomethyl ether 2000, sodium chloride, sodium hydrogen carbonate, sodium sulfate, triethylamine, and tris(hydroxymethyl)aminomethane were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Acetonitrile, activated alumina, copper(I) bromide, diethylether, *N,N,N',N'',N''*-pentamethyldiethylenetriamine, and tetrahydrofuran with stabilizer for gel permeation chromatography (GPC) were purchased from FUJIFILM Wako Pure Chemical Co. (Osaka, Japan). Laurdan was purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). The samples were used as received, without further purification.

1.2. Instrumentation

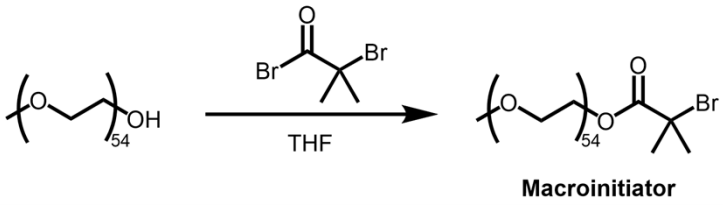
NMR spectra were obtained using an ECA-500 or ECA-400 Fourier-transform spectrometer (JEOL Co., Tokyo, Japan). Chemical shifts were based on the trimethylsilane peak at 0 ppm. Mass spectra were obtained using a timsTOF instrument (Bruker Co., Billerica, MA, USA). The samples for mass spectroscopy were prepared by dissolving the polymers in acetonitrile.

The molecular-weight distribution of the polymers was measured using a GPC system using stabilizer-included THF as the eluent at 1 mL/min. The GPC system consisted of a liquid transfer pump (LC-20AD; Shimadzu Co., Kyoto, Japan), refractive index detector (RID-20A; Shimadzu Co., Kyoto, Japan), and TSKgel GMH_{HR}-M column (Tosoh Co., Tokyo, Japan).

The morphology of the polymersomes was observed using a transmission electron microscope (Tecnai T12, FEI Co., Hillsboro, OR, USA) operated at 120 kV. The size distribution of the polymersomes was determined using dynamic light scattering (DLS; FDLS-3000, Otsuka Electronics Co., Ltd., Osaka, Japan). Infrared (IR) spectra were recorded using an IR spectrometer (ALPHA, Bruker Co., Billerica, MA, USA) in the attenuated total reflection (ATR) mode. Raman spectra were obtained using a LabRAM HR Evolution Raman spectrometer (Horiba Advanced Techno, Co., Ltd., Kyoto, Japan). Fluorescence spectra were obtained using an RF-6000 fluorescence spectrometer (Shimadzu Co., Kyoto, Japan).

1.3. Synthetic procedures

Synthesis of the macroinitiator



Scheme S1. Synthesis of the macroinitiator.

Polyethylene glycol monomethyl ether 2000 (2.00 g, 1.0 mmol) and triethylamine (0.42 mL, 3.0 mmol) were added to anhydrous tetrahydrofuran (20 mL). The mixture was cooled at 0 °C under an Ar atmosphere, and a solution of 2-bromoisobutyryl bromide (0.25 mL, 2.0 mmol) in anhydrous tetrahydrofuran (4 mL) was added dropwise. The resulting reaction mixture was stirred at 0 °C for 1 h and then at room temperature (23–25 °C) for 2 days under Ar. After the reaction, the solvent was evaporated, and the crude sample was filtered using toluene. The filtrate was evaporated and precipitated using chloroform as a good solvent and diethyl ether as a poor solvent. The precipitate was collected via filtration to obtain the macroinitiator as a white solid (2.06 g, 0.805 mmol, 81%). Regarding ^{1}H NMR analysis, the integration value of the singlet peak (3.38 ppm) derived from three hydrogens at the methoxy group was set as 3.00.

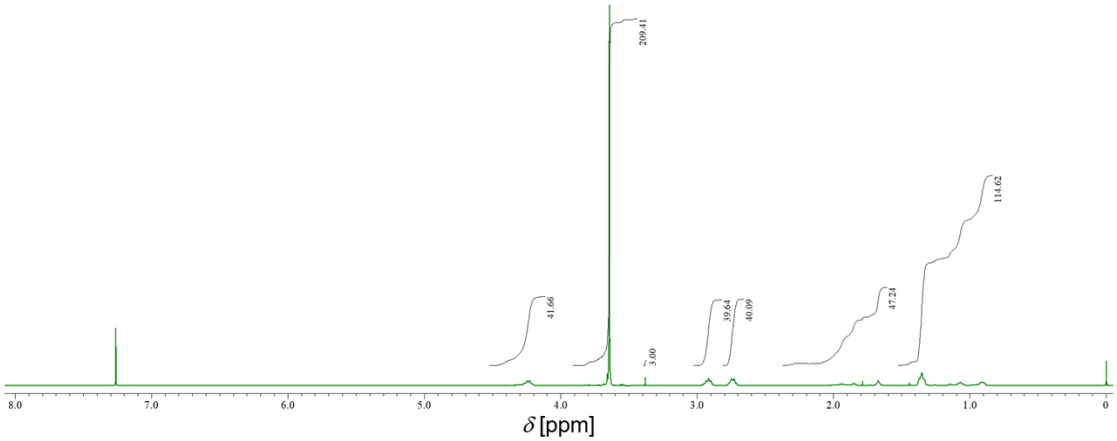


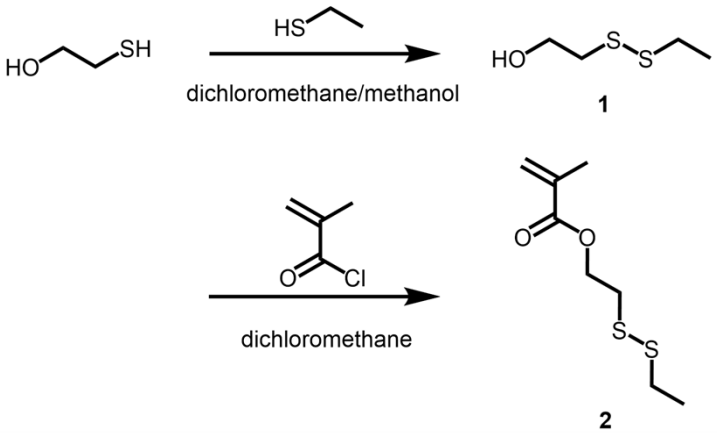
Figure S1. ^{1}H NMR spectrum of macroinitiator (500 MHz, $CDCl_3$).

