Supplementary Information

for

Bioorthogonal Micellar Nanoreactors for Prodrug Cancer Therapy using an Inverse-electron-demand Diels-Alder Reaction

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Materials

Materials for the synthesis of the amphiphilic block copolymers.

Poly(ethylene glycol) methyl ether (4-cyano-4-pentanoate dodecyltrithiocarbonate) (average molecular weight = 5.4×10^3 g mol^{-1}, M_w/M_n(SEC) = 1.1) was purchased from Sigma-Aldrich Co. LLC (Sigma-Aldrich) and used as received. 2,2'-Azobis(isobutyronitrile) (AIBN; purity > 98%), *N*-succinimidyl methacrylate (purity > 98%), and triethylamine (purity > 99%) were purchased from Tokyo Chemical Industry Co., Ltd. (TCI) and used as received. Methyltetrazine amine (purity > 95%) was purchased from Click Chemistry Tools (Scottsdale, AZ). *N*,*N*-Dimethylformamide (DMF; super dehydrated for organic synthesis) were purchased from FUJIFILM Wako Pure Chemical Corporation (Wako). DMF was used after drying over activated molecular sieves (4 Å) overnight.

Materials for the synthesis of doxorubicin prodrug

Cyclooctene (purity > 95.0%), *N*-bromosuccinimide (NBS; purity > 98%), methyl benzoate (purity > 99%), *N*,*N*-diisopropylethylamine (purity > 99%), and 4-nitrophenyl chloroformate (purity > 98%) were purchased from TCI and used as received. Sodium hydrogen carbonate ($NaHCO_3$; purity > 99.5%), carbon tetrachloride (CCl_4; purity > 99.5%), silver nitrate (purity > 99.8%) acetone (super dehydrated for organic synthesis; purity > 99.5%), hexane (super dehydrated for organic synthesis; purity > 99.5%), and pyridine (super dehydrated for organic synthesis; purity > 99.5%) were purchased from Wako. Doxorubicin hydrochloride was purchased from Sigma-Aldrich.

Materials for the synthesis of Cy5-functionalized amphiphilic block copolymers

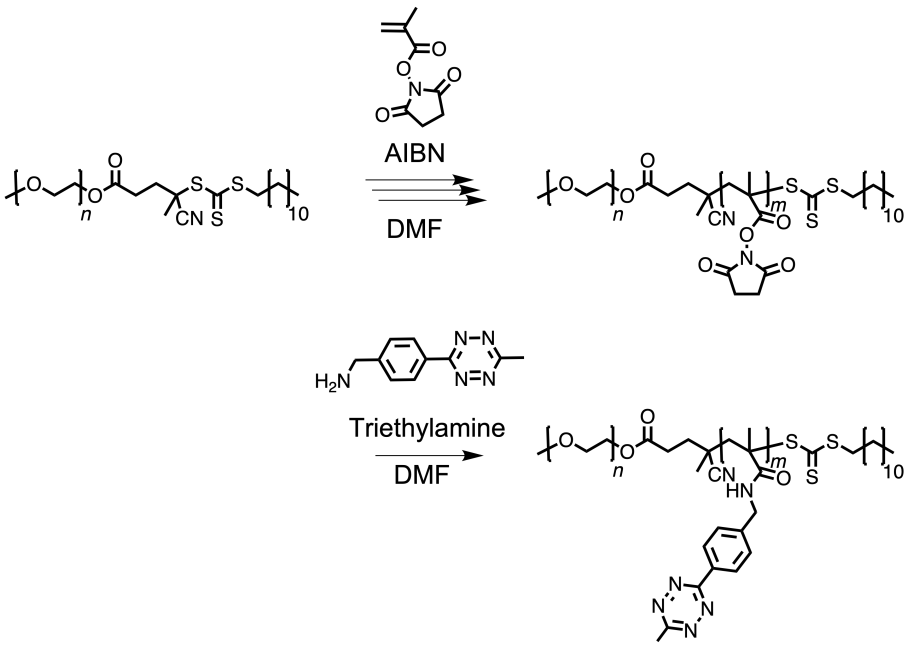
(1-Cyano-2-ethoxy-2-oxoethylidenaminooxy) dimethylaminomorpholinocarbenium hexafluorophosphate (COMU; purity > 98%) and ethyl cyano(hydroxyimino) acetate (Oxyma; purity > 98%) were purchased from TCI. Cy5-amine (purity = 99.0%) was purchased from Broadpharm.

Synthesis

NMR spectroscopy

^{1}H NMR spectra were recorded in chloroform-*d*, or dimethylsulfoxide (DMSO)-d_6 using a Bruker Avance III OneBay 400 MHz spectrometer. Chemical shifts (δ) are expressed in parts per million (ppm) and reported relative to tetramethylsilane (TMS; 0 ppm) as the internal standard.

The synthesis of the polymers was carried out using a syringe technique under an atmosphere of argon in a Schlenk tube equipped with a three-way stopcock. The amphiphilic block copolymer was synthesized as outlined in **scheme S1**.



Scheme S1. Scheme for the synthesis of tetrazine-bearing amphiphilic block copolymer. Step 1. RAFT polymerization of *N*-succinimidyl methacrylate in the presence of PEG-RAFT agent and AIBN in DMF. Step 2. Coupling reaction of methyl tetrazine amine and poly(ethylene glycol)-*block*-poly(*N*-succinimidyl methacrylate) in the presence of triethylamine in DMF.

Synthesis of poly(ethylene glycol)-*block*-poly(*N*-succinimidyl methacrylate)

Poly(ethylene glycol) methyl ether (4-cyano-4-pentanoate dodecyltrithiocarbonate) (0.5 g, 93 μmol), *N*-succinimidyl methacrylate (0.68 g, 3.7 mmol), AIBN (3 mg, 19 μmol), and DMF (3 mL) were added to a dry flask under an argon atmosphere, and the resulting solution was stirred for 24 h at 70 ºC. The reaction solution was poured into diethyl ether (1 L). The precipitate was collected by filtration and was washed wither diethyl ether. Subsequently, the solvent was removed under reduced pressure to yield a pale-yellow powder (1.0 g). The ^{1}H NMR spectrum in dimethyl sulfoxide-d_6 is shown in **Fig. S1**. ^{1}H NMR (400 MHz, DMSO-d_6, r.t.) δ 3.51 (—OC$\underline{H_2}$C$\underline{H_2}$—), 3.32 (—COC$\underline{H_2}$C$\underline{H_2}$CO—), 3.0–2.2 (—C$\underline{H_2}$C(CH$_3$)CO—), 1.7–0.9 (—CH$_2$C(C$\underline{H_3}$)CO—).

