Supporting information

Thioether-Based Polymeric Micelles with Fine-Tuned Oxidation Sensitivities for Chemotherapeutic Drug Delivery

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Materials

Pentafluorophenol, 3-methylthiopropylamine (TP), and thiomorpholine (TM), were purchased from Tokyo Chemical Industry (USA). 4-(methylthio)benzylamine·HCl salt (TPh·HCl) was purchased from Enamine (USA). 4-acryloylmorpholine (AM), acryloyl chloride, 2,2'-azobis(isobutyronitrile) (AIBN), 2-(dodecylthiocarbonothioylthio)-2-methylpropionic acid (CTA), aluminum oxide (Al_2O_3), 1-methyl-2-pyrrolidinone (NMP), 4-dimethylamiopyridine (DMAP), deuterated chloroform ($CDCl_3$), calcium hydride (CaH_2), trifluoroacetic acid (TFA), sodium dodecyl sulfate (SDS), phosphor pentoxide (P_4O_{10}) and 4Å molecular sieves were purchased from Sigma Aldrich (USA). 1,4-dioxane, anhydrous dimethylformamide (DMF), tetrahydrofuran (THF), acetonitrile (CH_3CN), ethyl acetate (EtOAc), diethyl ether (Et_2O), ethanol (EtOH), triethylamine (TEA), potassium hydroxide (KOH) pellets, acetic anhydride (Ac_2O), sodium bicarbonate ($NaHCO_3$), hydrochloric acid (HCl), phosphate buffered saline (10x, PBS), Nile red, Hoechst 33342, MEM, fetal bovine serum, penicillin-streptomycin, trypsin-EDTA, and *N*-acetyl-L-cysteine (NAC) were purchased from Fisher Scientific (USA). Fluorescein isothiocyanate isomer I was purchased from Acros Organics. Hydrogen peroxide (H_2O_2) solution (30 wt%) was purchased from VWR (USA). Tert-butyl *N*-(2-aminoethyl) carbamate was purchased from Oakwood Products. XPell pellets were purchased from Xplosafe (USA). Dialysis cassettes (MWCO 2 kDa) was purchased from Spectrum Laboratories (USA). Sephadex LH20 and G20 were purchased from GE Healthcare (USA). ResiPore PL1113-6300 SEC column was purchased from Agilent (USA). Sepax SRT SEC-500 was purchased from Sepax Technologies (USA) XBridge Protein BEH SEC 200 Å column and BEH200 SEC Protein Standard were purchased from Waters (USA). Doxorubicin·HCl was purchased from Cayman Chemical Company (USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from MP Biomedical (USA). Human liver cancer HepG2 cells were purchased from ATCC (USA). Human umbilical vein endothelial cells (HUVEC, pooled donor) was purchased from Corning (USA). Transparent and black 96 well plates were purchased from ThermoFisher Scientific (USA). The glass-bottom dishes were purchased from Matsunami Glass (USA)

AIBN was recrystallized from MeOH. 4-acryloylmorpholine was passed through a plug of Al_2O_3 to remove inhibitor. 1,4-dioxane was distilled from CaH_2 and kept over molecular sieves and XPell pellets. Petafluorophenyl acrylate (PFPA) was synthesized using the reported procedure [Woodfield et al. Macromolecules 2014, 47 (2), 750-762]. Other reagents were used as received without further purification.

Instrumentation

Proton NMR (^{1}H NMR) spectroscopy. ^{1}H NMR spectra were measured with a Bruker NEO-400MHz NMR spectrometer. A number of 32 scans was collected and the delay time (D1) was set to 10 s for polymers and 1 s for small compounds. The chemical shifts are referenced to the residual undeuterated NMR solvent signal at 7.26 ppm ($CDCl_3$).

Fluorine NMR (^{19}F NMR) spectroscopy. ^{19}F NMR spectra in $CDCl_3$ were measured with a Bruker Bruker AV-III-HD-500 spectrometer. A number of 64 scans was collected and the delay time (D1) was set to 1 s. The chemical shifts are referenced against CF_3COOH at -76.55 ppm.

Attenuated total reflection infrared spectroscopy (ATR-IR). Attenuated total reflection infrared (ATR-IR) spectra were obtained using a Bruker vertex 70 spectrometer equipped with a Diiamax diamond ATR accessory set at a fixed incident angle of 45 degrees.

Gel permeation chromatography (GPC). Elution profiles of the polymers were obtained on a Waters Alliance e2695 HPLC system equipped with a 2414 refractive index (RI) detector and a Wyatt DAWN 8 multiangle light scattering detector.

For Polymers **7**-**9**, a ResiPore PL1113-6300 column was used. THF was used as the eluent with a flow rate of 1.0 mL/min. The temperature of the column oven and the RI detector was 40 and 45°C, respectively.

The M_w and M_n values of polymers were determined by Zimm plot using the refractive index increment (*dn*/*dc*) values of 0.052 mL/g for PPFPA homopolymer and 0.155 mL/g for PAM homopolymers to determine absolute molecular weight of polymers. For the PAM-PPFPA polymer, the dn/dc value was calculated using the following equation:

$$\frac{dn}{dc} = w_{PPFPA}\left(\frac{dn}{dc}\right)_{PPFPA} + w_{PAM}\left(\frac{dn}{dc}\right)_{PAM}$$

where w_i is the weight fraction of the component *i*.

For the micelles, a Sepax SRT SEC-500 or a XBridge Protein BEH SEC 200 Å column was used. PBS (pH 7.4) was used as the eluent with a flow rate of 0.4 mL/min. The temperature of the column oven and the RI detector was 40 and 45°C, respectively. The molar mass of the micelles (M_w) was determined by Zimm plot. The *dn*/*dc* values were estimated using the peak area detected by the RI detector assuming 100% mass recovery and the mass of the micelles injected for the measurement. For TP micelles, the M_p value of each peak was determined using the calibration curve of BEH200 SEC Protein Standard.

UV-Vis spectroscopy. The UV/Vis spectra were measured on a Thermo Scientific Nanodrop One^c spectrophotometer.