^{1}H NMR (500 MHz, $CDCl_3$) δ 4.34–4.32 (m, 2H, -C<u>H</u>$_2$-O-(C=O)-), 3.79–3.49 (m, 211H, -(O-C<u>H</u>$_2$-C<u>H</u>$_2$)$_n$-), 3.38 (s, 3H, C<u>H</u>$_3$-O-CH$_2$-CH$_2$-), 1.94 (s, 6H, -O-(C=O)-C-(C<u>H</u>$_3$)$_2$).

Table S1. Molecular weight distribution of macroinitiator.

	NMR	GPC		M_w/M_n
	M_n	M_n	M_w	
Macroinitiator	2560	2932	3050	1.04

Synthesis of the disulfide-containing monomer



Scheme S2. Synthesis of 2-(ethyldisulfaneyl)ethyl methacrylate (**2**), the disulfide-containing monomer.

*Step 1: synthesis of 2-(ethyldisulfaneyl)ethan-1-ol (**1**)*

2-Mercaptoethanol (6.00 g, 76.8 mmol), ethanethiol (4.78 g, 76.8 mmol), and pyridine (12.4 mL, 154 mmol) were added to a mixture of anhydrous dichloromethane and methanol (1/1 v/v, 260 mL). Then, iodine (19.5 g, 76.8 mmol) was gradually added, and the resulting mixture was stirred at room temperature (23–25 °C) for 1 h. After the reaction, the solvent was evaporated, and the crude product was extracted using ethyl acetate and washed with water (×2) and brine (×1). After extraction, anhydrous sodium sulfate was added to the organic layer for dehydration, and the organic phase was collected by filtration. The filtrate was evaporated and purified using silica gel column chromatography (hexane/ethyl acetate = 2/1 v/v, R_f = 0.39) to obtain compound **1** as a colorless oil (6.94 g, 50.2 mmol, 65%).

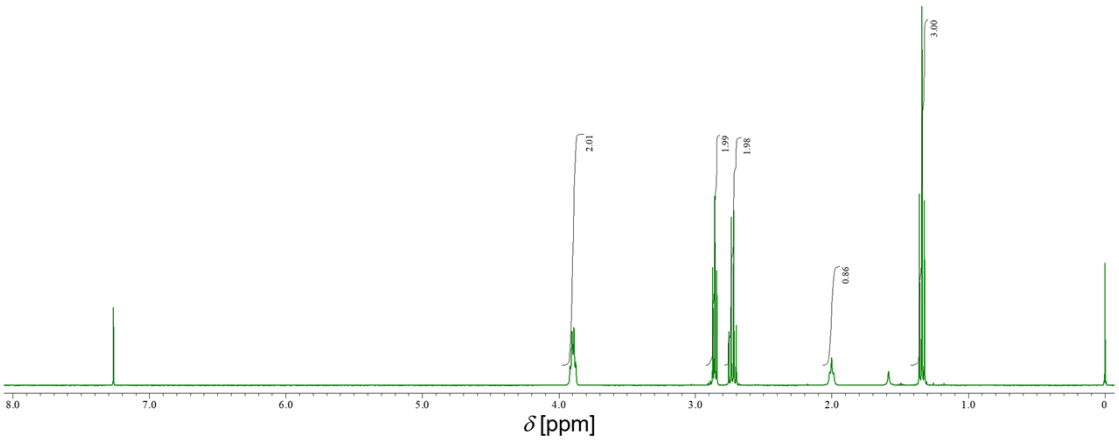


Figure S2. ^{1}H NMR spectrum of compound **1** (400 MHz, $CDCl_3$).

^{1}H NMR (400 MHz, $CDCl_3$) δ 3.90 (q, J = 5.7 Hz, 2H, HO-C$\underline{H}_2$-), 2.86 (t, J = 5.8 Hz, 2H, HO-CH_2-C$\underline{H}_2$-), 2.73 (q, J = 7.3 Hz, 2H, -S-S-C$\underline{H}_2$-CH_3), 2.00 (t, J = 5.7 Hz, 1H, $\underline{H}$O-CH_2-), 1.34 (t, J = 7.2 Hz, 2H, -S-S-CH_2-C$\underline{H}_3$).

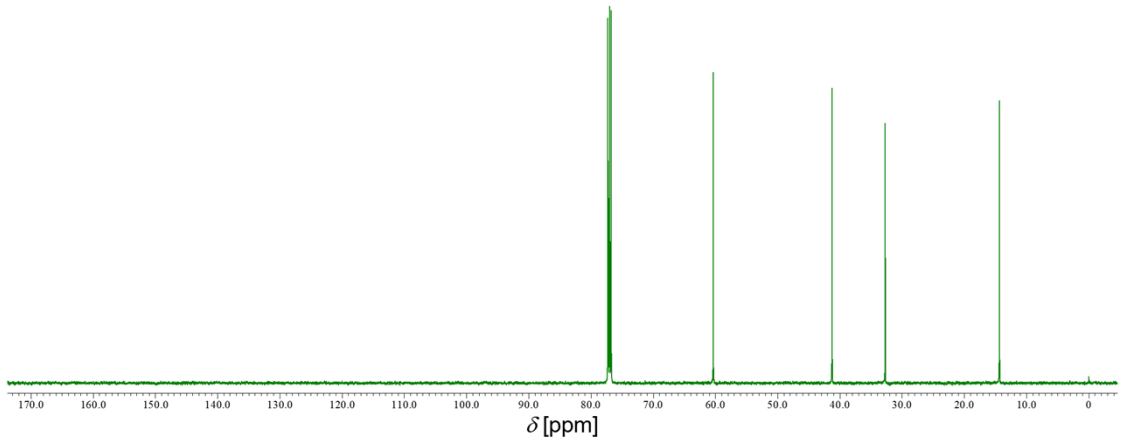


Figure S3. ^{13}C NMR spectrum of compound **1** (126 MHz, $CDCl_3$).