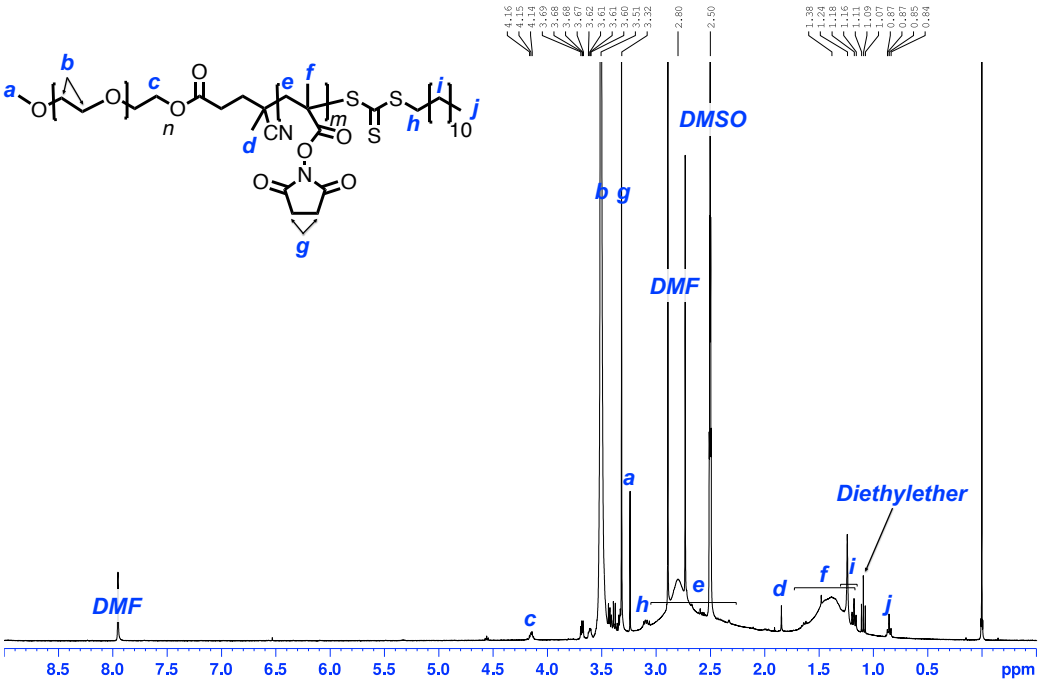


Fig. S1 ^{1}H NMR spectrum of poly(ethylene glycol)-*block*-poly(*N*-succinimidyl methacrylate) in DMSO-d_6.

Synthesis of the tetrazine bearing amphiphilic copolymer

Poly(ethylene glycol)-*block*-poly(*N*-succinimidyl methacrylate) (0.35 g, 1.0 mmol), methyltetrazine amine (0.25 g, 1.24 mmol), triethylamine (19 mg, 1.3 mmol), and DMF (10 mL) were added to a dry flask under an argon atmosphere, and the resulting solution was stirred for 72 h at 45 ºC. The crude product was purified by dialysis (regenerated cellulose membrane; Spectra/Por® 6; MWCO 1000) against methanol for 72 h and then double distilled water for 48 h. Subsequently, the solvent was lyophilized to yield a pink powder (0.32 g). The ^{1}H NMR spectrum in dimethyl sulfoxide-d_6 is shown in **Fig. S2**. ^{1}H NMR (400 MHz, DMSO-d_6, r.t.) δ 11.7 (—$CH_2CCOOH(CH_3)$—), 8.5 (—$CONHCH_2$—), 8.33 and 7.45 (phenyl), 4.35 (—$CONHCH_2$—), 3.51 (—OCH_2CH_2—), 2.94 (tetrazine), 3.0–2.2 (—$CH_2C(CH_3)CO$—), 1.5–0.7 (—$CH_2C(CH_3)CO$—).

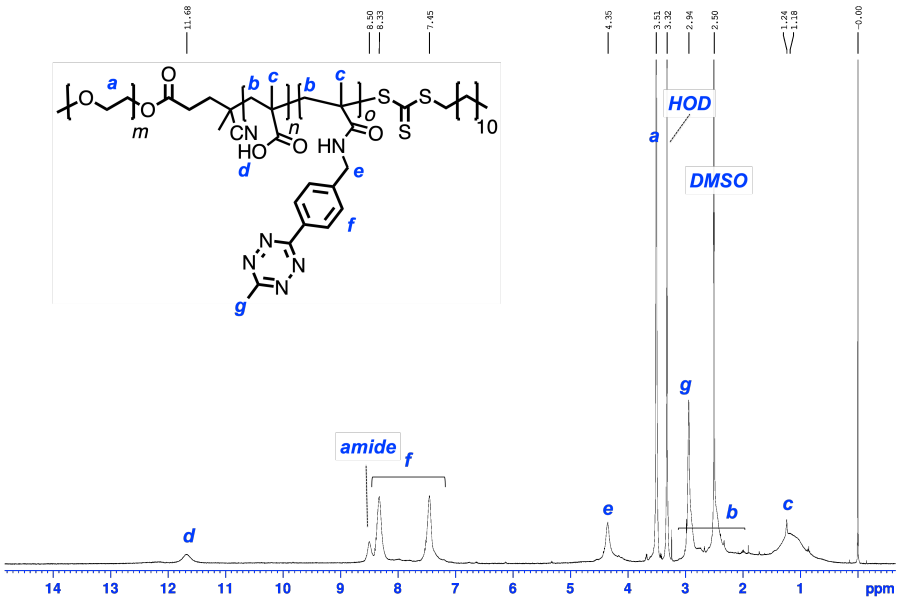


Fig. S2 ^{1}H NMR spectrum of tetrazine-functionalized amphiphilic block copolymer in DMSO-d_6.

Synthesis of (*Z*)-cyclooct-2-enol

Cyclooctene (8.56 g, 77.7 mmol), NBS (12.2 g, 68.4 mmol), AIBN (7.7 mg, 47 μmol), and CCl_4 (400 mL) was added to a dry flask under an argon atmosphere. The mixture solution was stirred for 2 h at 90 ºC. The solution was cooled to 0 ºC with an ice bath, and the precipitate was removed by filtration. The solvent was removed under reduced pressure to give (*Z*)-3-bromocyclooctene as pale-yellow oil. The crude product was dissolved in a mixture solution of acetone (80 mL) and water (40 mL), followed by addition of $NaHCO_3$ (10.2 g, 121 mmol). The mixture solution was stirred for 2 h at 65 ºC. The precipitate was removed by filtration, and the filtrate was extracted with diethyl ether. The organic layer was dried over sodium sulfate and was removed under reduced pressure. The crude product was purified using column chromatography over silica gel (hexane/ethyl acetate = 9/1 to 7/3, v/v, eluent) to give 4.5 g (46%) of (*Z*)-cyclooct-2-enol as a pale-yellow oil. The ^{1}H NMR spectrum is shown in **Fig. S3**. ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.66–5.52 (m, 2H), 4.68–4.65 (m, 1H), 2.24–2.06 (m, 2H), 1.95–1.89 (m, 1H), 1.71–1.35 (m, 9H).