Dynamic light scattering (DLS). Dynamic light scattering measurements were done using a Malvern Zetasizer Nano ZS Series instrument and disposable micro cuvettes (ZEN0040, Malvern). The Z-average diameter and polydispersity index (PDI) of the micelles were determined by the cumulant method. The CONTIN analysis was used to determine the size distribution.

Cryogenic transmission electron microscopy (cryoTEM). The morphology of the micelles was observed by cryoTEM. Quantifoil Holey Carbon Grids, 300 mesh, with 1 μm hole size and 2 μm spacings (Quantifoil MicroTools, Jena, Germany) were used for vitrification of polymer micelle solutions (1 mg/mL in water). The grids were glow discharged in a plasma cleaner for about five minutes with the carbon side of the grids facing the plasma. FEI Vitrobot (FEI Company, Hillsboro, OR) was used for vitrification of the micelle solutions. The vitrification was done using filter papers mounted to the blotting pads with 3 seconds blotting time. Cryogen ethane was used for vitrification of the samples. The samples were preserved in vitreous ice in liquid nitrogen before acquiring the cryoTEM images. High resolution cryogenic experiments were performed on the FEI Titan Krios in the Huck Institute of the Life Sciences at the Pennsylvania State University. The measurements were conducted using a 300 kV electron source and a Falcone 3ec direct electron detector in linear mode. The nanoprobe mode of the microscope with a 70 μm C2 aperture, with a spot size of 3, and illuminated area of 1.20 μm at nominal magnification of 59,000x was employed to collect the TEM images. Binning of 1 (no camera pixel binning) was used for the images with 4096×4096 pixels.

UV-Vis/fluorescence spectroscopy with a plate reader. The UV-Vis absorbance and fluorescence intensities were measured with a Tecan infinite M200 plate reader.

Confocal laser scanning fluorescence microscopy (CLSFM). Fluorescence images were acquired on an Olympus Fluoview FV1000-D confocal microscope equipped with 405, 473, 559, and 632 nm lasers.

Synthesis

Synthesis of *N*-(3-(methylthio)propyl)acetamide (TPAM, 1). 3-(methylthio)propan-1-amine (564 mg, 5.4 mmol) and Et_3N (948 μL, 6.8 mmol, 1.3 eq) were dissolved in 5 mL CH_2Cl_2 and cooled on ice for 30 min. To the cold solution was added Ac_2O (643 μL, 6.8 mmol, 1.3 eq) in 5 mL CH_2Cl_2 dropwise over the course of 5 min. The solution was warmed to RT and stirred for 24 h. Since ninhydrin stain indicated the presence of unreacted amine, another 95 μL of Ac_2O (1.0 mmol, 0.18 eq) and 139 μL of Et_3N (1.0 mmol, 0.18 eq)

was added. After stirring for another 43 h, the reaction mixture was evaporated in a stream of air at 30°C. The clear oil was dissolved in 10 mL of 5% $NaHCO_3$ (aq) and the aqueous solution was extracted with CH_2Cl_2 (3 x 10 mL). After drying over $MgSO_4$, the solution was concentrated in a stream of air. The clear oil was dissolved in 10 mL of 1 M HCl (aq) and extracted with CH_2Cl_2 (3 x 10 mL). The clear organic phase was dried over $MgSO_4$, concentrated in stream of air and dried under vacuum to yield 576 mg of a viscous oil (3.9 mmol, 72%). ^{1}H NMR in $CDCl_3$ (**Figure S2a**, Supporting Information), δ (ppm): 5.87 (bs, 1H, NH), 3.33 (m, 2H, $C\underline{H}_2NH$), 2.52 (t, 2H, CH_2S), 2.08 (s, 3H, SCH_3), 1.97 (s, 3H, CH_3CO), 1.80 (m, 2H, $CH_2C\underline{H}_2CH_2$).

Synthesis of 1-thiomorpholinoethan-1-one (TMAM, 2). Thiomorpholine (531 mg, 5.2 mmol), 767 µL of Et_3N (5.5 mmol, 1.1 eq) and 9.7 mg of DMAP (0.08 mmol, 0.02 eq) were dissolved in 5 mL of CH_2Cl_2 and put on ice for 30 min. To the cooled solution was added Ac_2O (520 µL, 5.5 mmol, 1.1 eq). After stirring for 43 h the mixture was concentrated in a stream of air. The clear oil was dissolved in 10 mL of 5% $NaHCO_3$ (aq) and the aqueous solution was extracted with CH_2Cl_2 (3 x 10 mL). After drying over $MgSO_4$, the solution was concentrated in a stream of air. The clear oil was dissolved in 10 mL of 1 M HCl (aq) and the solution extracted with CH_2Cl_2 (3 x 10 mL). The clear organic phase was dried over $MgSO_4$, concentrated in flow of air and dried under vacuum to yield 443 mg of a clear oil (3.1 mmol, 59%). ^{1}H NMR in $CDCl_3$ (**Figure S2b**, Supporting Information), δ (ppm): 3.86 (m, 2H, CH_2NCH_2), 3.72 (m, 2H, CH_2NCH_2), 2.61 (m, 4H, CH_2SCH_2), 2.09 (s, 3H, CH_3CO).

Synthesis of *N*-(3-(methylthio)benzyl)acetamide (TPhAM, 3). (3-(methylthio)phenyl)methanamine·HCl salt (952 mg, 5.0 mmol) was suspended in 5 mL of CH_2Cl_2 and cooled on ice for 3 min before adding 1.46 mL of Et_3N (10.5 mmol, 2.1 eq). To homogenize the mixture, another 5 mL of CH_2Cl_2 was added. After stirring for 5 min at 0°C, 520 µL of Ac_2O (5.5 mmol, 1.1 eq) was added. After adding 13.3 mg of DMAP (0.11 mmol, 0.02 eq), the mixture was stirred for 23 h. The reaction mixture was evaporated in a stream of air at 30°C. The residue was suspended in 10 mL of 5% $NaHCO_3$ (aq) and the mixture was extracted with CH_2Cl_2 (3 x 10 mL). After drying over $MgSO_4$, the solution was concentrated in a stream of air. The white solid was suspended in 10 mL of 1 M HCl (aq) and the mixture was extracted with CH_2Cl_2 (3 x 10 mL). The clear organic phase was dried over $MgSO_4$, concentrated in a flow of air and dried under vacuum to yield 862 mg of a white solid (4.4 mmol, 87%). ^{1}H NMR in $CDCl_3$ (**Figure S2c**, Supporting Information), δ (ppm): 7.21 (m, 4H, CH_{aromat}), 5.75 (bs, 1H, NH), 4.39 (d, 2H, $C\underline{H}_2NH$), 2.47 (s, 3H, CH_3S), 2.01 (s, 3H, CH_3CO).