^{13}C NMR (126 MHz, $CDCl_3$) *d* 60.4 (HO-$\underline{C}H_2$-), 41.3 (HO-CH_2-$\underline{C}H_2$-), 32.7 (-S-S-$\underline{C}H_2$-CH_3), 14.4 (-S-S-CH_2-$\underline{C}H_3$).

*Step 2: synthesis of 2-(ethyldisulfaneyl)ethyl methacrylate (**2**)*

Compound **1** (6.00 g, 43.4 mmol) and triethylamine (13.8 mL, 100 mmol) were added to anhydrous dichloromethane (200 mL), and the mixture was cooled at 0 °C under an Ar atmosphere. Then, a solution of methacryloyl chloride (8.3 mL, 86.5 mmol) in anhydrous dichloromethane (30 mL) was added dropwise. The resulting mixture was stirred at room temperature (23–25 °C) for 2 days under an Ar atmosphere. Thereafter, the solvent was evaporated, and the crude product was extracted using chloroform (20 mL) and washed with a saturated $NaHCO_3$ solution (20 mL, ×3) and brine (20 mL, ×1). After extraction, anhydrous sodium sulfate was added to the organic layer for dehydration, and the organic phase was collected by filtration. The filtrate was evaporated and purified using silica gel column chromatography (hexane/ethyl acetate = 97/3 v/v, R_f = 0.21) to obtain compound **2** as a colorless oil (5.42 g, 26.3 mmol, 61%).

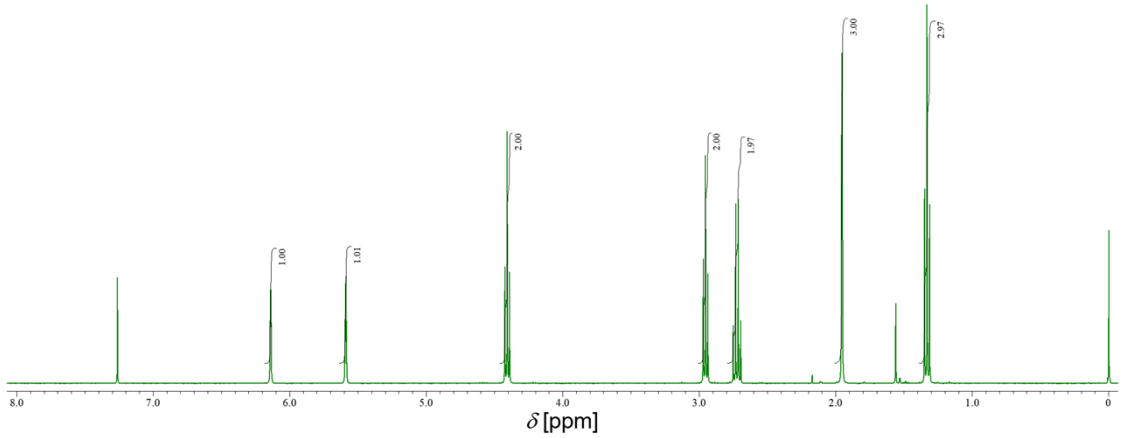


Figure S4. ^{1}H NMR spectrum of compound **2** (400 MHz, $CDCl_3$).

^{1}H NMR (400 MHz, $CDCl_3$) δ 6.18–6.14 (m, 1H, $\underline{H}_2C$=C-), 5.63–5.58 (m, 1H, $\underline{H}_2C$=C-), 4.41 (t, J = 6.8 Hz, 2H, -(C=O)-O-C$\underline{H}_2$-), 2.95 (t, J = 6.6 Hz, 2H, -(C=O)-O-CH_2-C$\underline{H}_2$-), 2.72 (q, J = 7.3 Hz, 2H, -S-S-C$\underline{H}_2$-CH_3), 1.96 (s, 1H, H_2C=C-C$\underline{H}_3$), 1.33 (t, J = 7.4 Hz, 3H, -S-S-CH_2-C$\underline{H}_3$).

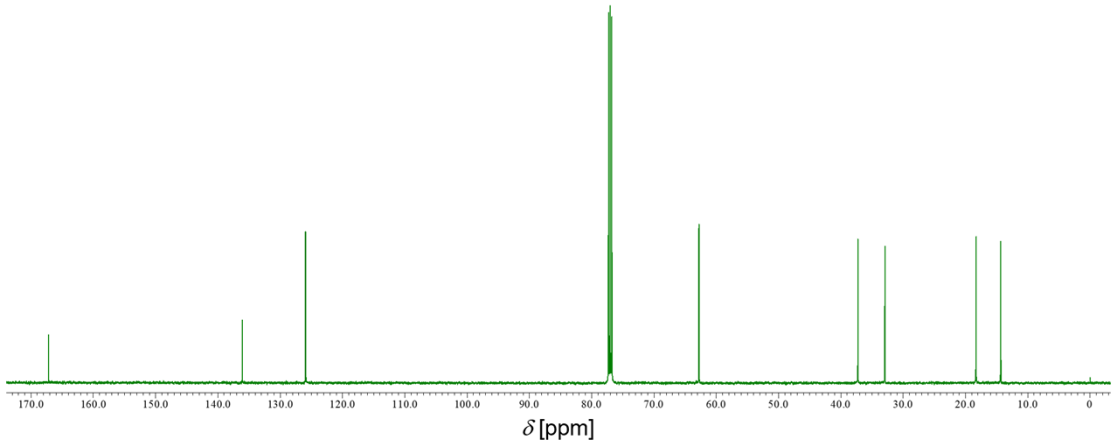
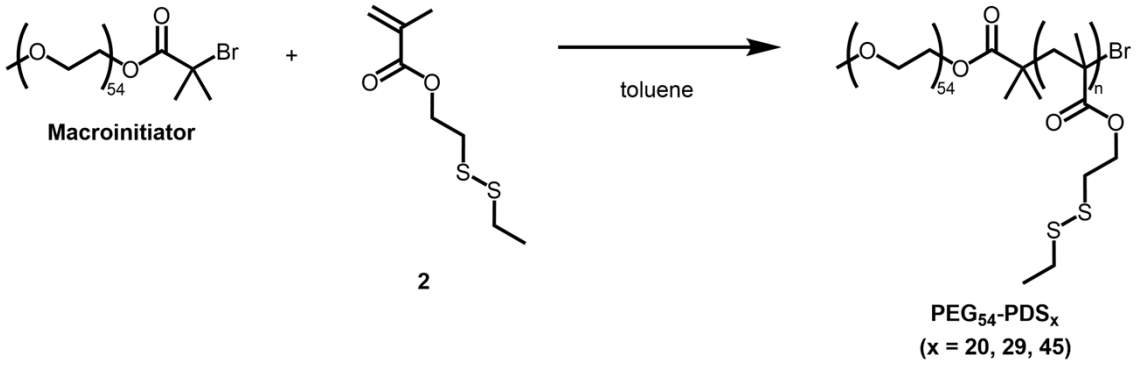