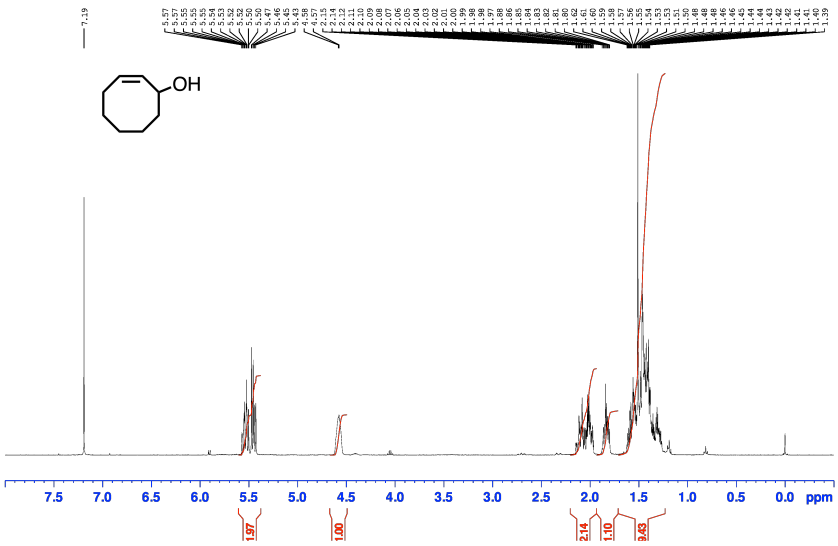


Fig. S3 ^{1}H NMR spectrum of (*Z*)-cyclooct-2-enol in chloroform-*d*.

Synthesis of (*E*)-cyclooct-2-enol

(*Z*)-cyclooct-2-enol (2.0 g, 15.8 mmol), methyl benzoate (2.2 g, 15.5 mmol), diethyl ether (400 mL), and hexane (400 mL) were added to a photochemical reaction vessel (VG300, SEN LIGHTS Corp., Japan). The solution was irradiated at 254 nm for 48 h, while it was continuously led through a column filled with silica gel (40 g), topped with silica gel / silver nitrate (35 g, 10% (w/w)). The reaction vessel and column were placed in the dark during the irradiation. The column was eluted with dichloromethane to remove unreacted materials. The silica gel was stirred with dichloromethane/ aqueous ammonia = 1/1, (v/v; 300 mL) for 1 h. The organic layer was separated, dried over sodium sulfate, and removed under reduced pressure. The crude product was purified using column chromatography over silica gel (hexane/ethyl acetate = 100/1 to 25/1, v/v, eluent) to give 172 mg (8%) of axial isomer of (*E*)-cyclooct-2-enol and 170 mg (8%) of equatorial isomer of (*E*)-cyclooct-2-enol as pale-yellow oil. The ^{1}H NMR spectra are shown in **Figs. S4 and S5**, respectively. ^{1}H NMR (axial isomer, 400 MHz; $CDCl_3$): δ 5.98 (ddd, J = 16.1, 11.6, 4.1 Hz, 1H), 5.60 (dd, J = 16.5, 2.2 Hz, 1H), 4.64 (s, 1H), 2.51 (dd, J = 6.3, 5.0 Hz, 1H), 2.01–1.96 (m, 2H), 1.92–1.84 (m, 1H), 1.74–1.47 (m, 7H), 1.18–1.10 (m, 1H), 0.97–0.89 (m, 1H), 0.84–0.75 (m, 1H). ^{1}H NMR (equatorial isomer, 400 MHz; $CDCl_3$): δ 5.69 (ddd, J = 15.7, 11.2, 4.1 Hz, 1H), 5.57 (dd, J = 16.2, 9.1 Hz, 1H), 4.29 (td, J = 9.5, 5.6 Hz, 1H), 2.44–2.40 (m, 1H), 2.18 (dt, J = 12.8, 5.8 Hz, 1H), 2.03–1.76 (m, 5H), 1.56–1.40 (m, 3H), 0.96–0.87 (m, 2H), 0.82–0.74 (m, 1H).

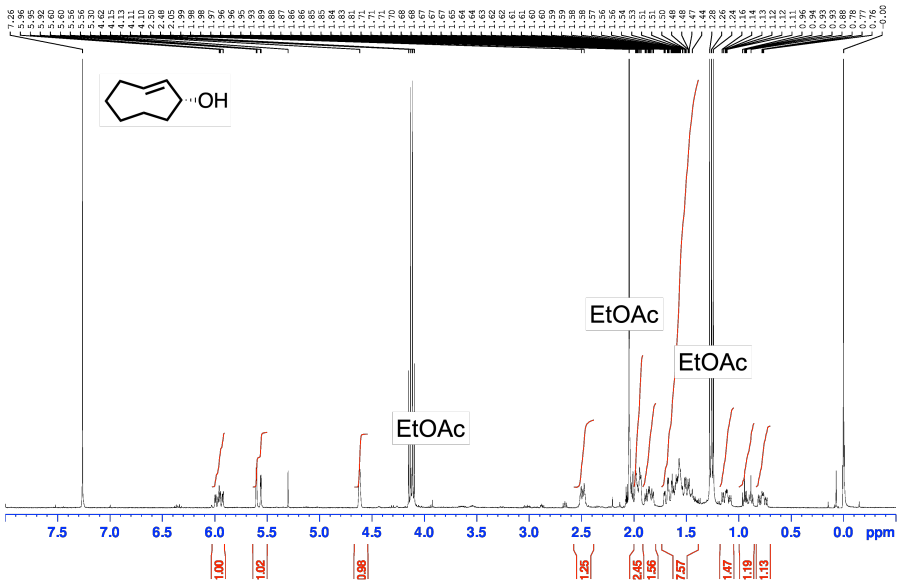


Fig. S4. ^{1}H NMR spectrum of axial isomer of (*E*)-cyclooct-2-enol in chloroform-*d*.

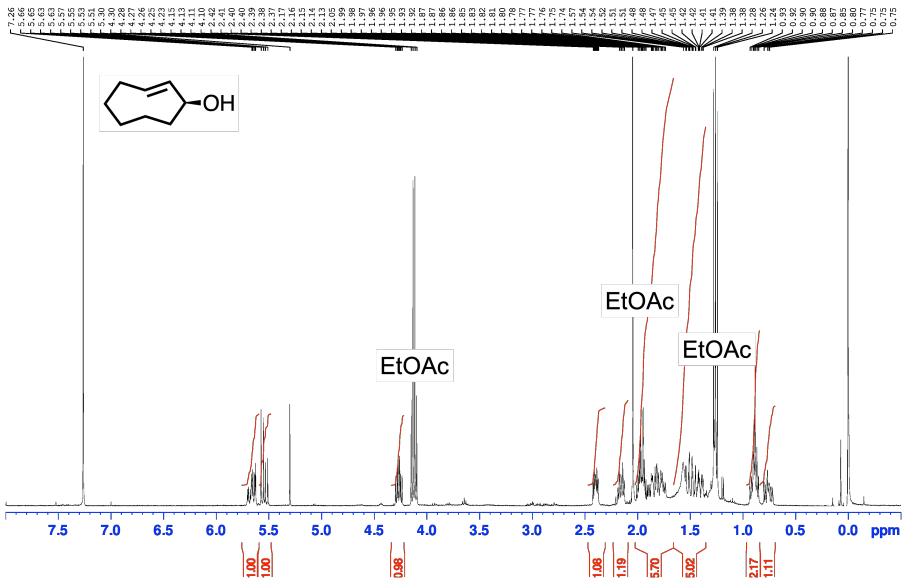


Fig. S5. ^{1}H NMR spectrum of equatorial isomer of (*E*)-cyclooct-2-enol in chloroform-*d*.