Synthesis of *N*-(3-(methylsulfinyl)propyl)acetamide (TPAM-SO, 4). TPAM (90 mg, 0.6 mmol) was dissolved in 540 µL of water in a glass vial and 68 µL of 9.0 M H_2O_2 (0.6 mmol, 1 eq) was added. The mixture was rotated at RT for 28 h, frozen and lyophilized to yield 99 mg of a white solid. ^{1}H NMR showed complete disappearance of the signals of TPAM and the appearance of new signals assigned to be those of the sulfoxide as shown by FTIR (**Figure 3a** manuscript). ^{1}H NMR in $CDCl_3$ (**Figure S2d**, Supporting Information), δ (ppm): 6.55 (bs, 1H, NH), 3.38 (m, 2H, CH_2-NH), 2.74 (m, 2H, $CH_2S(O)$), 2.57 (s, 3H, $S(O)CH_3$), 2.05 (m, 2H, $CH_2C\underline{H}_2CH_2$), 2.02 (s, 3H, CH_3CO). In addition, some sulfone formed as suggested by the signals at 6.40 (bs, NH), 3.07 (m, 2H), 2.91 (s, $S(O_2)CH_3$), 1.97 (s, $COCH_3$). Other signals of the sulfone were not observed due to overlap with the sulfoxide signals.

Synthesis of 1-(1-oxidothiomorpholino)ethan-1-one (TMAM-SO, 5). TMAM (119 mg, 0.8 mmol) was dissolved in 817 µL of water in a glass vial and 91 µL of 9.0 M H_2O_2 (0.8 mmol) was added. The mixture was rotated for 28 h, frozen and lyophilized to yield 119 mg of a white solid. ^{1}H NMR showed complete disappearance of TMAM and the appearance of new signals assigned to be the sulfoxide as shown by FTIR (**Figure 3b** manuscript). ^{1}H NMR in $CDCl_3$ (**Figure S2e**, Supporting Information), δ (ppm): 4.48 (m, 1H, CH_2NCH_2), 4.13 (m, 1H, CH_2NCH_2), 3.73 (m, 2H, CH_2NCH_2), 2.84 (m, 2H, $CH_2S(O)CH_2$), 2.67 (m, 2H, $CH_2S(O)CH_2$), 2.13 (s, 3H, $COCH_3$). In addition, some sulfone had formed as suggested by the signals at 3.94 (m, CH_2), 3.03 (m, CH_2), 2.16 (s, $COCH_3$). Other signals of the sulfone were not observed due to overlap with the sulfoxide signals.

Synthesis of *N*-(3-(methylsulfinyl)benzyl)acetamide (TPhAM-SO, 6). TPhAM (23 mg, 0.1 mmol) was dissolved in 180 µL of dioxane in a glass vial and to the clear solution was added 76 µL of 1.75 M H_2O_2 (aq) (0.1 mmol) and the mixture was stirred for 7 d at RT. The reaction mixture was diluted with water and the clear solution was lyophilized to yield 25 mg of a white solid. ^{1}H NMR showed 90% of TPhAM to be oxidized to the sulfoxide as shown by FTIR (**Figure 3c** manuscript). ^{1}H NMR in $CDCl_3$ (**Figure S2f**, Supporting Information), δ (ppm): 7.57 (d, 2H, CH_{aromat}), 7.42 (d, 2H, CH_{aromat}), 6.25 (bs, 1H, NH), 4.47 (d, 2H, $PhCH_2S(O)$), 2.68 (s, 3H, $S(O)CH_3$), 2.05 (s, 3H, $COCH_3$).

Synthesis of 5-[(2-aminoethyl)thioureidyl]fluorescein TFA salt (FITC-NH_2·TFA, 16). FITC-NH_2·TFA was synthesized as reported previously with slight modifications [Simirnov et al., J. Am. Chem. Soc. 2013, 135, 2887-2890]. Tert-butyl *N*-(2-aminoethyl)carbamate (50 mg, 0.314 mmol) was dissolved in 50 mL of 0.1 M $NaHCO_3$ (aq) and 2.5 mL of CH_3CN on ice. To this solution, fluorescein isothiocyanate (100 mg, 0.257 mmol) suspended in 3.5 mL of CH_3CN was added. The reaction mixture was stirred for 1 d in the dark. The clear orange solution was neutralized with 1 M HCl (aq) and evaporated under reduced pressure at 40°C to remove CH_3CN. The orange-brown precipitate was collected by centrifugation, washed with deionized water (2 x 10 mL) and lyophilized. The crude product was dissolved in 25 mL EtOAc and centrifuged to remove an insoluble brown solid. The clear yellow supernatant was evaporated at 40°C under reduced pressure. The solid was dissolved in 2 mL of TFA/water (95/5 v/v%) and stirred at RT for 2 h. The reaction mixture was evaporated under air flow at RT. The residue was dissolved in 1 mL DMF and added dropwise to 50 mL Et_2O. The precipitate was collected on a glass filter, dissolved in water and lyophilized. ^{1}H NMR of the product in d_6-DMSO was measured to confirm the successful synthesis of FITC-NH_2·TFA.