Figure S5. ^{13}C NMR spectrum of compound **2** (126 MHz, $CDCl_3$).

^{13}C NMR (126 MHz, $CDCl_3$) δ 167.2 (-(C=O)-O-), 136.1 (H_2C=C-), 125.9 (H_2C=C-), 62.8 (-(C=O)-O-CH_2-), 37.3 (-(C=O)-O-CH_2-CH_2-), 32.9 (-S-S-CH_2-CH_3), 18.3 (H_2C=C-CH_3), 14.3 (-S-S-CH_2-CH_3).
HR-ESI-MS (*m/z*): calcd. for $C_8H_{14}O_2S_2$ 229.0333 $[M+Na]^+$; found 229.0338 $[M+Na]^+$.

*Synthesis of disulfide-containing amphiphilic polymers (**PEG_{54}-b-PDS_x**)*



Scheme S3. Synthesis of PEG_{54}-*b*-PDS_x.

The macroinitiator (100 mg, 0.0391 mmol), CuBr (6 mg, 0.039 mmol), and compound **2** (203 mg, 0.984 mmol) were added to anhydrous toluene (500 μL). Then, *N,N,N',N'',N''*-pentamethyldiethylenetriamine (24 μL, 0.12 mmol) dissolved in anhydrous toluene (500 μL) was added, followed by anhydrous toluene (500 μL). The mixture was cooled at 0 °C under Ar gas flow for 30 min to remove oxygen. Thereafter, the flask was sealed tightly, and the mixture was stirred at 80 °C for 1 day under an Ar atmosphere. After the reaction, the crude product was purified using activated alumina column chromatography (chloroform). The obtained syrup was precipitated using chloroform as a good solvent (0.5 mL) and methanol or hexane as a poor solvent (10 mL) to obtain a viscous liquid (278 mg, n = 20). Using similar procedures, compound **2** (285 mg, 1.38 mmol for n = 29, 369 mg, 1.79 mmol for n = 45) was used instead to obtain a viscous liquid (348 mg for n = 29, 420 mg for n = 45). The compounds were identified using ^{1}H NMR spectroscopy (**Figure S6-S8**). The degree of polymerization (n) was calculated based on the ^{1}H NMR results. The molecular-weight distribution was determined using GPC (**Table S2**). Regarding ^{1}H NMR analysis, the integration value of the singlet peak (3.38 ppm) derived from three hydrogens at the methoxy group was set as 3.00.

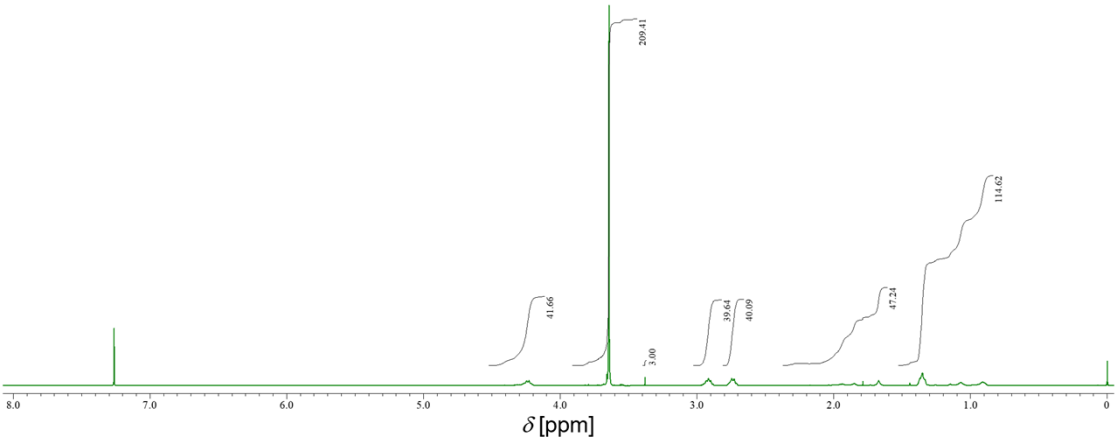


Figure S6. ^{1}H NMR spectrum of PEG_{54}-*b*-PDS_{20} (400 MHz, $CDCl_3$).

