Supporting Information

Garlic-inspired trisulfide linkers for thiol-stimulated H_2S release

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Experimental Details

Materials

DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine) and PEG(2000)-phospholipid (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (ammonium salt) were purchased from Avanti Polar Lipids. mPEG-amine MW 2000g/mol (Linear Monofunctional PEG Amine NH_2) was purchased from Creative PEG Works. Sodium sulfide (Na_2S) anhydrous was purchased from Alfa Aesar (Cat. No. 65122) and hydrochloric acid 32% was purchased from Ajax Finechem. Solvents (except anhydrous solvents) were purchased from Merck Millipore and used as received. All other chemicals such as reagents and anhydrous solvents for synthesis were purchased from Sigma-Aldrich at the highest purity available and used without further purification (unless otherwise stated). A Reveleris® Flash Chromatography System fitted with GRACE® silica cartridges was used for purification of monomer and intermediates. TLC was performed on Merck Silica $60F_{254}$ plates.

PBS 7.4 (phosphate buffered saline, pH 7.4) was reconstituted from Aldrich powder which when dissolved in 1 L of deionized water yields 0.01 M phosphate buffered saline (NaCl 0.138 M, KCl 0.0027 M) pH 7.4 at 25 °C.

Characterization

^1H (400 MHz) and ^{13}C NMR (100 MHz) Spectroscopy

Spectra were obtained with a Bruker UltraShield 400 MHz spectrometer at 25°C running Bruker Topspin Software. Spectra were recorded for samples dissolved in deuterated solvent and chemical shifts are reported as parts per million from external tetramethylsilane.

High Resolution Mass Spectrometry Time-of-Flight (HRMS TOF) Analysis.

All analyses were performed on an Agilent 6224 TOF LC/MS Mass Spectrometer coupled to an Agilent 1290 Infinity (Agilent, Palo Alto, CA). All data were acquired and reference mass corrected via a dual-spray electrospray ionisation (ESI) source. Each scan or data point on the

Total Ion Chromatogram (TIC) is an average of 13,700 transients, producing a spectrum every second. Mass spectra were created by averaging the scans across each peak and background subtracted against the first 10 seconds of the TIC. Acquisition was performed using the Agilent Mass Hunter Data Acquisition software version B.05.00 Build 5.0.5042.2 and analysis was performed using Mass Hunter Qualitative Analysis version B.05.00 Build 5.0.519.13.

Dynamic Light Scattering (DLS)

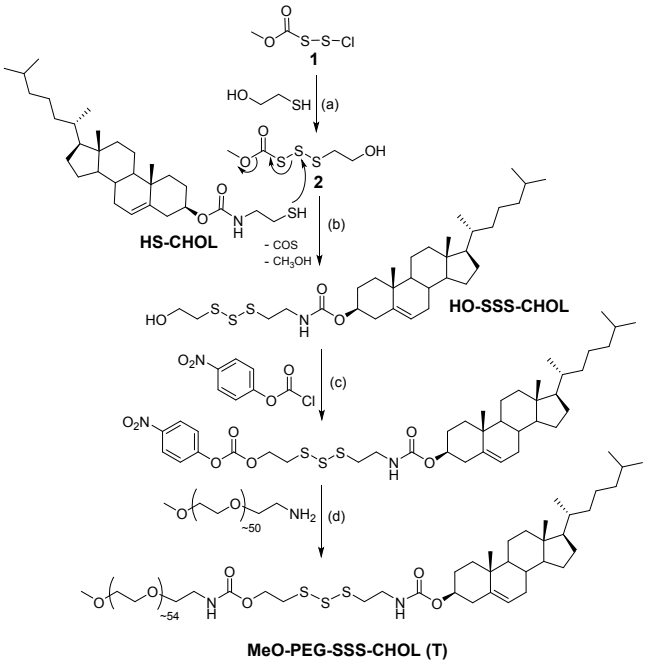
DLS measurements were carried out at 25 °C on a Malvern Zetasizer Nano ZS Series running DTS software (laser, 4 mW, $\lambda = 633$ nm; angle 173°). The dispersant chosen for measurements was water with assumed viscosity (cP) of 0.8872. The polydispersity index (PDI) used to describe the average diameters and size distribution of prepared micelles and liposomes, was determined via a cumulants analysis of the measured intensity autocorrelation function using the DTS software. Samples were filtered using 0.45 μm PTFE syringe filter to remove contaminants /dust prior to measurement.