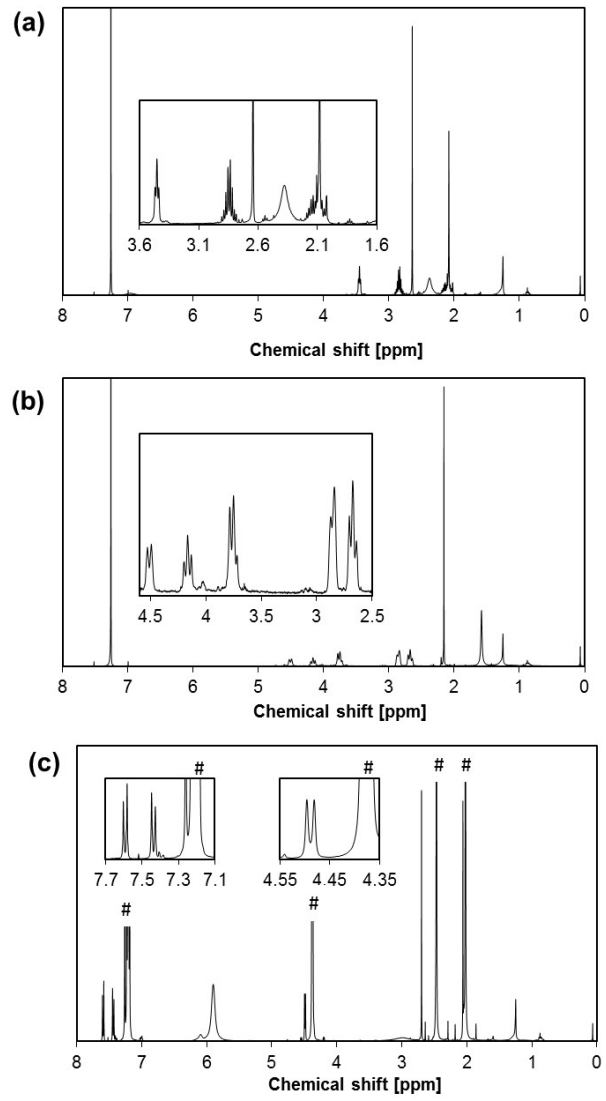


Figure S1. ^{1}H NMR spectra of the reaction mixture of the time course experiment. After 48 h the reaction mixtures were lyophilized, extracted with chloroform, concentrated and dried under vacuum at 40ºC. ^{1}H NMR spectra of the samples showed the presence of the sulfoxide of TPAM (**a**), TMAM (**b**) and TPhAM (**c**). In case of TPAM and TMAM, the starting thioether signals are not present due to evaporation during lyophilization and drying. In the case of TPhAM, signals of the starting material have been indicated with #.

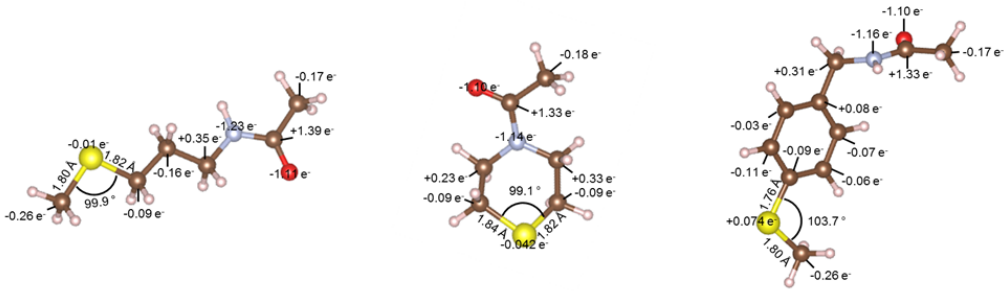


Figure S2. Molecular structures of TPAM, TMAM, and TPhAM. Color code: brown-carbon, yellow-sulfur, blue-nitrogen, red-oxygen, white-hydrogen.

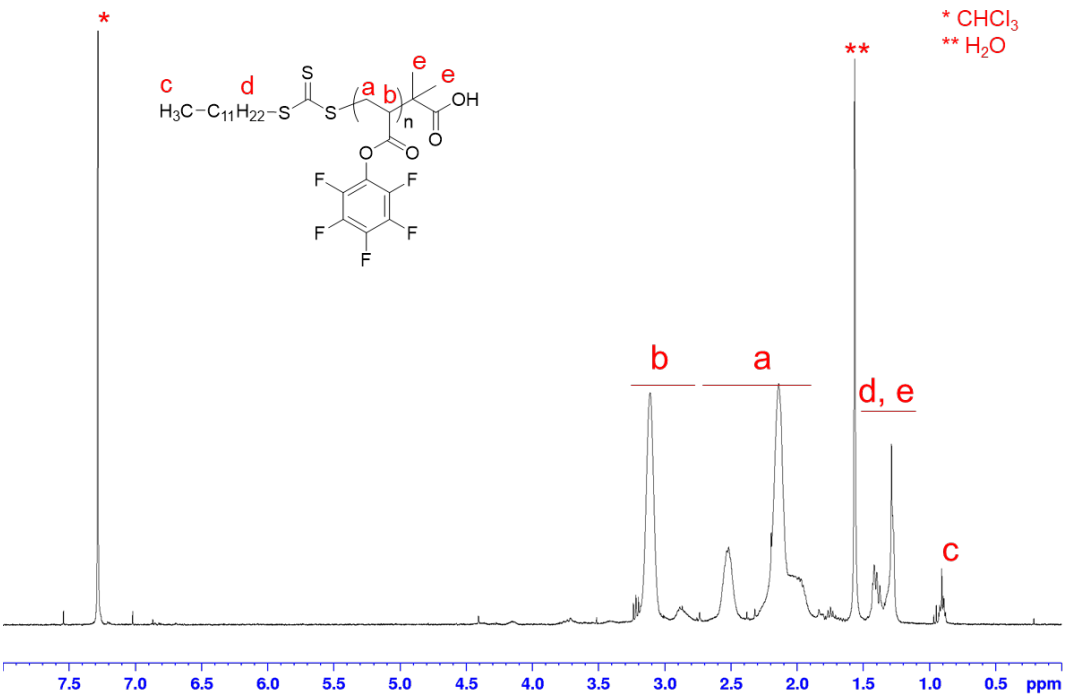


Figure S3. ^{1}H NMR spectrum of PPFPA (**7**) in $CDCl_3$.