^{1}H NMR (400 MHz, $CDCl_3$) δ 4.49–4.21 (m, 42H, -C$\underline{H}_2$-CH_2-S-S-CH_2-CH_3, -C$\underline{H}_2$-O-(C=O)-), 3.91–3.45 (m, 209H, -(O-C$\underline{H}_2$-C$\underline{H}_2$)$_n$-), 3.38 (s, 3H, C$\underline{H}_3$-O-CH_2-CH_2-), 3.01–2.84 (m, 40H, -C$\underline{H}_2$-S-S-CH_2-CH_3), 2.75–2.66 (m, 40H, -S-S-C$\underline{H}_2$-CH_3), 2.33–1.67 (m, -O-(C=O)-C-(C$\underline{H}_3$)$_2$, -C(C$\underline{H}_3$)- in polymer chains), 1.51–0.87 (m, -S-S-CH_2-C$\underline{H}_3$, -C$\underline{H}_2$- in polymer chains).

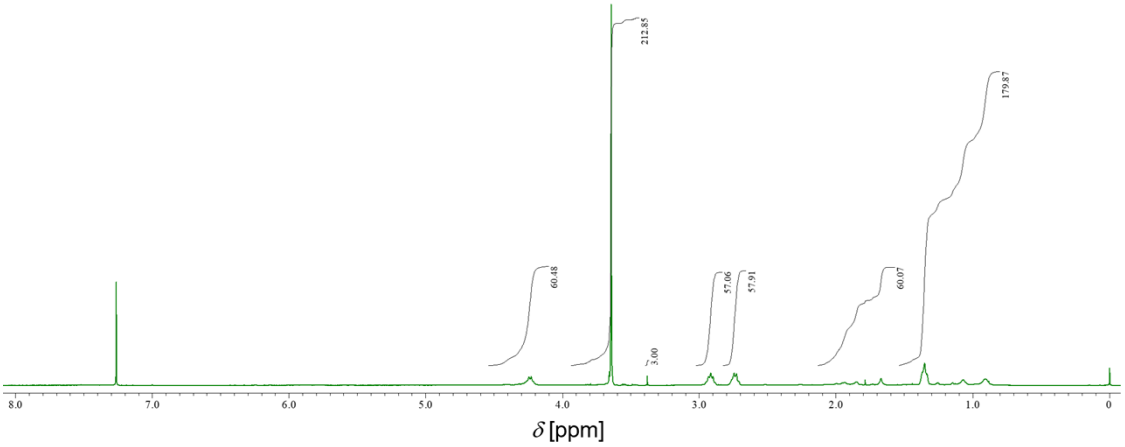


Figure S7. ^{1}H NMR spectrum of PEG_{54}-*b*-PDS_{29} (400 MHz, $CDCl_3$).

^{1}H NMR (400 MHz, $CDCl_3$) δ 4.43–4.14 (m, 60H, -C$\underline{H}_2$-CH_2-S-S-CH_2-CH_3, -C$\underline{H}_2$-O-(C=O)-), 3.91–3.45 (m, 213H, -(O-C$\underline{H}_2$-C$\underline{H}_2$)$_n$-), 3.38 (s, 3H, C$\underline{H}_3$-O-CH_2-CH_2-), 2.93–2.84 (m, 57H, -C$\underline{H}_2$-S-S-CH_2-CH_3), 2.75–2.68 (m, 58H, -S-S-C$\underline{H}_2$-CH_3), 2.11–1.67 (m, -O-(C=O)-C-(C$\underline{H}_3$)$_2$, -C(C$\underline{H}_3$)- in polymer chains), 1.51–0.87 (m, -S-S-CH_2-C$\underline{H}_3$, -C$\underline{H}_2$- in polymer chains).

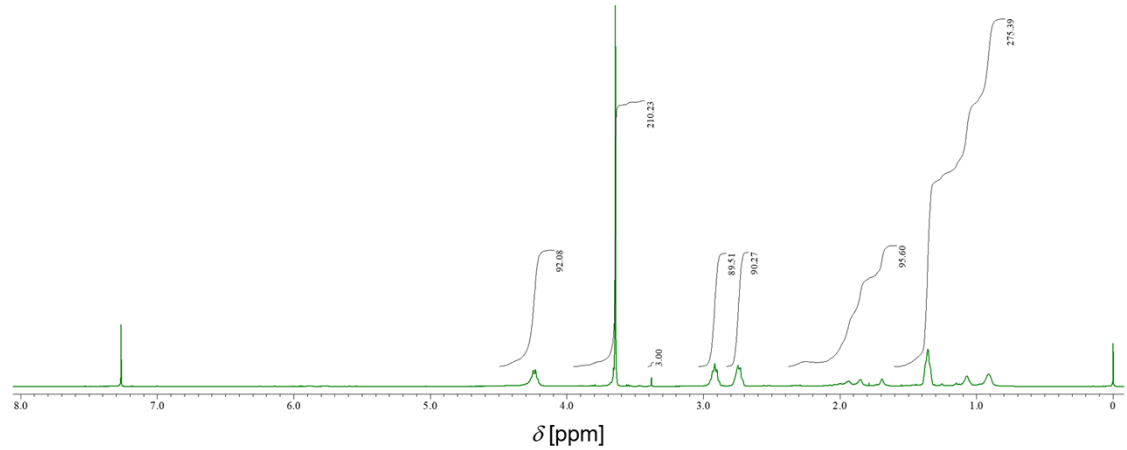


Figure S8. ^{1}H NMR spectrum of PEG_{54}-*b*-PDS_{45} (400 MHz, $CDCl_3$).

^{1}H-NMR (400 MHz, $CDCl_3$) δ 4.49–4.12 (m, 92H, -C$\underline{H}_2$-CH_2-S-S-CH_2-CH_3, -C$\underline{H}_2$-O-(C=O)-), 3.91–3.45 (m, 210H, -(O-C$\underline{H}_2$-C$\underline{H}_2$)$_n$-), 3.38 (s, 3H, C$\underline{H}_3$-O-CH_2-CH_2-), 3.04–2.84 (m, 90H, -C$\underline{H}_2$-S-S-CH_2-CH_3), 2.75–2.68 (m, 90H, -S-S-C$\underline{H}_2$-CH_3), 2.36–1.66 (m, -O-(C=O)-C-(C$\underline{H}_3$)$_2$, -C(C$\underline{H}_3$)- in polymer chains), 1.55–0.91 (m, -S-S-CH_2-C$\underline{H}_3$, -C$\underline{H}_2$- in polymer chains).