Synthesis of (*E*)-cyclooct-2-en-1-yl (4-nitrophenyl) carbonate

Axial isomer of (*E*)-cyclooct-2-enol (0.17 g, 1.37 mmol), pyridine (0.17 mL, 2.08 mmol), and dry dichloromethane (15 mL) was added to a dry flask under an argon atmosphere. The solution was cooled to 0 ºC with an ice bath. 4-Nitrophenyl chloroformate (0.33 g, 1.65 mmol) was added to the mixture solution. The resulting solution was stirred for 20 h at room temperature. The solvent was removed under reduced pressure. The crude product was purified using column chromatography over silica gel (hexane/ethyl acetate = 10/1, v/v, eluent) to give 0.19 g (47%) of (*E*)-cyclooct-2-en-1-yl (4-nitrophenyl) carbonate as a pale-yellow powder. In a similar fashion, equatorial (*E*)-cyclooct-2-en-1-yl (4-nitrophenyl) carbonate was also synthesized. The ^{1}H NMR spectra are shown in **Figs. S6 and S7**, respectively. ^{1}H NMR (axial isomer, 400 MHz; $CDCl_3$): δ 8.30 (d, J = 9.3 Hz, 2H), 7.42 (d, J = 9.3 Hz, 2H), 6.04–5.97 (m, 1H), 5.61–5.46 (m, 2H), 2.57–2.54 (m, 1H), 2.27–2.22 (m, 1H), 2.14–1.72 (m, 5H), 1.65–1.45 (m, 1H), 1.24–1.16 (m, 1H), 0.91–0.82 (m, 1H). ^{1}H NMR (equatorial isomer, 400 MHz; $CDCl_3$): δ 8.32–8.28 (m, 2H), 7.43–7.39 (m, 2H), 5.92–5.84 (m, 1H), 5.68 (dd, J = 16.3, 9.5 Hz, 1H), 5.20 (td, J = 10.0, 5.6 Hz, 1H), 2.52–2.47 (m, 1H), 2.38–2.33 (m, 1H), 2.09–1.89 (m, 4H), 1.78–1.69 (m, 1H), 1.50–1.45 (m, 1H), 1.00–0.85 (m, 2H).

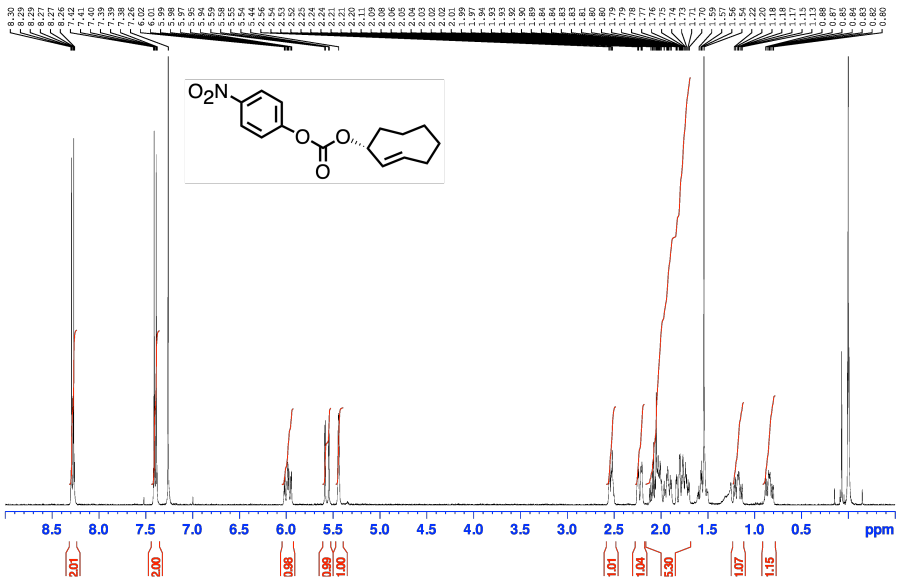


Fig. S6. [1]H NMR spectrum of axial isomer of (*E*)-cyclooct-2-en-1-yl (4-nitrophenyl) carbonate in chloroform-*d*.

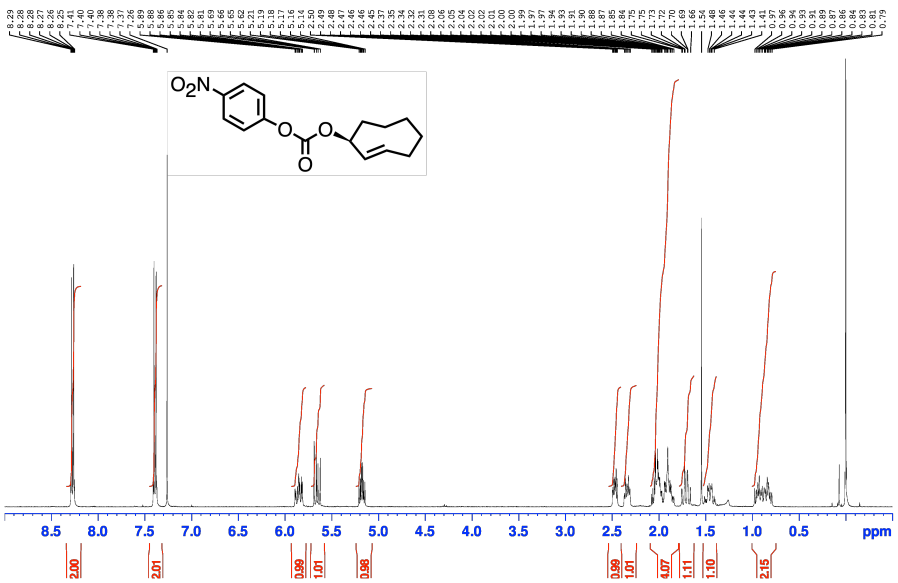


Fig. S7. [1]H NMR spectrum of equatorial isomer of (*E*)-cyclooct-2-en-1-yl (4-nitrophenyl) carbonate in chloroform-*d*.

