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Supporting Information

Dual location stimuli-responsive degradation strategy of block copolymer nanocarriers for accelerated release

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I. Instrumentation and materials

Instrumentation and analyses. ^{1}H-NMR spectra were recorded using a 500 MHz Varian spectrometer. $CDCl_3$ singlet at 7.26 ppm was selected as the reference standard. Spectral features are tabulated in the following order: chemical shift (ppm); multiplicity (s - singlet, d - doublet, t - triplet, m - complex multiple); number of protons; position of protons. Monomer conversion was determined using ^{1}H NMR of aliquots taken during polymerization. Molecular weight and molecular weight distribution were determined by gel permeation chromatography (GPC). A Viscotek GPC was equipped with a VE1122 pump and a refractive index (RI) detector. Two PolyAnalytik columns (PAS-103L and 106L) were used with THF as an eluent at 30 °C at a flow rate of 1.0 mL/min. In addition, an Agilent GPC was equipped with a 1260 Infinity Isocratic Pump, and a RI detector. Two Agilent columns (PLgel mixed-D and mixed-C) were used with DMF containing 0.1 mol% LiBr at 50 °C at a flow rate of 1.0 mL/min. Linear poly(methyl methacrylate) (PMMA) standards from Fluka were used for calibration. Aliquots of polymer samples were dissolved in either THF or DMF/LiBr. The clear solutions were filtered using a 0.25 μm PTFE filter to remove any solvent-insoluble species. A drop of anisole was added as a flow rate marker. The size of micelles in hydrodynamic diameter by volume was measured by dynamic light scattering (DLS) at a fixed scattering angle of 173° at 25 °C with a Malvern Instruments Nano S ZEN1600 equipped with a 633 nm He-Ne gas laser. Fluorescence spectra on a Varian Cary Eclipse Fluorescence spectrometer and UV/Vis spectra on an Agilent Cary 60 UV/Vis spectrometer were recorded using a 1 cm wide quartz cuvette.

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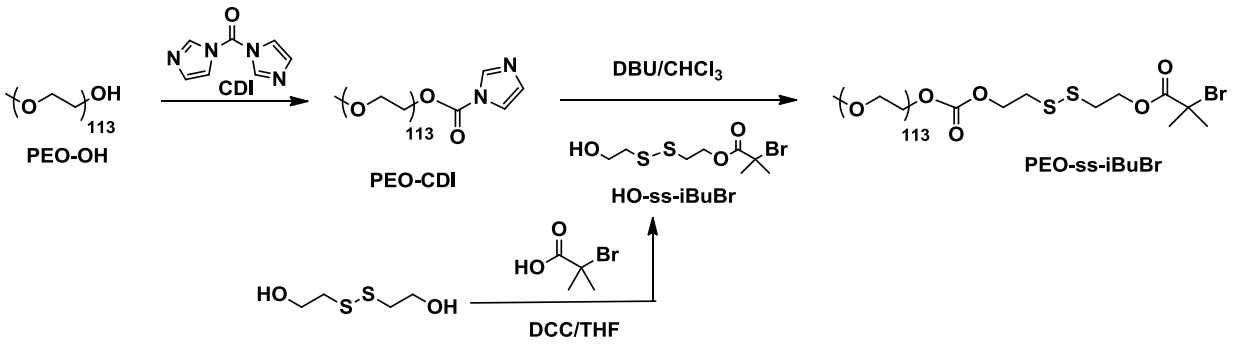
Transmission Electron Microscopy (TEM). TEM images were taken using a Philips Tecnai 12 TEM, operated at 120 kV and equipped with a thermionic LaB6 filament. An AMT V601 DVC camera with point to point resolution and line resolution of 0.34 nm and 0.20 nm respectively was used to capture images at 2048 by 2048 pixels. To prepare specimens, the micellar dispersions were dropped onto copper TEM grids (400 mesh, carbon boated), blotted and then allowed to air dry at room temperature.