Table S2. Molecular weight distribution of PEG_{54}-*b*-PDS_x (x = 20, 29, and 45). DP represents degree of polymerization.

Polymer	Conversion (%)	DP	NMR	GPC		M_w/M_n
			M_n	M_n	M_w	
PEG_{54}-*b*-PDS_{20}	79	20	6700	2733	4024	1.47
PEG_{54}-*b*-PDS_{29}	82	29	8500	2797	4103	1.47
PEG_{54}-*b*-PDS_{45}	98	45	12000	3984	7448	1.87

1.4. Preparation of polymersomes

A Tris-HCl buffer solution (10 mM, pH 7.4, 1 mL) was gradually added to a solution of PEG_{54}-PDS_x (10 mg/mL, 1 mL) in THF/1,4-dioxane (4/1 v/v) at 1 mL/h using a syringe pump (Legato 180, KD Scientific Inc., Holliston, MA, USA) with vigorous stirring. The obtained suspension was then loaded into a dialysis bag (2-316-08, Japan Medical Science Co., Osaka, Japan, MWCO: *ca.* 3500) and dialyzed overnight against a Tris-HCl buffer solution (10 mM, pH 7.4, 1 L, ×3) to remove the organic solvent. After dialysis, the polymersome suspension was collected.

1.5. Characterization of polymersomes

The obtained polymersomes were observed using a TEM. The polymersome suspensions were diluted 10 times using a Tris-HCl buffer solution (10 mM, pH 7.4), and each diluted suspension (3 μL) was deposited onto a Cu grid coated with collodion (6511; NISSHIN EM Co., Ltd., Tokyo, Japan) and left overnight at room temperature (23–25 °C) for solvent evaporation. The samples were observed at an operating voltage of 120 kV. The size distribution of the polymersomes was analyzed by conducting the DLS of the polymer suspensions without dilution. For the characterization of the sample reacted with GSH, a GSH solution (250 mM, 40 μL) was added to polymersome suspensions (1.96 mL), and they were allowed to react at 37 °C for 6 h. Note that the final GSH concentration was 5 mM. After the reaction, the suspensions were analyzed by TEM and DLS using the aforementioned procedures.

1.6. Reaction analysis

A GSH solution (250 mM, 20 μL) was added to polymersome suspensions (980 μL), and the samples were allowed to react at 37 °C for 6 h. After the reaction, the obtained samples were lyophilized, and THF (1 mL) was added. Only the supernatants were collected for GPC analyses. The residue was removed, because it was water soluble and was estimated to be derived from the Tris-HCl salt. The molecular-weight distributions of the collected supernatants were analyzed using GPC. In addition, the supernatant was evaporated and analyzed using infrared (IR) and Raman spectroscopy. Control experiments were carried out without adding GSH.

1.7. Evaluation of the changes in the membrane structures

A GSH solution (250 mM, 10 μL) was added to each polymersome suspension (490 μL), and the samples were allowed to react at 37 °C for 6 h. After the reaction, an ethanol solution of Laurdan (4 μL) was added to the suspensions (400 μL), and the samples were analyzed using fluorescence spectroscopy. Further, an ethanol solution of Nile Red (5 μL) was added to another batch of suspensions (500 μL), and the resulting samples were analyzed using fluorescence spectroscopy. Control experiments were carried out without the addition of GSH. The generalized polarization (GP) of Laurdan was calculated to estimate the membrane fluidity, using the following equation:

$$GP = \frac{I_{440} - I_{490}}{I_{440} + I_{490}} \qquad (S1)$$

where I_{440} is the fluorescence intensity of Laurdan at 440 nm and I_{490} is the fluorescence intensity at 490 nm.[53] Control experiments were carried out without adding GSH.

1.8. Measurement of the cargo release

A Tris-HCl buffer solution (10 mM, pH 7.4, 1 mL) containing calcein (2 mM) was gradually added to the PEG_{54}-*b*-PDS_x solution (10 mg/mL, 1 mL) in tetrahydrofuran/1,4-dioxane (4/1 v/v) at 1 mL/h rate using a syringe pump (Legato 180, KD Scientific Inc., Holliston, MA, USA) under vigorous stirring. The obtained suspension was then loaded into a dialysis bag (2-316-08, Japan Medical Science Co., Osaka, Japan, MWCO: ca. 3500) and dialyzed overnight against Tris-HCl buffer (10 mM, pH 7.4, 1 L, ×3) to remove the organic solvent and calcein dissolved in water. After dialysis, the calcein-incorporated polymersome suspensions were collected.

The polymersome suspensions (2 mL) were added to a dialysis bag (2-316-08, Japan Medical Science Co., Osaka, Japan; MWCO: *ca.* 3500), and the bag was immersed in a Tris-HCl buffer solution (10 mM, pH 7.4, 30 mL) containing GSH (5 mM) and allowed to react at 37 °C with stirring. The external solution (500 μL) was collected and replaced with a fresh Tris-HCl buffer solution (10 mM, pH 7.4, 500 μL) with GSH (5 mM) every 30 min at 0−3 h and every 1 h at 3−6 h. The collected solution was analyzed using fluorescence spectroscopy. Control experiments were carried out without adding GSH. GSH solutions of different concentrations (1.0 and 2.5 mM) were used to compare the release rate of calcein in response to the GSH concentration. Encapsulation efficiency (EE, wt%) and loading content (LC, wt%) were calculated[1] in the following:

$$EE\ [wt\%] = \frac{Weight\ of\ calcein\ in\ polymersomes}{Weight\ of\ feeding\ calcein} \times 100 \qquad (S2)$$

$$LC\ [wt\%] = \frac{Weight\ of\ calcein\ in\ polymersomes}{Weight\ of\ amphiphilic\ polymer} \times 100 \qquad (S3)$$