Synthesis of (*E*)-cyclooctene doxorubicin conjugates

Axial (*E*)-cyclooct-2-en-1-yl (4-nitrophenyl) (13 mg, 0.038 mmol), DIPEA (44 mg, 0.34 mmol), doxorubicin hydrochloride (28.6 mg, 0.043 mmol), and dry DMF (2 mL) was added in this order to a dry Schlenk tube under an argon atmosphere. The mixture solution was stirred for 96 h at 30 ºC. The solvent was removed under reduced pressure. The crude product was purified using column chromatography over silica gel (dichloromethane/methanol = 95/5, v/v, eluent) to give 19 mg (71%) of axial (*E*)-cyclooctene doxorubicin conjugate as a red powder. In a similar fashion, equatorial (*E*)-cyclooctene doxorubicin conjugate was also synthesized. The ^{1}H NMR spectra are shown in **Figs. S8 and S9**, respectively. ^{1}H NMR (axial isomer, 400 MHz; $CDCl_3$): δ 13.95 (s, 1H), 13.20 (s, 1H), 8.00 (s, 1H), 7.77 (q, *J* = 8.1 Hz, 1H), 7.38 (t, *J* = 6.5 Hz, 1H), 5.83–5.71 (m, 1H), 5.49–5.45 (m, 2H), 5.22 (dd, *J* = 30.8, 11.6 Hz, 3H), 4.77 (d, *J* = 4.6 Hz, 3H), 4.59 (s, 1H), 4.17 (d, *J* = 6.1 Hz, 1H), 4.08 (s, 4H), 3.89–3.88 (m, 1H), 3.70 (d, *J* = 6.1 Hz, 1H), 3.25 (d, *J* = 18.8 Hz, 1H), 3.08 (t, *J* = 4.7 Hz, 1H), 2.98 (d, *J* = 13.5 Hz, 3H), 2.90 (s, 1H), 2.44 (s, 1H), 2.34 (d, *J* = 14.6 Hz, 1H), 2.20–2.16 (m, 3H), 2.01–1.81 (m, 11H), 1.63 (d, *J* = 0.8 Hz, 3H), 1.48–1.44 (m, 2H), 1.32 (d, *J* = 6.3 Hz, 4H), 1.04 (d, *J* = 1.5 Hz, 1H), 0.77 (d, *J* = 0.5 Hz, 1H). ^{1}H NMR (equatorial isomer, 400 MHz; $CDCl_3$): δ 13.97 (s, 1H), 13.23 (s, 1H), 8.04 (d, *J* = 7.7 Hz, 1H), 7.81–7.78 (m, 1H), 7.40 (d, *J* = 8.5 Hz, 1H), 5.70 (ddd, *J* = 15.9, 11.2, 4.4 Hz, 1H), 5.49 (dt, *J* = 19.1, 6.4 Hz, 2H), 5.30 (s, 1H), 5.07-4.99 (m, 2H), 4.78 (d, *J* = 4.3 Hz, 2H), 4.57 (s, 1H), 4.16–4.15 (m, 1H), 4.09 (t, *J* = 2.6 Hz, 4H), 3.88 (t, *J* = 0.6 Hz, 1H), 3.69 (d, *J* = 6.3 Hz, 1H), 3.27 (d, *J* = 19.0 Hz, 1H), 3.05–2.99 (m, 3H), 2.39-1.76 (m, 12H), 1.66 (s, 2H), 1.48–1.37 (m, 2H), 1.31 (d, *J* = 6.5 Hz, 4H), 0.88-0.76 (m, 2H).

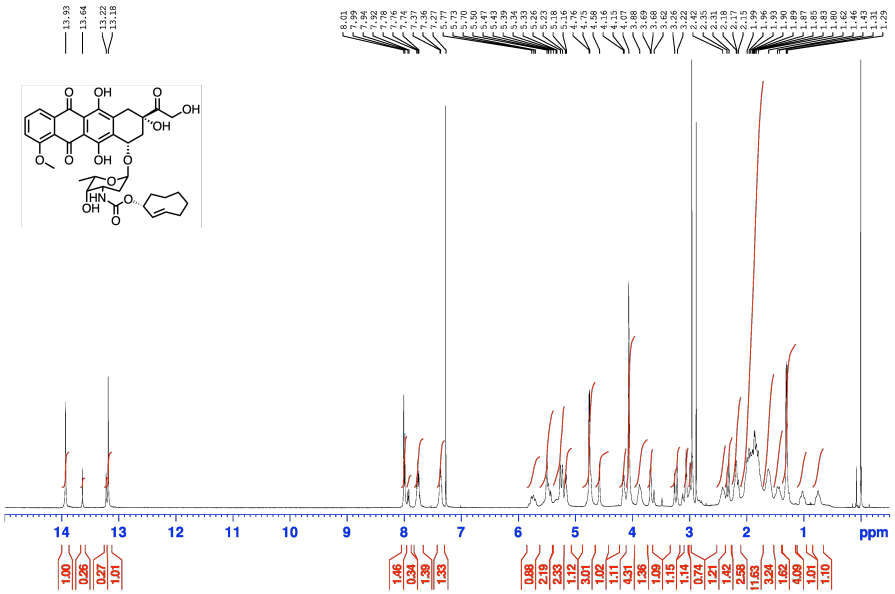


Fig. S8. ^{1}H NMR spectrum of axial isomer of (*E*)-cyclooctene doxorubicin conjugate in chloroform-*d*.

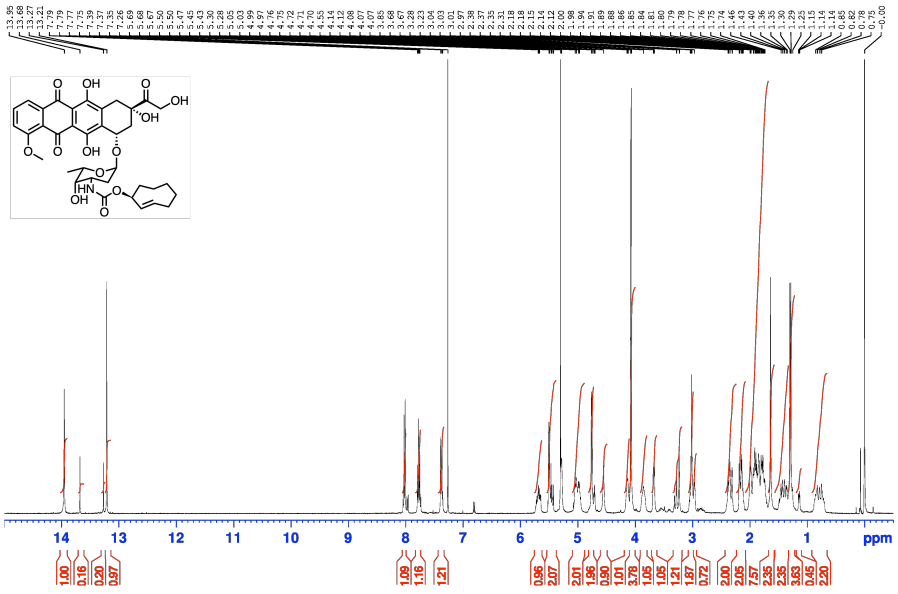


Fig. S9. ^{1}H NMR spectrum of equatorial isomer of (*E*)-cyclooctene doxorubicin conjugate in chloroform-*d*.