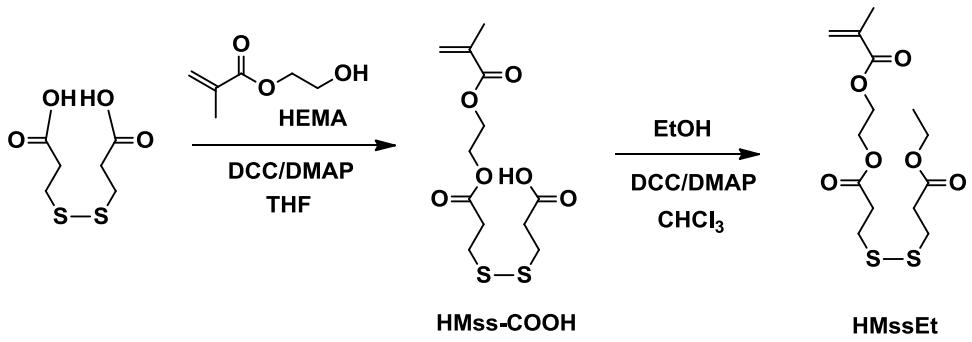
Materials. 3,3′-Dithiodipropionic acid (ss-DCOOH), 2-hydroxyethyl disulfide (ss-DOH), 2-hydroxyethyl methacrylate (HEMA), poly(ethylene oxide) methyl ether (PEO-OH, MW = 5000 g/mol and # of ethylene oxide (EO) units = 113), α-bromoisobutyl bromide (Br-iBuBr), 1,1′-carbonyldiimidazole (CDI, reagent grade), 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU, 98%), N,N'-dicyclohexyl carbodiimide (DCC), 4-dimethylaminopyridine (DMAP), triethylamine (Et_3N, > 99.5 %), copper(II) bromide ($CuBr_2$, >99.99%), anisole (anhydrous, >99%), tin(II) 2-ethylhexanoate ($Sn(EH)_2$, 95%), doxorubicin hydrochloride (DOX, $-NH_3^+Cl^-$ salt form, >98%), and glutathione ethyl ester (GSH-OEt, reduced form) from Aldrich, and DL-dithiothritol (DTT, 99%) and 2-bromo-2-methylpropionic acid (Br-MPC, 98%) from Acros Organics were purchased and used as a received, unless otherwise specified. Tris(2-pyridylmethyl)amine (TPMA) was synthesized according to literature procedure.[1]

Synthesis of PEO-ss-iBuBr. A water-soluble PEO ATRP macroinitiator labeled with a disulfide linkage (PEO-ss-Br) was synthesized in three steps.[2] The first step is the synthesis of 2-hydroxyethyl-2′-(bromoisobutyrl)ethyl disulfide (HO-ss-iBuBr) by the reaction of ss-DOH (5.0 g, 32.4 mmol) with Br-iBuBr (8.2 g, 35.7 mmol) in the presence of Et_3N (6.0 mL, 42.8 mmol) in THF (200 mL). The second step is the synthesis of PEO-CDI by the reaction of PEO-OH (18.9 g, 3.8 mmol) with CDI (3.7 g, 22.7 mmol) in chloroform (40 mL) at room temperature under stirring for 3 days. The third step is the reaction of the purified PEO-CDI (6.0 g, 1.18 mmol) with the purified HO-ss-iBuBr (0.5 g, 1.47 mmol) in the presence of DBU (32.6 mg, 0.21 mmol) in chloroform (20 mL). Yield = 5.9 g (93%). ^{1}H-NMR ($CDCl_3$, ppm) 4.44 (t, 2H, $–CH_2OC(O)C(CH_3)_2Br$), 4.39 (t, 2H, $–CH_2OC(O)O–$), 4.30 (t, 2H, $–OC(O)OCH_2–$), 3.4– 3.9 (m, EO proton $–CH_2–$), 3.38 (s, 3H, $H_3CO–$), 2.97 (m, 4H, $–CH_2SSCH_2–$), 1.94 (s, 6H, $–C(CH_3)_2Br$).



Scheme S1. Synthesis of PEO-ss-Br.

Synthesis of a methacrylate bearing a pendant disulfide linkage (HMssEt). As described in our previous report,[3] HEMA (10.5 g, 80.9 mmol) was reacted with ss-DCOOH (70.0 g, 332.9 mmol) in the presence of DCC (18.0 g, 87.2 mmol) and DMAP (0.88 g, 7.2 mmol) in THF (700 mL), yielding residues containing HMss-COOH. After the removal of THF, the intermediate HMss-COOH was reacted with EtOH (19 g, 412.4 mmol) in the presence of DCC (20.0 g, 96.9 mmol) and DMAP (0.12 g, 0.98 mmol) dissolved in chloroform (110 mL). Column chromatography was used to purify HMssEt as an oily residue. Yield = 11.9 g (42%). R_f = 0.36 on silica (3/7 ethyl acetate/hexane). ^{1}H-NMR ($CDCl_3$, ppm) 6.12 (s, 1H, H_2C=C-), 5.59 (s, 1H, H_2C=C-), 4.35 (s, 4H, -C(O)O$\underline{CH_2}\underline{CH_2}$O(O)C-), 4.15 (q, 2H,-C(O)O$\underline{CH_2}CH_3$), 2,91 (t, 4H, -$\underline{CH_2}SS\underline{CH_2}$-), 2.76 (t, 2H, -O(O)C$\underline{CH_2}CH_2SS$-), 2.71 (t, 2H, -SSC$H_2\underline{CH_2}$C(O)O-), 1.94 (s, 3H, H_2C=C($\underline{CH_3}$)-), 1.26 (t, 3H, -C(O)OC$H_2\underline{CH_3}$). ^{13}C-NMR ($CDCl_3$, ppm): 171.5, 171.3, 166.9, 135.8, 126.0, 62.4, 62.2, 60.7, 34.1, 33.9, 33.2, 32.9, 18.2, 14.1. Mass calculated for ($C_{14}H_{22}O_6S_2Na^+$): 373.07555. Found: 373.07554.