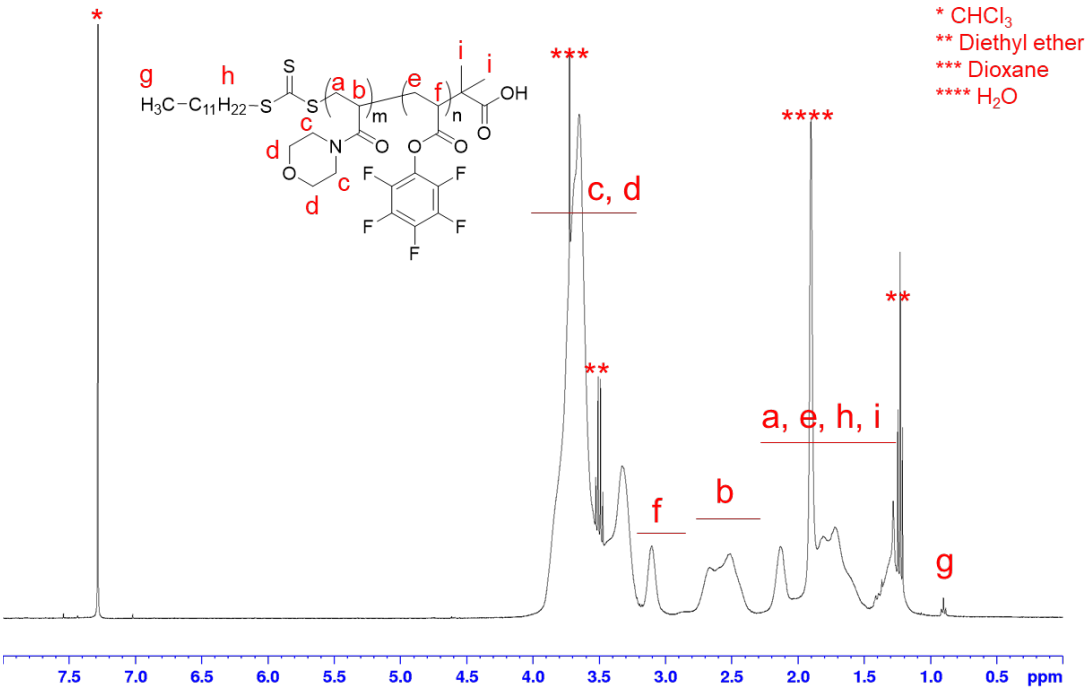


Figure S4. ^{1}H NMR spectrum of PAM-PPFPA (**8**) in $CDCl_3$.

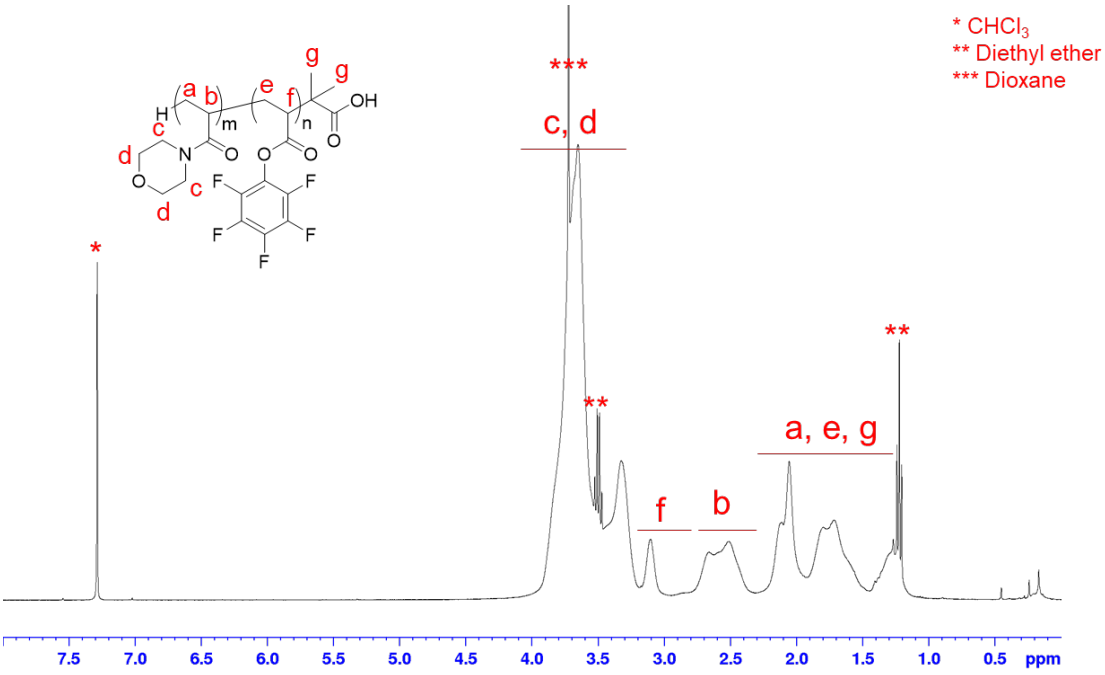


Figure S5. ^{1}H NMR spectrum of PAM-PPFPA after CTA removal (**9**) in $CDCl_3$.