Synthesis of Cy5-labeled amphiphilic block copolymer

The amphiphilic block copolymer (38 mg, 3.3 μmol), Cy5-amine (5.0 mg, 6.6 μmol), COMU (7.4 mg, 14.4 μmol), oxyma (6.4 mg, 14.4 μmol), and dry DMF (5 mL) was added to a dry flask under an argon atmosphere. The solution was stirred for 72 h at 45 ºC. The crude product was purified with dialysis (regenerated cellulose membrane; Spectra/Por 7; MWCO 1000) against DMF for 2 days, then water for 1 day. Subsequently, the solvent was lyophilized to yield Cy5-functionalized block copolymer as a blue powder. The degree of the modification was one Cy5 molecule per polymer.

Characterization of the molecular assemblies

Preparation of polymer solutions

Distilled water (0.9 mL) was added to a glass vial containing DMSO solution of polymer (10 mg mL^{-1}, 0.1 mL). Subsequently, the solutions were incubated at room temperature for at least 30 minutes. The resulting solution was dialysis (regenerated cellulose membrane; Spectra/Por 7; MWCO 1000) against water to remove DMSO.

Dynamic Light Scattering (DLS)

DLS measurements were carried out using a nanoPartica SZ-100 instrument (Horiba, Japan) operating at a wavelength of 532 nm and a detection angle of 173°. The obtained data were collected at 25 °C unless otherwise stated and analyzed using the cumulant method.

Fluorescence measurements

The fluorescence spectra of pyrene were recorded using a fluorescence spectrophotometer (FP-8350, JASCO Corp, Japan). The excitation wavelength was 285 nm, and the emission spectra were recorded in the range of 295–500 nm. The scan speed and the slit width were 100 nm nm^{-1} and 2.5 nm, respectively. The measurement temperature was kept constant at 25 ºC.

Transmission Electron Microscopy (TEM)

A solution of the polymer in water (5 μL; 1.0 mg mL^{-1}) was placed on a copper grid coated with an elastic carbon film (ELS-C10 STEM Cu100P, Okenshoji Co., Ltd. Japan). Excess sample solution was

removed using a filter paper. A solution of 0.1 wt% phosphotungstic acid (5 μL; pH = 7.4) was added as the staining agent and then removed prior to drying the sample in a desiccator. The grid was placed in a JEM-2100 (JEOL Ltd., Japan) electron microscope, which was operated at 100 kV.

Cryogenic Transmission Electron Microscopy (Cryo-TEM)

Cryo-TEM measurements were carried out on a JEM-2100Plus (JEOL, Japan), operated at 200 kV. The samples were vitrified in a controlled environment vitrification system with relative humidity close to 80% at 23 ºC. Three micro-litter of polymer solution (10 mg mL^{-1}) was deposited on holey carbon support film grids (Quantifoil R2/1, EM-Japan Co., Ltd.) and excess fluid was gently removed by blotting. The grids were then directly plunged into liquid ethane. Grids were stored in liquid nitrogen until being transferred to the electron microscope.

Small Angle X-ray Scattering (SAXS)

SAXS measurements were performed at the BL40B2 beamline of SPring-8 (Japan). A 25.4 × 28.9 cm^2 photon-counting detector (PILATUS3 S 2M) or a 30 × 30 cm^2 imaging-plate detector (Rigaku R-axis VII) was placed 2.1 m from the sample. The wavelength (λ) of the incident X-rays was 1.0 Å. The X-ray transmittance values of the sample and of water were measured using ion chambers located in front of and behind the sample. The polymer solutions or water were placed in a quartz capillary (diameter: 2 mm; Hilgenberg GmbH). The sample temperature was controlled using a Peltier thermostat cell holder system (TS-62, Instec Inc., USA) and the samples were incubated at the target temperature for 10 min before the measurements. SAXS images were collected with an exposure time of 180 s. The resulting 2D SAXS images were converted into one-dimensional intensity versus q profiles by circular averaging using the FIT2D software package. The background intensity of the capillary filled with distilled water was subtracted. The scattering curves from the polymer solutions were fitted with a form factor using core-shell sphere model as follows.

The form factor of the core-shell sphere model can be expressed by:

$$I(q) = NVs^2\left\{(\rho_{core}-\rho_{shell})\frac{V_c}{V_s}\frac{3[\sin(qR_c)-qR_c\cos(qR_c)]}{(qR_c)^3}+(\rho_{shell}-\rho_{solvent})\frac{3[\sin(qR_s)-qR_s\cos(qR_s)]}{(qR_s)^3}\right\}^2$$

where, ρ_{core}, ρ_{shell}, $\rho_{solvent}$, V_c, V_s, R_c, and R_s are the electron densities of the hydrophobic core, hydrophilic shell, and solvent, the volume of the core and overall particle, the radius of the core, and overall radius, respectively. A gaussian distribution of the overall radius was used to take the polydispersity into account.

IEDDA reaction of the block copolymer and doxorubicin prodrugs

A solution of axial or equatorial doxorubicin prodrug solution in DMSO (2 μL, 10 mM) was added to the polymer solution in water (1998 μL, 50 μg mL^{-1}). The solution was incubated at 25 ºC. At various time interval, 100 μL of the solution was sampled, and the reaction was monitored using a shimadzu prominence HPLC system equipped with a UV-Vis detector (SPD-20A) in acetonitrile/water = 7/3, (v/v) using a reverse phase column (TOSOH, TSKgel ODS-100V).

Cell culture and anticancer prodrug activation by the block copolymer micelles

1. Cell viability assay of doxorubicin and doxorubicin prodrug for CT26 cells

CT26 cells were purchased from the RIKEN BRC through the National Bio-Resource Project of the MEXT/AMED, Japan. The cytotoxicity of doxorubicin and the doxorubicin prodrug with different concentrations were evaluated with cell counting kit-8 (CCK-8, DOJINDO, Japan). CT26 cells were seeded in 96-well dishes at a density of 5×10^3 cells/well and were cultured in RPMI1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin for 24 h at 37 ºC in 5% CO_2. After the medium was removed, 100 μL of fresh medium containing doxorubicin or Dox-TCO were added. After the cells were cultured at 37 ºC in 5% CO_2 for 24 h, CCK-8 solutions were added to the cells. After 90 min, the absorbance at 450 nm was measured using a microplate reader (Multiskan FC Advance, Thermo Fisher Scientific K. K.).

2. Cell viability assay of prodrug activation by WST-8 assay and Live-dead assay

WST-8 assay

CT26 cells were seeded in 96-well dishes at a density of 5×10^3 cells/well and were cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS and 1% penicillin-streptomycin for 24 h at 37 ºC in 5% CO_2. The medium was removed and the fresh culture medium (0.15 mL) containing the block copolymer (50 μg mL^{-1}) and axial isomer of Dox-TCO (10 μM) were then added. CT26 cells treated doxorubicin or doxorubicin prodrug, and CT26 cells with the amphiphilic block copolymer were used as control. After the cells were cultured at 37 ºC in 5% CO_2 for 24 h, CCK-8 solutions were added to the cells. After 90 min, the absorbance at 450 nm was measured using a microplate reader (Multiskan FC Advance, Thermo Fisher Scientific K. K.).