Scheme S2. Synthesis of of HMssEt.

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II. Synthesis of PEO-ss-PHMssEt block copolymers by ARGET ATRP

ARGET ATRP of HMssEt mediated with $CuBr_2$/TPMA complex in the presence of PEO-ss-Br macroinitiator was carried out in anisole at 40 °C. Typically, PEO-ss-Br (0.25 g, 48 μmol), HMssEt (1.0 g, 2.85 mmol), TPMA (2.0 mg, 7.1 μmol), $CuBr_2$ (0.5 mg, 2.4 μmol), and anisole (3.5 g) were mixed in a 10 mL Schlenk flask. The mixture was deoxygenated by purging under nitrogen for 1 hr and placed in an oil bath at 40 °C. A pre-purged solution of $Sn(EH)_2$ (7.8 mg, 19 μmol) dissolved in anisole (0.5 g) was injected into the Schlenk flask to initiate polymerization. Polymerization was stopped by cooling the reaction vessel and exposing the contents to air. For kinetic studies, aliquots were withdrawn at different time intervals for analysis of monomer conversion by ^{1}H NMR and molecular weight by GPC.

For purification, the polymer solution was diluted with acetone and then passed through a basic alumina column to remove residual copper. The acetone was quickly removed under rotary evaporation at room temperature, and the polymer was isolated by precipitation from hexane three times, and then dried under vacuum at room temperature for 18 hrs.

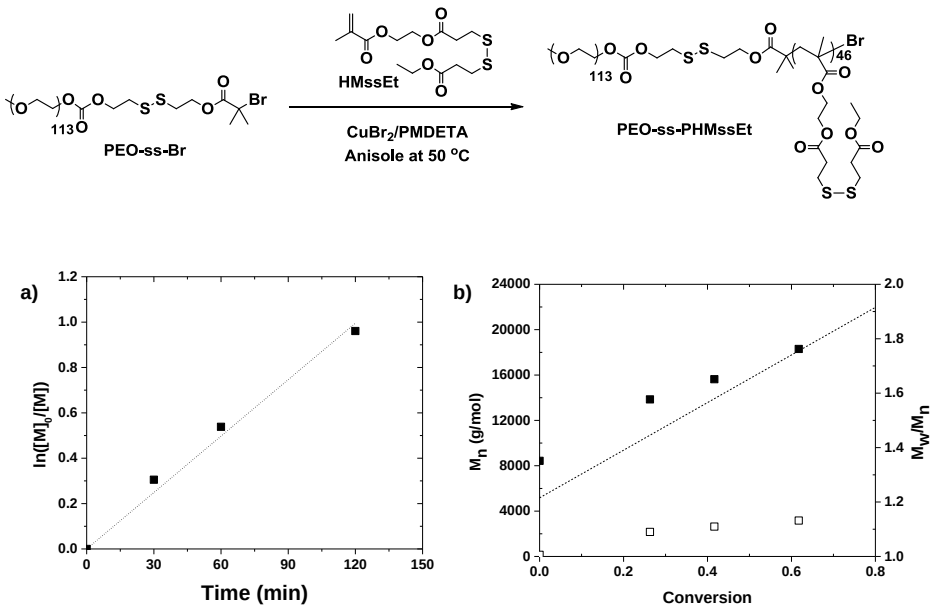

Figure S1. First-order kinetic plot (a) and evolution of molecular weight and molecular weight distribution over conversion (b) for ARGET ATRP of HMssEt in anisole at 40 °C. Conditions: $[HMssEt]_0/[PEO\text{-}ss\text{-}Br]_0/[CuBr_2]_0/[TPMA]_0/[Sn(II)EH_2]_0$ = 60/1/0.05/0.15/0.4; HMssEt/anisole = 0.25/1 wt/wt. The dotted lines are linear fit in (a) and theoretically predicted molecular weight with respect to conversion in (b). Theoretical molecular weights = $[HMssEt]_0/[PEO\text{-}ss\text{-}Br]_0$ x conversion x MW (HMssEt = 350 g/mol) + MW (PEO-ss-Br). GPC with THF as an eluent was used for kinetic studies.