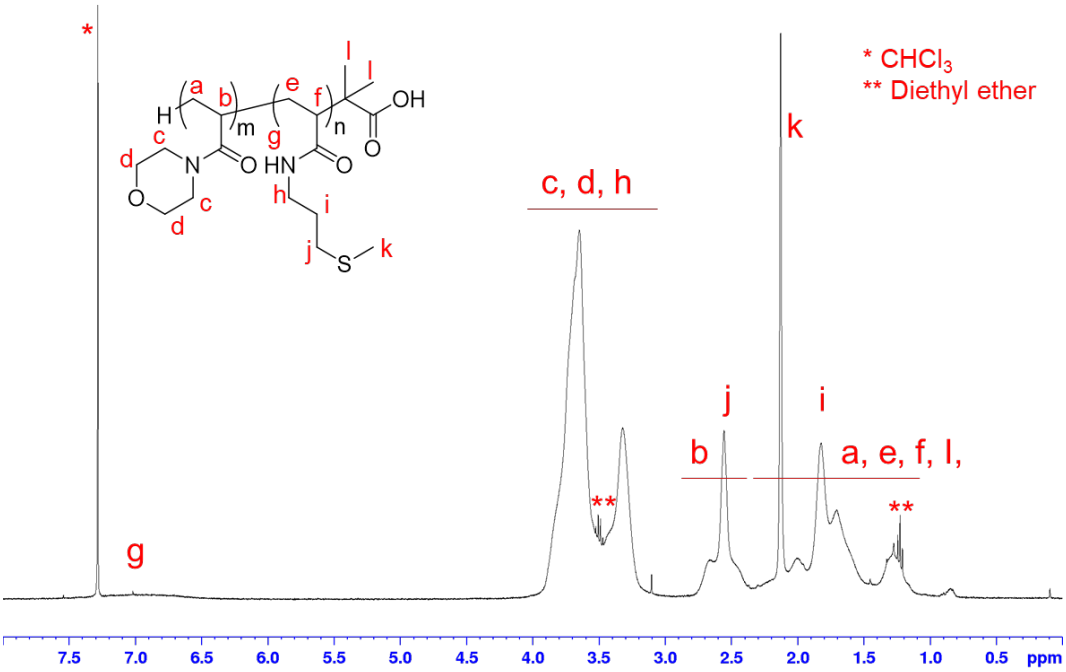


Figure S6. ^{1}H NMR spectrum of TP (**10**) in $CDCl_3$.

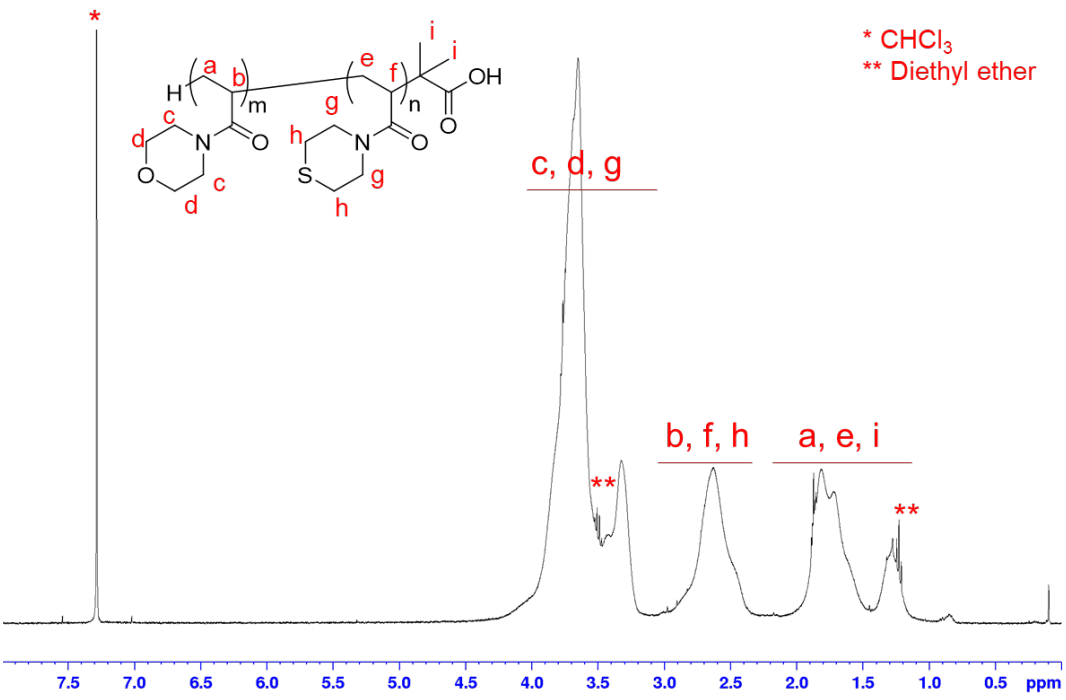


Figure S7. ^{1}H NMR spectrum of TM (**11**) in $CDCl_3$.

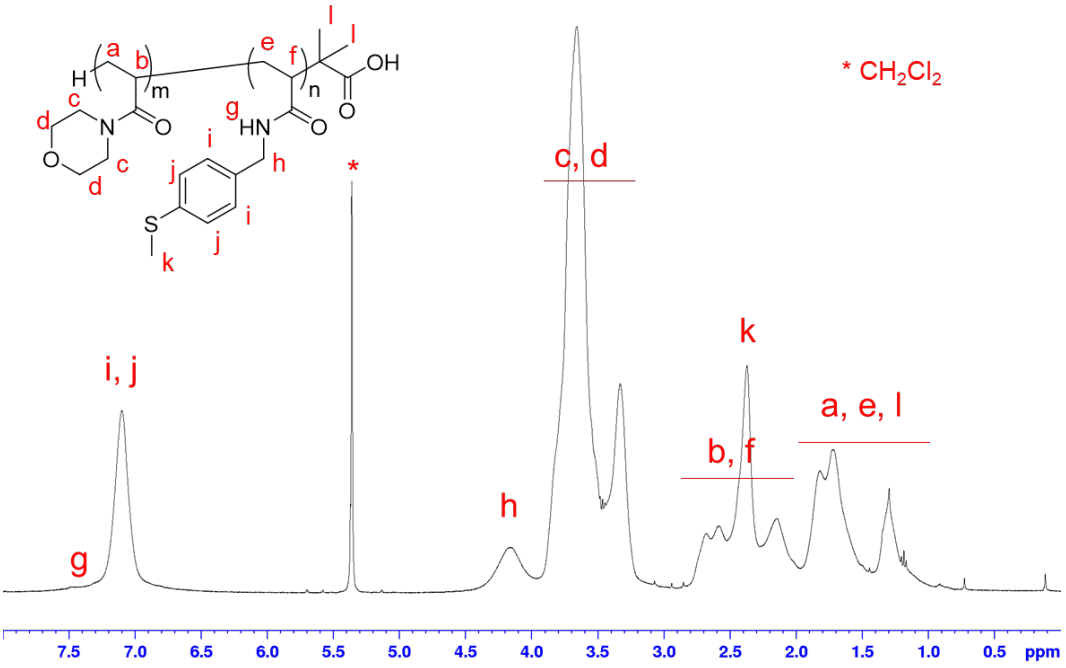


Figure S8. ^{1}H NMR spectrum of TPh (**12**) in CD_2Cl_2.