Live-dead assay

CT26 cells were seeded in 8-well μ-slide (ibid) at a density of 1×10^4 cells/well and were cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS and 1% penicillin-streptomycin for 24 h at 37 ºC in 5% CO_2. The medium was removed and the fresh culture medium (0.25 mL) containing the block copolymer (50 μg mL^{-1}) and axial doxorubicin prodrug (10 μM) were then added. CT26 cells treated doxorubicin or axial isomer of Dox-TCO, and CT26 cells with the amphiphilic block copolymer were used as control. The cells were incubated at 37 ºC for 24 hours. After washing the cells with PBS buffer, the solution of live/dead assay dyes (abcam) was added into wells, and the images of the cells were taken using a fluorescence microscope (BZ-X800; Keyence, Tokyo, Japan or EVOS M7000 Imaging system; Thermo Fisher Scientific).

Animal experiments

All animal experiments were approved by the ethics committee for animal welfare of the University of Kitakyushu.

Blood circulation and bio-distribution of the block copolymer micelles

Male BALB/c mice aged 6 weeks were purchased from Japan SLC Co. (Shizuoka, Japan). Each clipped mouse was subcutaneously inoculated in the right flank with a suspension of CT26 mouse colon carcinoma cells in a HBSS buffer (0.1 mL, 1×10^7 cells/ml). Tumours were allowed to grow for 7 days. The tumor-bearing mice were intravenously administrated with a 100 μL of Cy5 functionalized block copolymer (2.0 mg mL^{-1} in water). After various time intervals, the bloods were collected from the tail vein using heparinized syringes and centrifuged at 3000 × *g* for 5 min at 5 °C to obtain plasma. The fluorescence intensities of the blood samples were measured using Pharos FXTM Plus Molecular Imager (BioRad, USA). The total volume of blood was assumed to be 7% of body weight. [S1] After 24 h, the mice were anesthetized, and a blood sample was withdrawn from the posterior vena cava. Transcardiac perfusion with PBS was then performed. Subsequently, heart, lung, kidney, liver, spleen, and tumor were collected and weighted. A total of 1.0 mL of a PBS buffer was added before the samples were homogenized using a gentle MAXS dissociator with a standard C tubes program. The sample solution was centrifuged at 12,000 × *g* for 15 min at 4 ºC. The fluorescence intensities of the supernatant were measured using the microplate reader.

Anti-tumor effect for CT26 tumor bearing mice

The tumor-bearing mice were prepared as described above and were randomly divided into five group (n = 5) after 7 days of tumor implants. BALB/C mice were injected via intravenously with (1) PBS, (2) doxorubicin (10 mg/kg body weight), (4) doxorubicin prodrug (12 mg/kg body weight), (5) block copolymer micelle solution (3 mg mL^{-1}) and doxorubicin prodrug (12 mg/kg body weight) at day 0. For the group 5, the injection of doxorubicin prodrug was followed 6 hours after the first injection. The injection volume was fixed at 0.1 mL. The anti-tumor effect was assessed by measuring length and width of the tumors with a caliper for every other day. The tumor volume was calculated using the following equation:

tumor volume = $(a \times b^2)/2$

, where a is the longer and b is shorter dimension. Body weight change was also measured. The mice were monitored for up to 10 days after first treatment.

Table S1. Structural parameters of the polymer micelles in water obtained from the SAXS analysis

radius of core (nm)	overall radius of micelles (nm)	Standard deviation	electron density of core (e nm^{-3})	electron density of shell (e nm^{-3})	electron density of solvent (e nm^{-3})
10.0	19.0	4.5	371	365	333

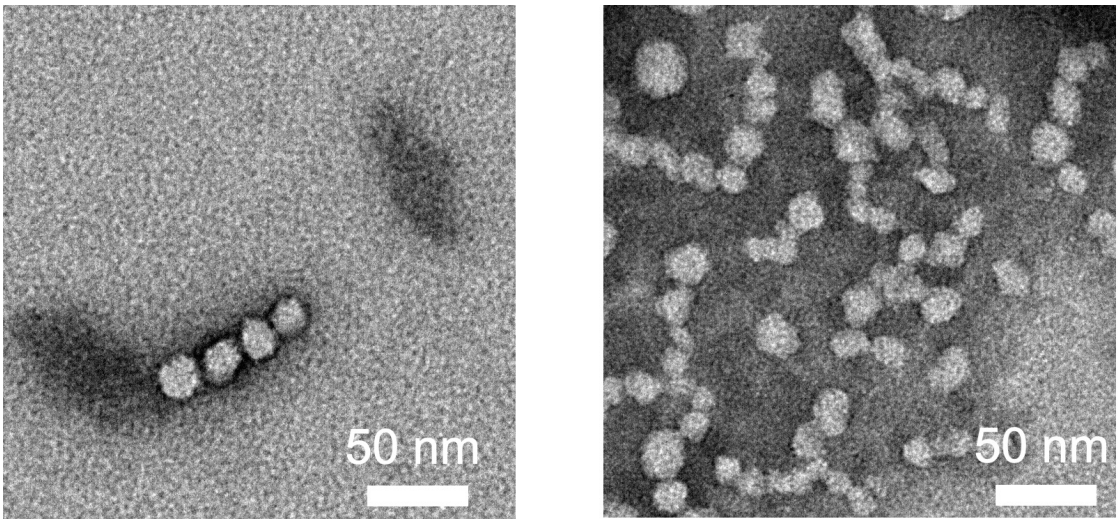


Fig. S10. TEM images of the self-assembles of tetrazine-functionalized amphiphilic block copolymer in water: [polymer] = 1.0 mg mL^{-1}.

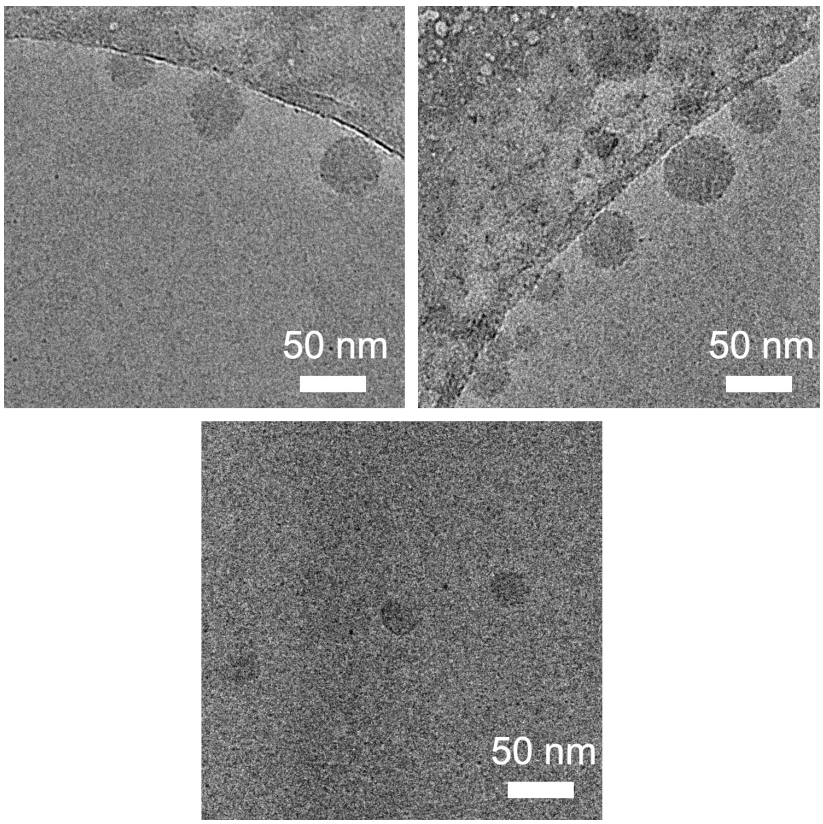


Fig. S11. Cryo-TEM images of the self-assembles of tetrazine-functionalized amphiphilic block copolymer in water: [polymer] = 5.0 mg mL^{-1}.