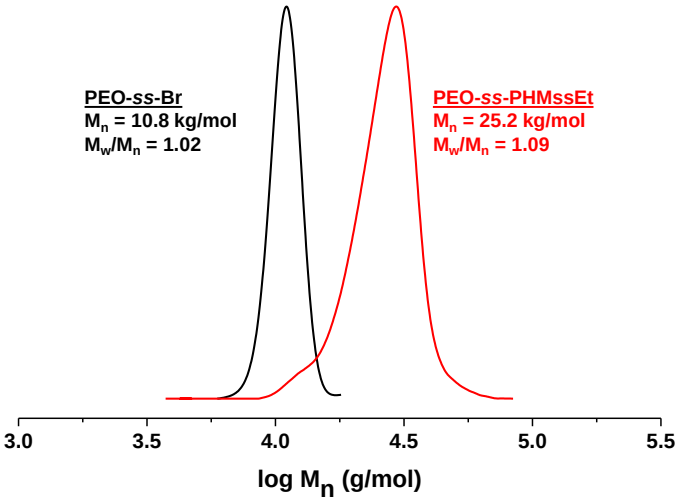

Figure S2. GPC traces of PEO-ss-Br and PEO-ss-PHMssEt in DMF.

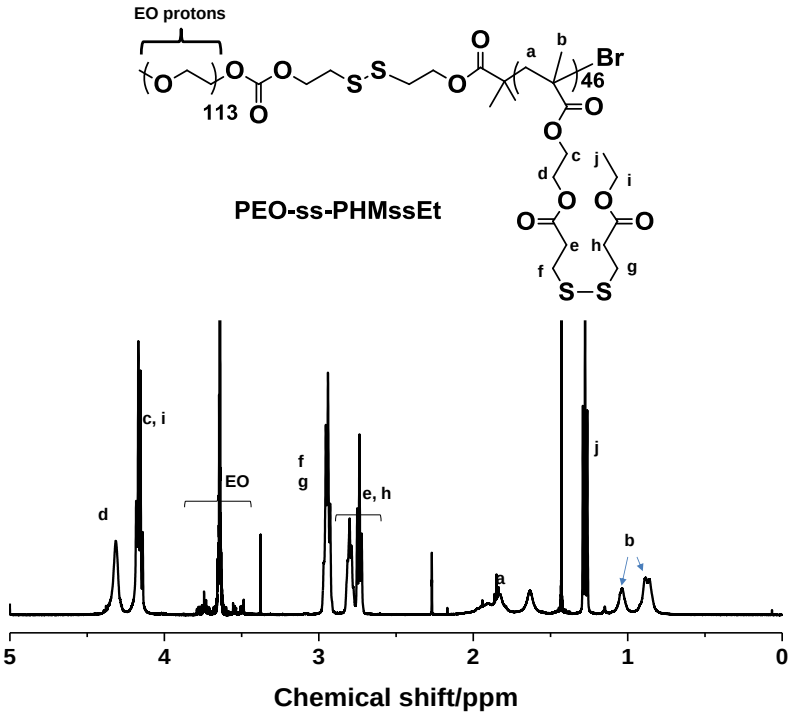

Figure S3. ^{1}H-NMR spectrum of PEO-ss-PHMssEt in $CDCl_3$.

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III. Formation and characterization of aqueous micellar aggregates

Determination of critical micellar concentration (CMC) using a Nile Red probe. A stock solution of Nile Red (NR) in THF at 1 mg/mL and a stock solution of PEO-ss-PHMssEt in THF at 1 mg/mL were prepared. Water (10mL) was then added drop-wise into a series of mixtures consisting of the same amount of the stock solution of NR (0.5 mL, 0.5 mg NR) and various amounts of the stock solution of PEO-ss-PHMssEt in 20 mL vials. The resulting dispersions were stirred for 24 hrs to evaporate THF. The dispersions were then filtered using 0.45 μm PES filters to remove excess NR. A series of NR-loaded micelles at various concentrations of PEO-ss-PHMssEt ranging from 10^{-6} to 0.1 mg/mL were formed. Their fluorescence spectra were recorded at λ_{ex} = 480 nm.