2. Supplementary figures and tables

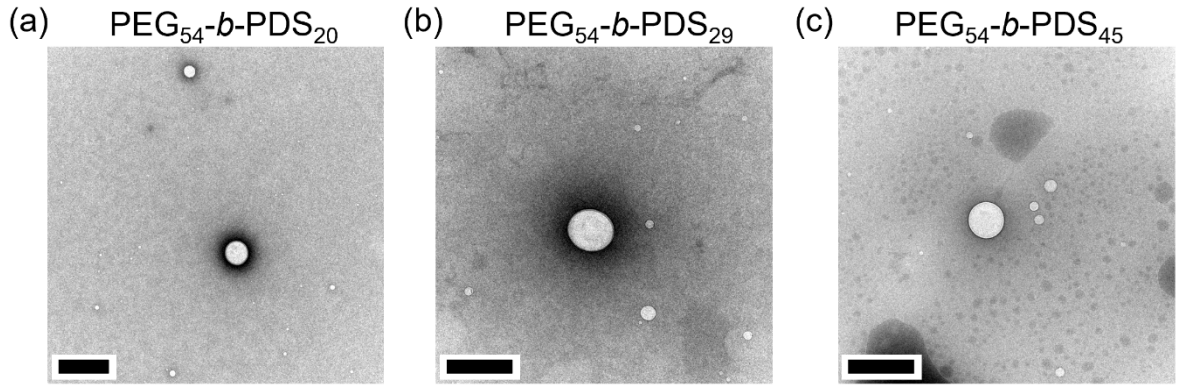


Figure S9. Wide-field TEM images of polymersomes comprising (a) PEG_{54}-*b*-PDS_{20}, (b) PEG_{54}-*b*-PDS_{29}, and (c) PEG_{54}-*b*-PDS_{45}. Scale bars: (a) 1 μm, (b, c) 0.5 μm.

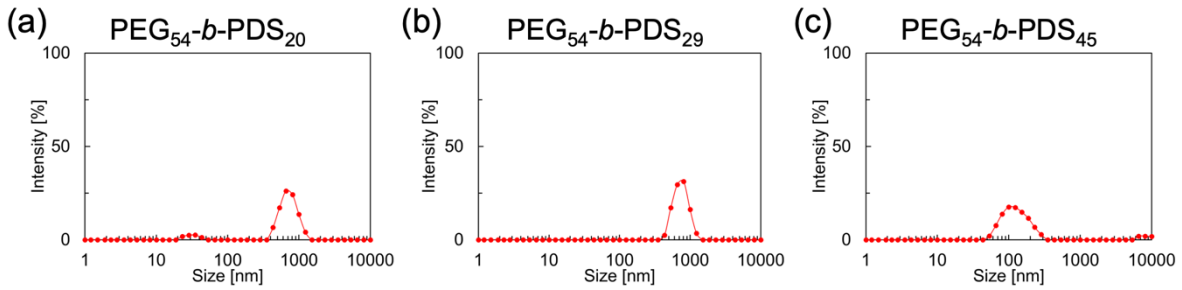


Figure S10. Size distribution obtained by DLS for the polymersomes comprising (a) PEG_{54}-*b*-PDS_{20}, (b) PEG_{54}-*b*-PDS_{29}, and (c) PEG_{54}-*b*-PDS_{45}.

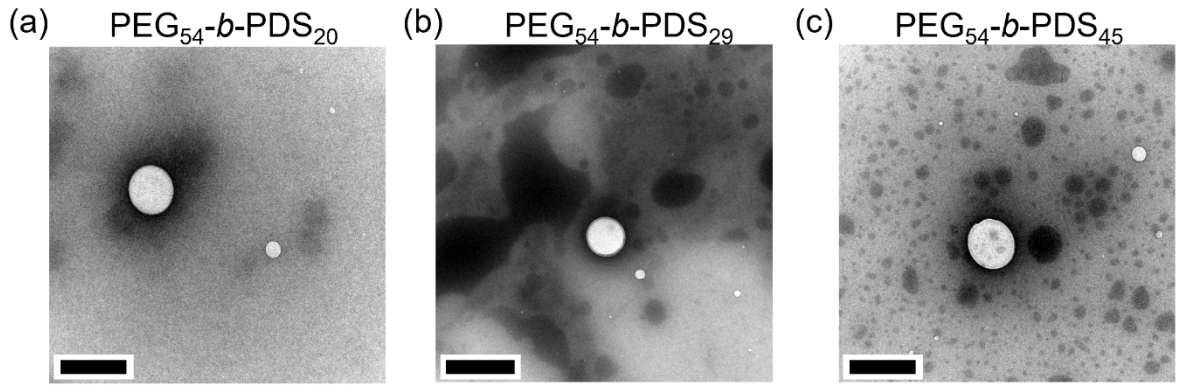


Figure S11. Wide-field TEM images of polymersomes comprising (a) PEG_{54}-*b*-PDS_{20}, (b) PEG_{54}-*b*-PDS_{29}, and (c) PEG_{54}-*b*-PDS_{45} after incubation with GSH (5 mM) in Tris-HCl buffer for 6 h. Scale bars: 200 nm.

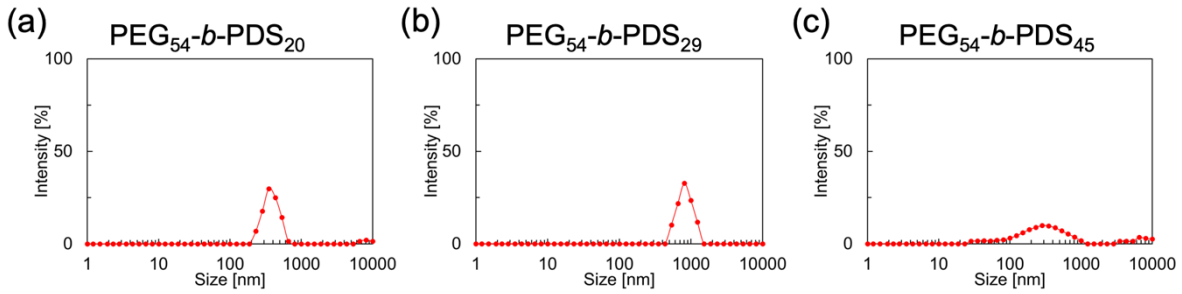


Figure S12. Size distribution of the polymersomes comprising (a) PEG_{54}-*b*-PDS_{20}, (b) PEG_{54}-*b*-PDS_{29}, and (c) PEG_{54}-*b*-PDS_{45}, as obtained by DLS after being incubated with GSH (5 mM) for 6 h.