Fluorescence spectroscopy

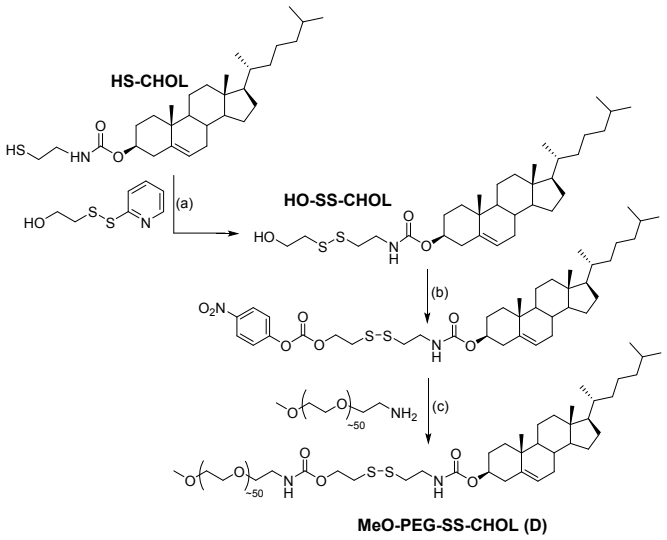
Fluorescence spectra were obtained using a fluorescence spectrophotometer (Shimadzu RF-5301 PC). Slit widths were set at between 3-5 mm for excitation and 3-15 nm for emission.

Schemes Relating to Discussion

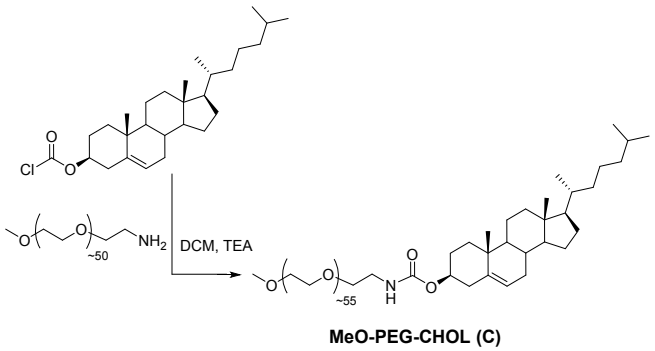
Scheme S1. Synthesis of MeO-PEG-SSS-CHOL (**T**) conjugate, showing thiol mediated fragmentation reaction of **2** (a) CH_2Cl_2 (b) $CHCl_3$, N-methylmorpholine (c) TEA, CH_2Cl_2 (d) DIPEA, DMAP, CH_2Cl_2



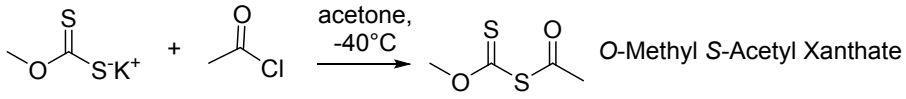
Scheme S2. Synthesis of MeO-PEG-SS-CHOL (**D**) conjugate: (a) THF, 0°C (b) TEA, CH_2Cl_2 (c) DIPEA, DMAP, CH_2Cl_2



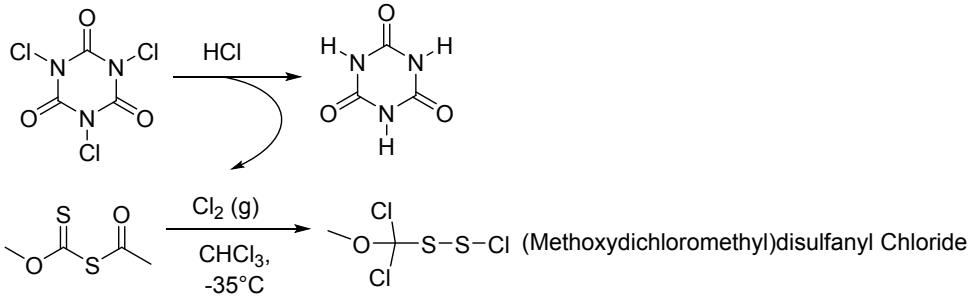
Scheme S3. Synthesis of MeO-PEG-CHOL (**C**) conjugate.