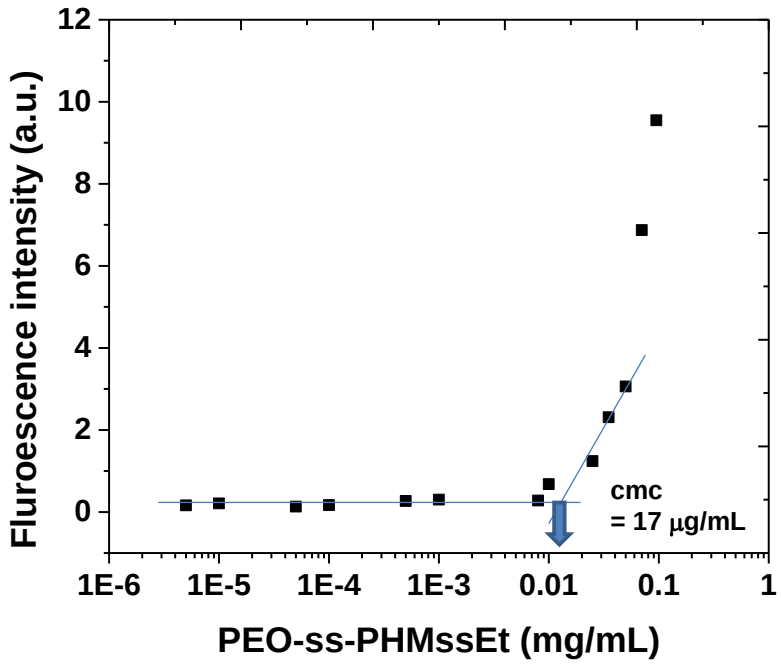

Figure S4. Fluorescence intensity of NR for aqueous mixtures consisting of NR with various amounts of PEO-ss-PHMssEt to determine CMC to be 17 μg/mL.

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Aqueous micellization of PEO-ss-PHMssEt using solvent evaporation method. For the preparation of a micellar dispersion at 1 mg/mL, water (15 mL) was added drop-wise to an organic solution consisting of the purified, dried PEO-ss-PHMssEt (15 mg) dissolved in THF (2.5 mL). The resulting dispersion was stirred at room temperature for 24 hrs, allowing for the removal of THF.

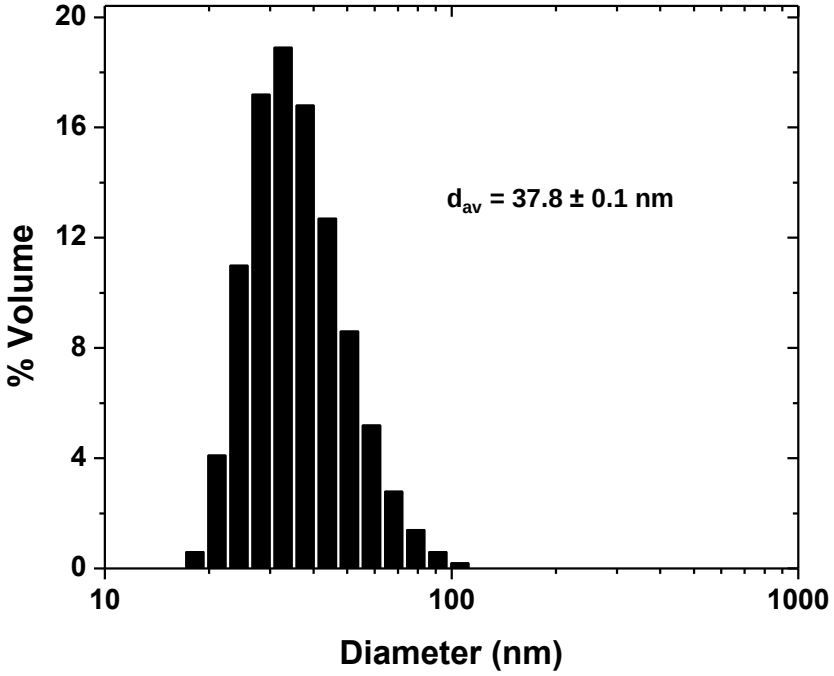

Figure S5. DLS diagram of micellar aggregates of PEO-ss-PHMssEt at 1.0 mg/mL prepare by a solvent evaporation method

IV. Reductive cleavage of disulfide linkages of PEO-ss-PHMssEt in DMF

To examine the cleavage of disulfide linkages of PEO-ss-PHMssEt in the presence of excess DTT, an aliquot of dried, purified PEO-ss-PHMssEt (20 mg) was mixed with DTT (33 mg, 0.22 mmol) in DMF (2.0 mL) under stirring at room temperature. Aliquots were taken periodically to analyze molecular weight distribution of degraded products using GPC.