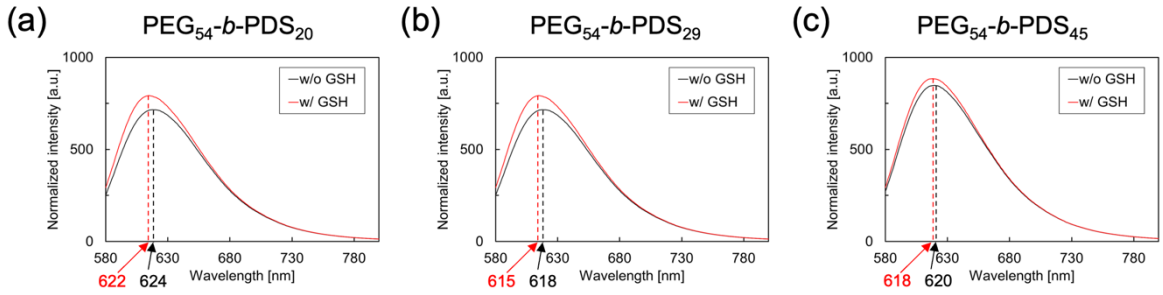


Figure S13. Changes in the fluorescent spectra of Nile Red incorporated in polymersomes comprising (b) PEG_{54}-*b*-PDS_{20}, (c) PEG_{54}-*b*-PDS_{29}, and (d) PEG_{54}-*b*-PDS_{45} with/without GSH.

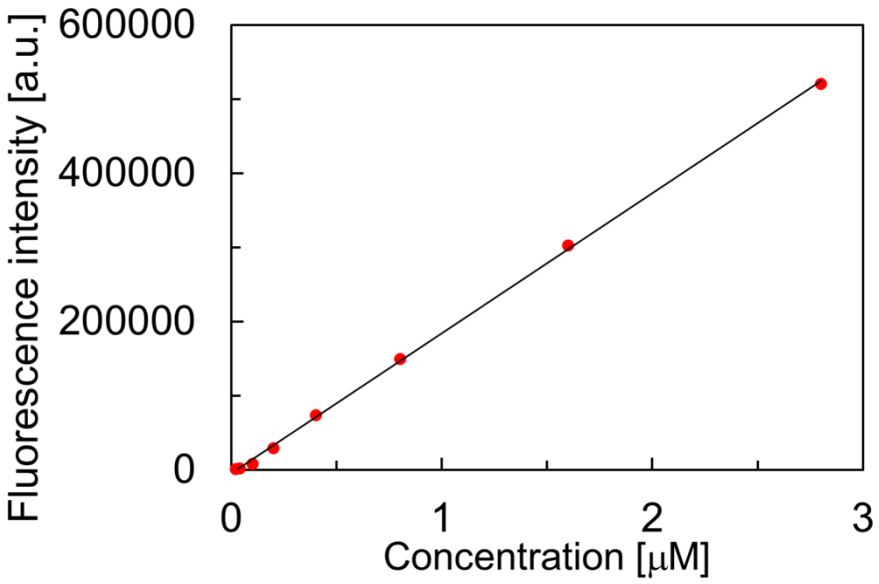


Figure S14. Calibration curve of fluorescence intensity of calcein.

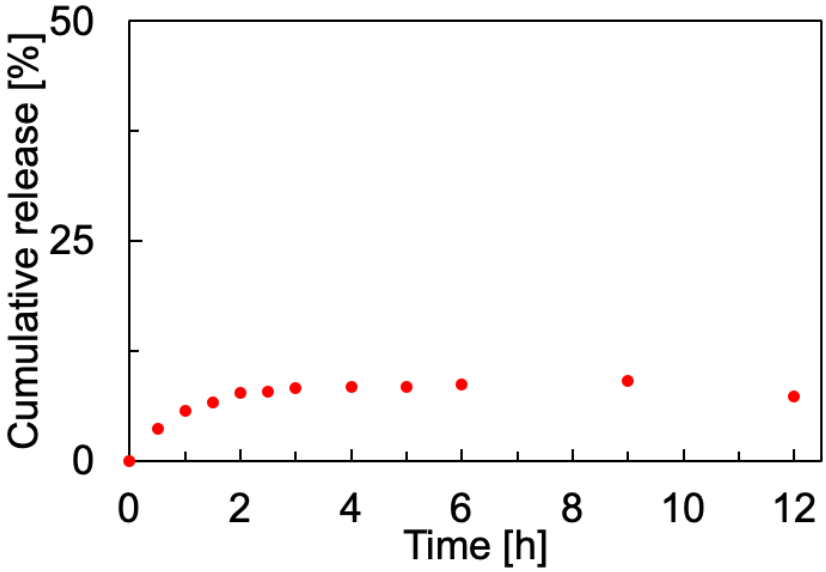


Figure S15. Release of calcein from polymersomes comprising PEG_{54}-*b*-PDS_{20} with/without GSH (5 mM) for 0-12 h.

Table S3. Encapsulation efficiency (EE) and loading content (LC) of calcein using polymersomes composed of PEG_{54}-*b*-PDS_x (x = 20, 29, and 45).

Polymer	EE [wt%]	LC [wt%]
PEG_{54}-*b*-PDS_{20}	26 ± 2	3.3 ± 0.2
PEG_{54}-*b*-PDS_{29}	28 ± 3	3.4 ± 0.4
PEG_{54}-*b*-PDS_{45}	28 ± 3	3.5 ± 0.4

3. References

1. C. Ferrero, M. Casas and I. Caraballo, *Pharmaceutics*, 2022, **14**, 1724.