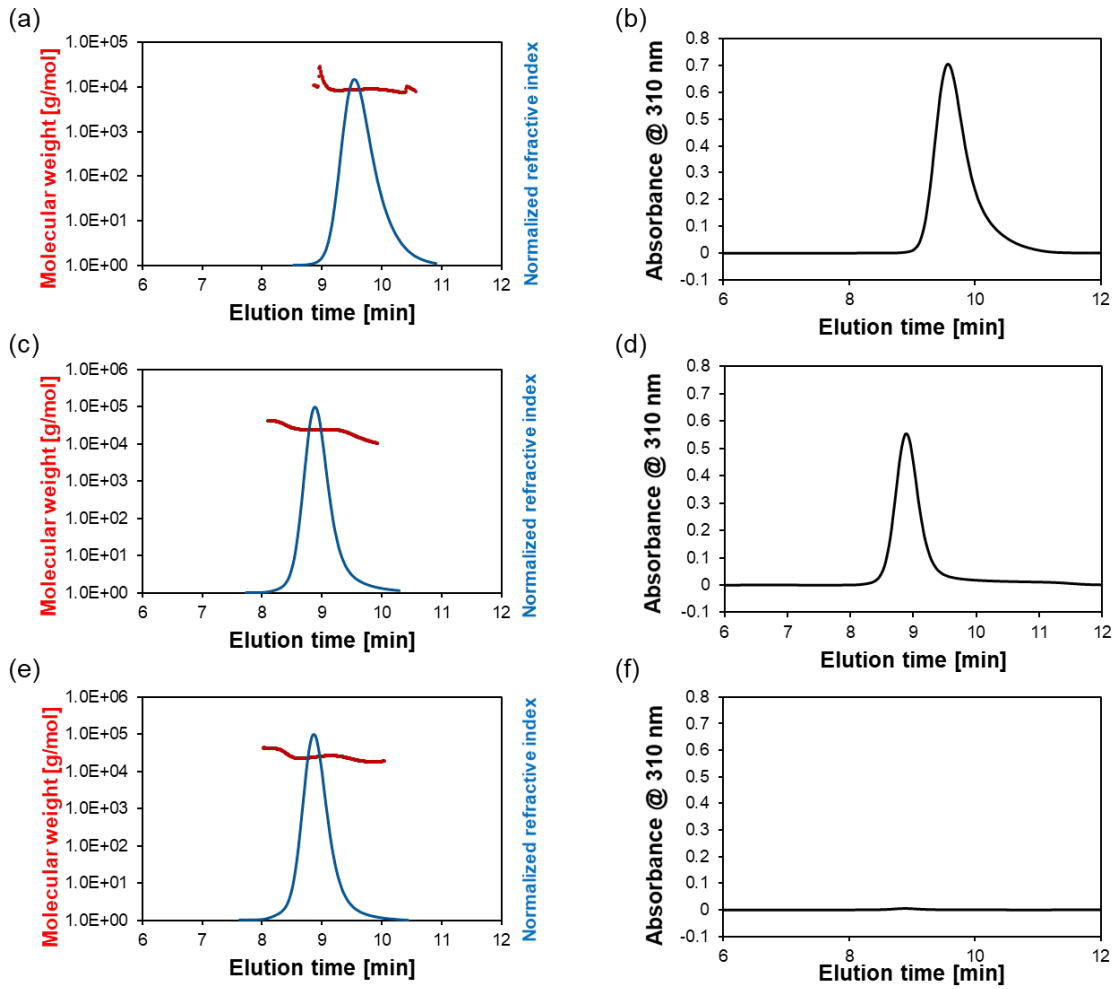
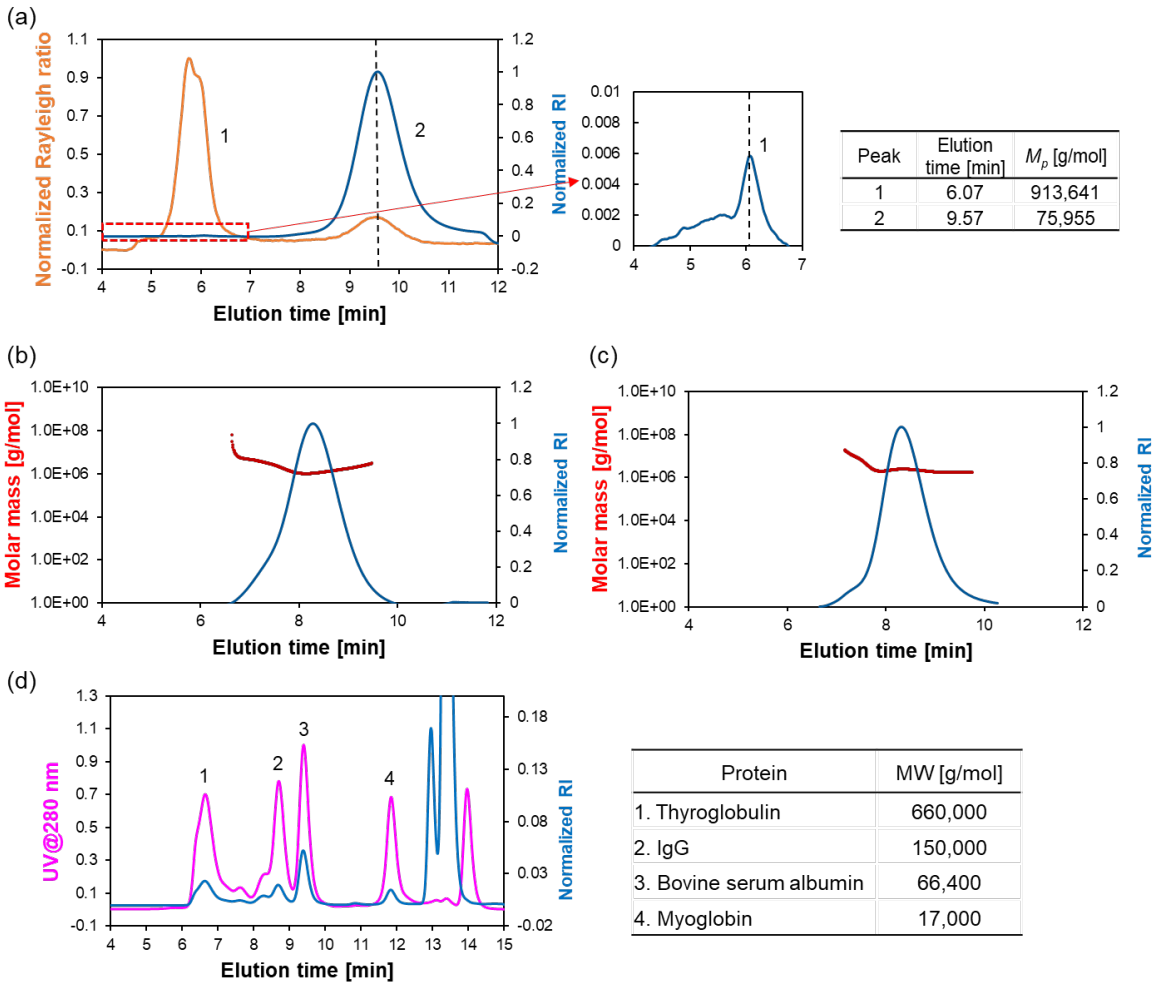