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V. Reductive cleavage of disulfide linkages of PEO-ss-PHMssEt micelles

To examine the cleavage of disulfide linkages of PEO-ss-PHMssEt micelles in the presence of excess DTT, PEO-ss-PHMssEt micelles were prepared at a concentration of 1 mg/mL via solvent-evaporation. 5 mL of the micellar dispersion was mixed with DTT (9.3 mg, 0.06 mmol) and kept stirring at room temperature for 24 hrs. Aliquots were taken periodically to analyze the particle size distribution by DLS and TEM.

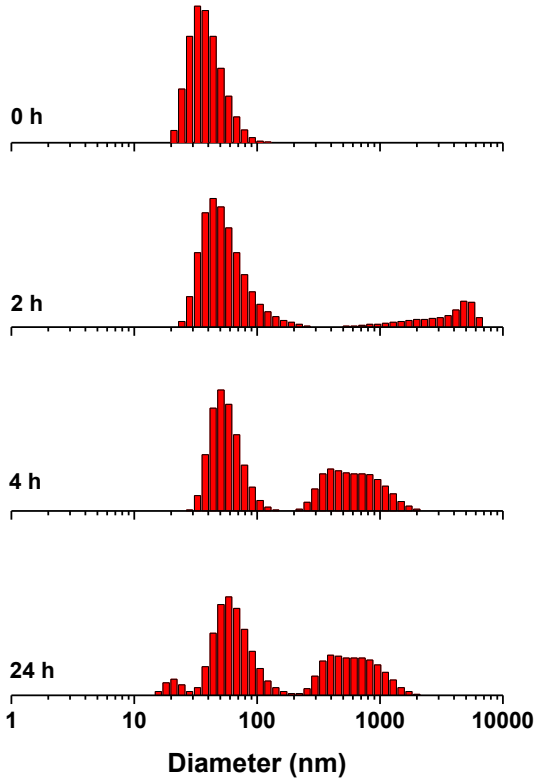

Figure S6. Degradation of micellar aggregates of PEO-ss-PHMssEt at 1.0 mg/mL in the presence of excess DTT as monitored by DLS over a 24 hr period.

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VI. DTT-triggered release of NR from NR-loaded micelles

To prepare NR-loaded micelles, clear organic solutions consisting of a stock solution of NR in THF (5 mg/mL, 310 μL), PEO-ss-HMssEt (15.5 mg), and THF (2.2 mL) were mixed with water (15 mL). The resulting mixtures were stirred for 24 hrs to remove THF and subjected to filtration using a 0.45μm PES filter to remove free NR, resulting in NR-loaded PEO-ss-PHMssEt micelles at 1.0 mg/mL concentration. Equivalent aliquots (3 mL each) of NR-loaded micellar dispersions were mixed with DTT (2.3 mg, 5 mM). As a control, another aliquot (3 mL) without DTT was also tested. Fluorescence spectra (λ_{ex} = 480 nm) of the three mixtures were measured at different time intervals.

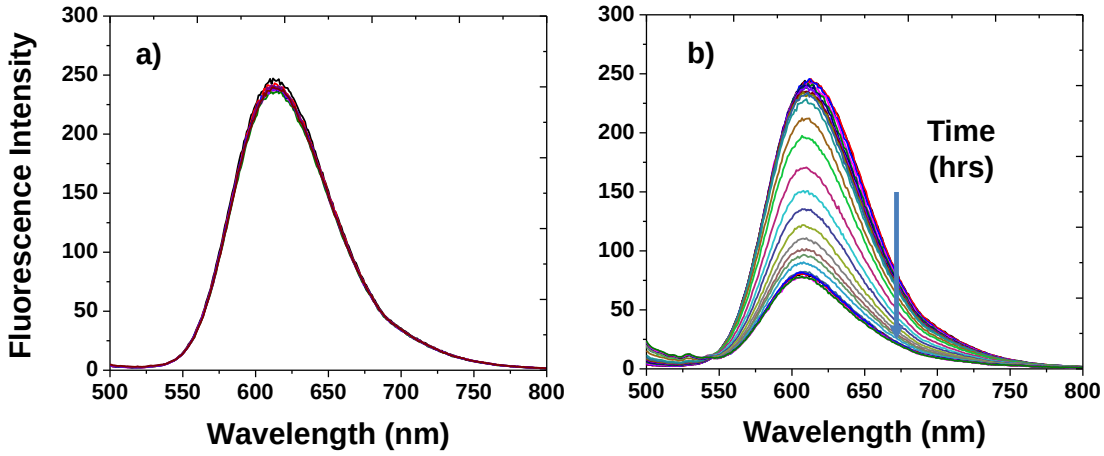

Figure S7. Overlaid fluorescence spectra of NR in the mixtures of NR-loaded micellar dispersions of PEO-ss-PHMssEt without as a control (a) and with 5 mM DTT (b).