Synthetic Protocols



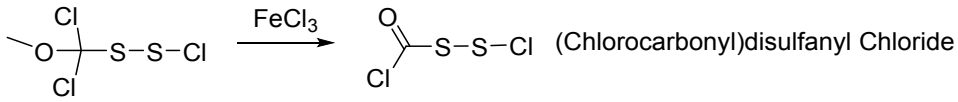
***O*-Methyl *S*-Acetyl Xanthate** Potassium methyl xanthate salt was synthesized by adding carbon disulphide (60 mL) dropwise to an ice-cooled solution of methanol (62 mL) and potassium hydroxide (17 g). The resulting yellow dispersion was left to stir overnight at room temperature followed by removal of CS_2 and excess methanol using rotary evaporation. The resulting pale yellow solid was collected by filtration using diethyl ether and dried under vacuum (isolated yield 85%). Acetyl chloride 13.3 g, 0.151 mol) was added dropwise under a nitrogen atmosphere to a dry ice-acetonitrile-cooled (-35 °C) solution of potassium methyl xanthate (20.0 g, 0.137 mol) in anhydrous acetone (400 mL). After addition was completed the reaction was left to warm up to room temperature and stirred for another 5 hours. The reaction mixture was then filtered to remove KCl, and then the salt rinsed with acetone. The filtrate was then concentrated at reduced pressure to provide a dark yellow liquid which was then purified by high vacuum distillation, bp 46 °C (0.1 mmHg); Yield: 17.10g (88%), yellow liquid. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 2.41 (s, 3H, CH_3CO-), 4.24 (s, 3H, CH_3OCS-) ppm. ^{13}C NMR (50 MHz, $CDCl_3$) δ: 30.9, 60.7, 190.0, 204.5 ppm.



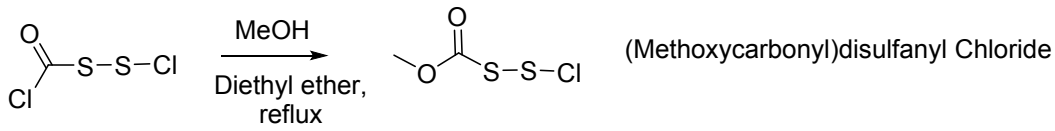
(Methoxydichloromethyl)disulfanyl chloride. Chlorine gas was generated by the reaction of trichlorocyanuric acid (TCCA) with dilute hydrogen chloride: (CAUTION: Cl_2 gas is highly toxic and a powerful irritant. Proper safety precautions and procedures should be employed during this reaction). Excess chlorine gas which is not taken up by the reaction is scavenged using a scrubber solution of dilute NaOH (producing bleach). A bubbler is used to monitor gas flow and relieve excess pressure.

Aqueous HCl solution was prepared (70 ml, a 1:1 mixture of 32% HCl solution with water) and slowly added using a dropping funnel to TCCA (26.5 g, 0.114 mol). The evolution of chlorine gas from TCCA was evident as a cloud of green gas which was then transferred to a

second flask which contained a stirring solution of *O*-methyl *S*-acetyl xanthate (17.1 g, 0.114 mol) in $CHCl_3$ (100 mL) (cooled previously to -35 °C using dry ice/acetonitrile bath). The gas was bubbled into the second solution using flexible tubing attached to a glass tube which was submerged in the xanthate solution. The flask containing the TCCA/Cl_2 mixture was stirred and occasionally shaken to encourage a positive flow of Cl_2 into the xanthate solution. Progress of the reaction was monitored by mass uptake and ^{1}H NMR, the amount of Cl_2 taken up expected to be 2 equivalents with respect to *O*-methyl *S*-acetyl xanthate (16.2 g). After approximately 30 minutes (time taken for the HCl addition to TCCA), *O*-methyl *S*-acetyl xanthate solution appeared to darken to a yellowish-orange colour. At this point the mixture was left to warm up to room temp and stirring was continued for a further 30 minutes. The solvent and acetyl chloride formed were then removed at reduced pressure to provide a yellow liquid, which was purified by high vacuum distillation, bp 42 °C (0.2 mmHg). Yield: 12.0 g (49%) ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.81 ppm. ^{13}C NMR (50 MHz, $CDCl_3$) δ: 57.3, 117.6 ppm.