Figure S9. GPC elution profiles of (a, b) PPFPA (**7**), (c, d) PAM-PPFPA (**8**), and (e, f) PAM-PPFPA after the CTA end group removal (**9**). (a, c, e) Molecular weight as determined by MALLS (red), refractive index (blue), (b, d, f) Absorbance at 310 nm as detected by a photodiode array (PDA) detector. Eluent: THF.



Peak	Elution time [min]	M_p [g/mol]
1	6.07	913,641
2	9.57	75,955

Protein	MW [g/mol]
1. Thyroglobulin	660,000
2. IgG	150,000
3. Bovine serum albumin	66,400
4. Myoglobin	17,000

Figure S10. GPC elution profiles of (a) TP, (b) TM, and (c) TPh and (d) BEH200 SEC Protein Standard. (a) Rayleigh ratio (orange) and RI (blue). Molecular weight of the peak maxima (M_p) was estimated based on the calibration curve of BEH200 SEC Protein Standard. (b) and (c) Molar mass (red) and RI (blue). (d) Absorbance at 280 nm (pink) and RI (blue). Eluent: PBS, pH7.4. The peak of RI and UV signals were aligned with the light scattering signal using ASTRA software.

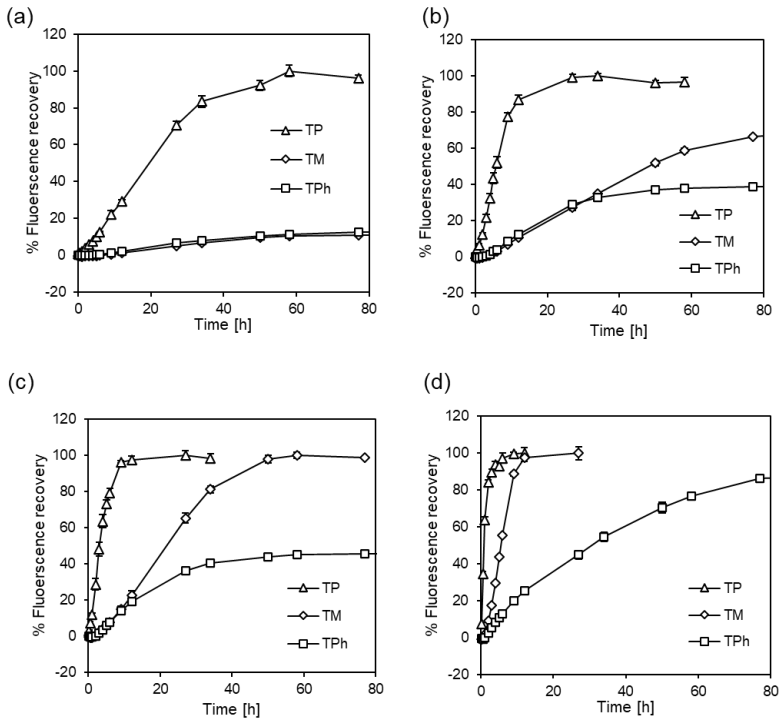


Figure S11. Dissociation of the thioether/FITC-containing micelles upon oxidation as determined by fluorescence recovery of self-quenched FITC fluorophores. The TP/FITC, TM/FITC and TPh/FITC micelles (0.05 mg/mL in PBS (pH7.4, 100 mM)) were incubated with (a) 1, (b) 5, (c) 10 and (d) 50 mM H_2O_2 at 37ºC and the fluorescence intensity (λ_{ex}=490 nm, λ_{em}=520 nm) was measured at different time points. TP/FITC: triangles, TM/FITC: diamonds, TPh/FITC: squares. *n*=3.

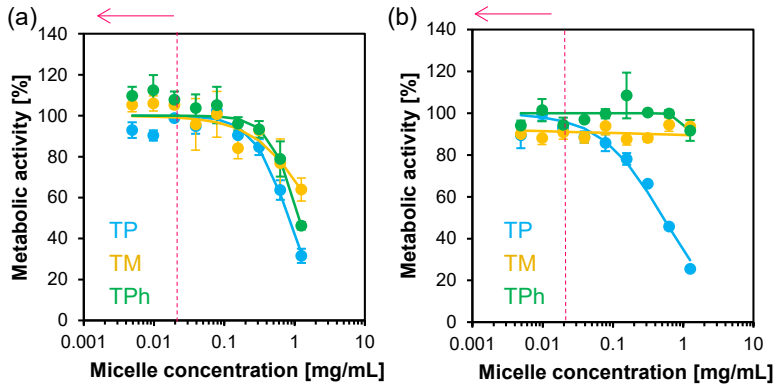


Figure S12. Cytotoxicity of micelles in (a) HepG2 cells and (b) HUVECs. Cells were cultured in the presence of micelles for 3 d. Metabolic activity was measured by MTT assay. *n*=3. The dotted line and the arrow indicate the concentration range of the micelles used in the MTT assay in **Figure 10** in the manuscript.

Table S1. The loading amount of Dox in the micelles (*n*=3).

Entry	wt% of Dox
TP	8.5 ±0.2
TM	10.5 ±0.3
TPh	10.8 ±0.4

wt% of Dox = $M_{Dox}/(M_{Dox} + M_{Micelles}) \times 100$

where M_i: Mass of component *i*