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VII. Preparation of DOX-loaded PEO-ss-PHMssEt micelles using dialysis method

Water (8 mL) was added drop-wise to a clear solution consisting of the purified, dried PEO-ss-PHMssEt (20 mg), DOX (2 mg), and Et_3N (3 molar equivalents to DOX) in DMF (2 mL). The resulting dispersion was dialyzed over water (500 mL) for 4 days, yielding DOX-loaded micelles of PEO-ss-PHMssEt at 2.0 mg/mL.

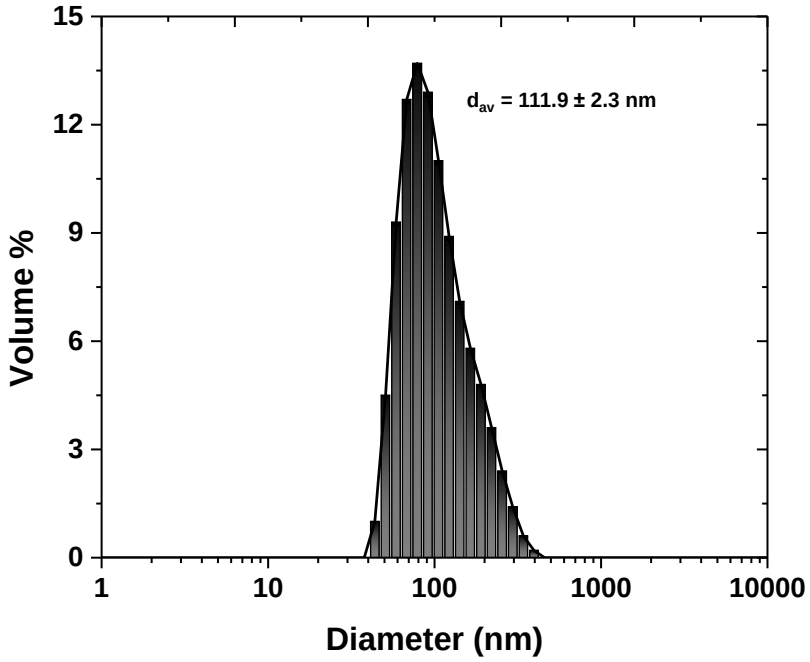

Figure S8. DLS diagram of DOX-loaded micelles of PEO-ss-PHMssEt at 2.0 mg/mL.

Determination of loading level of DOX using UV/Vis spectroscopy. Aliquots of DOX-loaded micellar dispersion (1 mL) were taken. After the removal of water, the residues were dissolved in DMF (2 mL) to form clear solutions. Their UV/Vis spectra were recorded and the loading level of DOX was calculated by the weight ratio of loaded DOX to dried polymers. The extinction coefficient of DOX at λ_{max} = 480 nm in DMF was 11,900 M^{-1} cm^{-1}.[4]

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VIII. Intracellular release of DOX upon degradation in response to GSH

Cell culture. HeLa cancer cells were cultured in DMEM (Dulbecco's modified Eagle's medium) containing 10% FBS (fetal bovine serum) and 1% antibiotics (50 units/mL penicillin and 50 units/mL streptomycin) at 37˚ C in a humidified atmosphere containing 5% CO_2.

Cell viability using MTT assay. Cells were plated at 5 x 10^5 cells/well into a 96-well plate and incubated for 24 hrs in DMEM (100 μL) containing 10% FBS. They were then incubated with various concentrations of micellar dispersions of PEO-ss-PHMssEt for 48 hrs. Blank controls without micelles were (cells only) run simultaneously. Cell viability was measured using CellTiter 96 Non-Radioactive Cell Proliferation Assay kit (MTT, Promega) according to manufacturer's instruction. Briefly, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solutions (15 μL) was added into each well. After 4 hrs of incubation, the medium containing unreacted MTT was carefully removed. A stop solution (100 μL) was added into each well in order to dissolve the formed formazan blue crystals, and then the absorbance at λ = 570 nm was recorded using a Powerwave HT Microplate Reader (Bio-Tek). Each concentration was 12-replicated. Cell viability was calculated as the percent ratio of absorbance of mixtures with micelles to control (cells only).