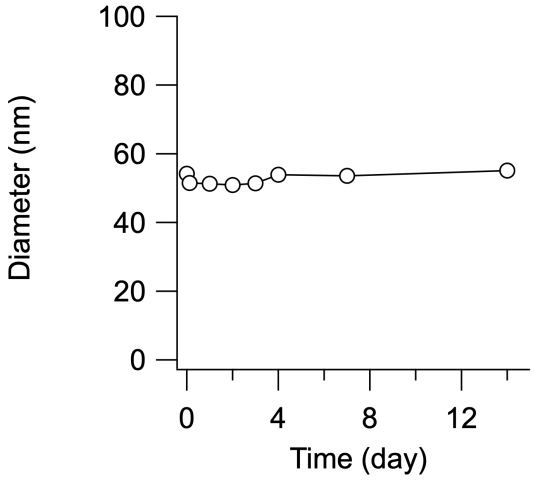


Fig. S12. Stability test for the polymer micelles. [polymer] = 1.0 mg mL^{-1}.

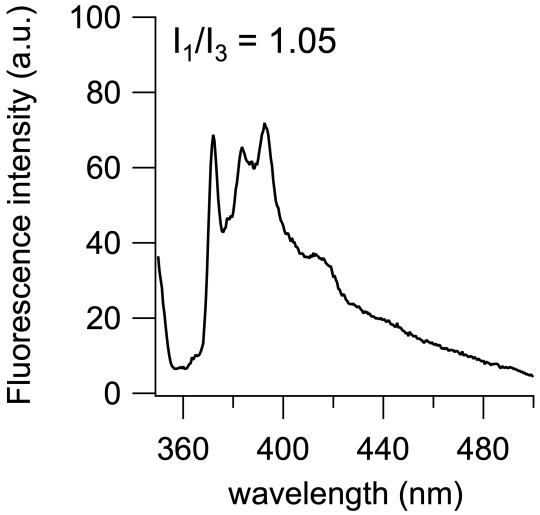


Fig. S13. fluorescence spectrum of pyrene in the presence of the polymer micelles in water at 25 ºC. Concentrations: [polymer] = 1.0 mg mL^{-1}, [pyrene] =1.0×10^{-6} M.

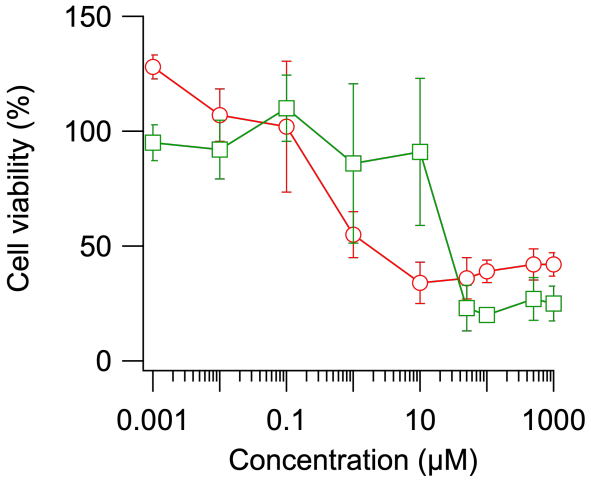


Fig. S14. Relative viability of CT26 cells treated with doxorubicin (red circles) or axial isomer of Dox-TCO (green squares) at various concentrations. The results represent mean values ± SD (n = 5).

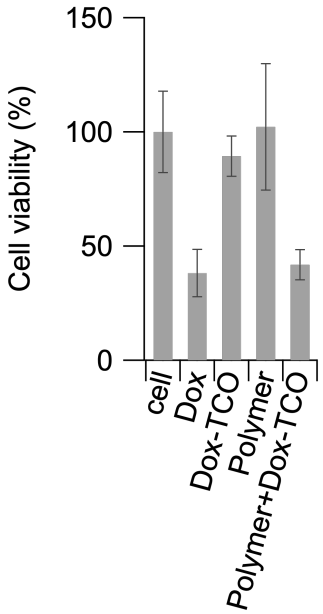


Fig. S15. Relative viability of CT26 cells treated with PBS, doxorubicin, Dox-TCO, the polymer micelles, and the combination of Dox-TCO and the polymer micelles. Concentrations: [Doxorubicin] = [Dox-TCO] = 10 μM, [polymer] = 43 μg mL^{-1}. The values represent mean ± SD (n = 5).

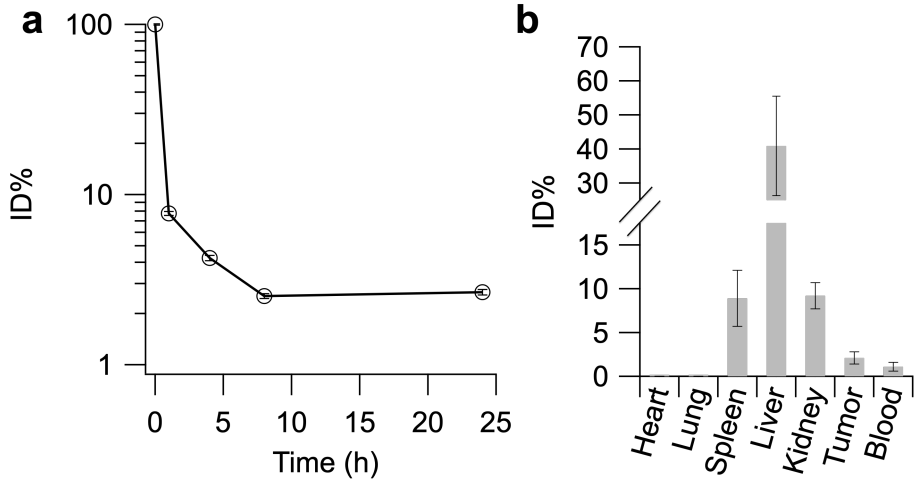


Fig. S16. (a) Blood circulation profile and (b) biodistribution of the Cy5-functionalized polymer micelles after 24 h intravenous injection.

Additional reference

1. H. S. S. Qhattal, T. Hye, A. Alali and X. Liu, *ACS Nano*, 2014, **8**, 5423-5440.