Intracellular DOX release and HeLa cells viability. Cells were plated at 5×10^5 cells/ well into a 96-well plate and incubated for 24 hrs in DMEM (100 μL). They were then mixed with and without GSH-OEt (10 mM in cell medium), and then incubated with different amounts of DOX-loaded micellar dispersion for 48 hrs. Blank controls without micelles (cells only) were run simultaneously to calculate viability as described above.

Cellular uptake using flow cytometry. Cells plated at 5×10^5 cells/ well into a 6-well plate and incubated for 24 hrs in DMEM (2 mL) were treated with and without GSH-OEt (10 mM in cell medium) for 12 hrs. Cells were washed with PBS buffer, and then incubated with DOX-loaded micelles (200 μL for DOX = 1.93 μg/mL) at 37 °C for 4 hrs. After culture medium was removed, cells were washed with PBS buffer three times and then treated with trypsin. The cells were suspended in DMEM (300 μL) for flow cytometry measurements. Data analysis was performed by means of a BD FACSCANTO II flow cytometer and BD FACSDiva software.

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Confocal laser scanning microscopy (CLSM). HeLa cells plated at 2×10^5 cells/ well into a 24-well plate and incubated for 24 hrs in DMEM (100 μL) were treated with GSH-OEt (10 mM in cell medium) for 12 hrs. Cells were washed with PBS buffer, and then incubated with DOX-loaded micelles (DOX = 3.2 μg/mL) at 37 °C for 24 hr. After culture medium was removed, cells were washed with PBS buffer three times. After the removal of supernatants, the cells were fixed with cold methanol (-20 °C) for 20 min at 4 °C. The slides were rinsed with TBST (tris-buffered saline Tween-20) for three times. Cells were stained with 2-(4-amidinophenl)-6-indolecarbamidine (DAPI) for 5 min. The fluorescence images were obtained using a LSM 510 Meta/Axiovert 200 (Carl Zeiss, Jena, Germany).

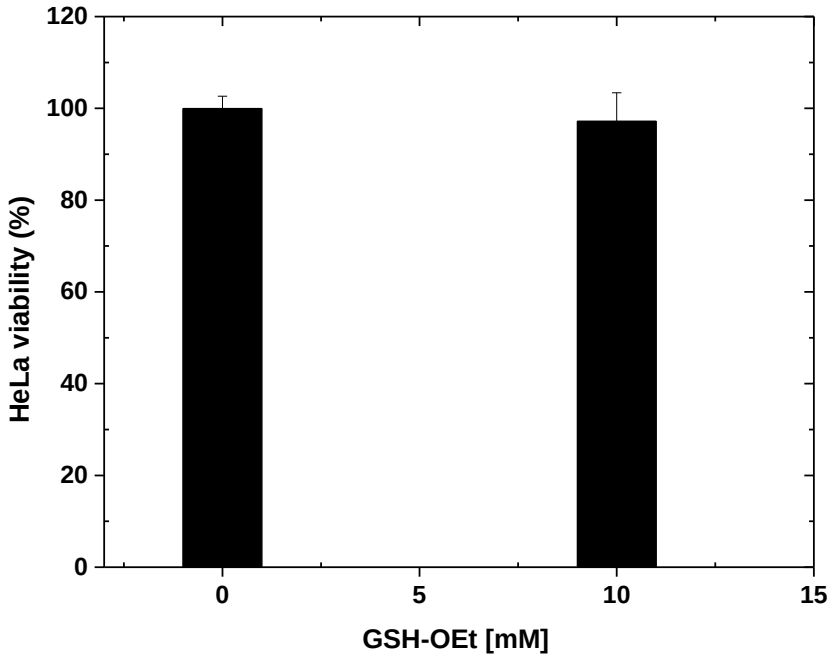

Figure S9. Viability of HeLa cells incubated with 10 mM GSH-OEt for 48 hrs determined by MTT assay. Data are presented as the average ± standard deviation (n = 12)

[1] G. J. P. Britovsek, J. England, A. J. P. White, *Inorg. Chem.* **2005**, *44*, 8125.
[2] Q. Zhang, N. R. Ko, J. K. Oh, *RSC Adv.* **2012**, *2*, 8079.
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[4] B. Khorsand, G. Lapointe, C. Brett, J. K. Oh, *Biomacromolecules* **2013**, *14*, 2103.