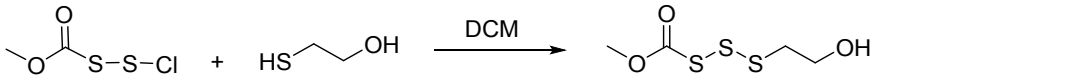


(Chlorocarbonyl)disulfanyl chloride. With stirring, anhydrous $FeCl_3$ (20 mg) was added carefully to (methoxydichloromethyl)disulfanyl chloride (11.7 g, 55 mmol) which was maintained at 0 °C under N_2. Vigorous gas evolution occurred, with weight loss >100% of the theoretical amount of MeCl (2.8 g) due to volatilization of the product). The title product was obtained as a yellow liquid upon distillation using a three-stage diaphram pump, bp 44 °C (12 mmHg) Yield: 3.0 g (34%). ^{13}C NMR (50 MHz, $CDCl_3$) δ: 163.0 ppm.



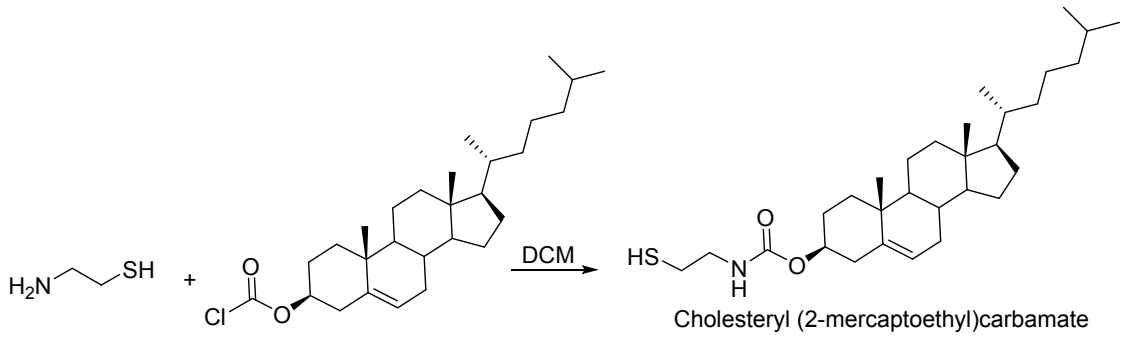
(Methoxycarbonyl)disulfanyl chloride. MeOH (760 μL, 0.018 mol) was added dropwise using a micro-syringe to a stirring solution of (chlorocarbonyl)disulfanyl chloride (2.9 g, 0.018 mol) in dry diethyl ether (20 mL). The solution was refluxed in a multi-necked round bottom flask fitted with a reflux condenser (left open to allow the escape of HCl), for at least 8 hours. The reaction was monitored using ^{13}C NMR. The ether was then removed under reduced pressure and the product isolated as a yellow liquid upon distillation using a three-stage

diaphragm pump, bp 40 °C (2 mmHg) Yield: 900 mg (32%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.01 ppm. ^{13}C NMR (50 MHz, $CDCl_3$) δ: 56.6, 165.9 ppm.



Methoxycarbonyl 3-(2-hydroxyethyl)trisulfane

Methoxycarbonyl 3-(2-hydroxyethyl)trisulfane. A solution of mercaptoethanol (158 mg, 142 µL, 2.03 mmol) in dry CH_2Cl_2 (2 mL) was added dropwise to an ice-cooled solution of (methoxycarbonyl)disulfanyl chloride (290 mg, 1.84 mmol) in 5 mL of dry CH_2Cl_2. Hydrogen chloride is evolved with the disappearance of the yellow colour. The reaction mixture was then left to stir for 1 hour at room temperature. The CH_2Cl_2 was then removed to yield 300 mg of crude product (50% pure). The product was purified using a Reveleris® Flash Chromatography System fitted with a GRACE® silica cartridge, with the gradient solvent system, 1:9 ethyl acetate/cyclohexane → 1:1 ethyl acetate/cyclohexane. The product was afforded as an oil (150 mg, 41%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.13 (t, 2H, *J* = 5.8Hz), 3.93 (s, 3H), 4.01 (t, 2H, *J* = 5.8Hz) ppm. ^{13}C NMR (50 MHz, $CDCl_3$) δ: 42.0, 56.0, 60.4, 169.6 ppm.



Cholesteryl (2-mercaptoethyl)carbamate

Cholesteryl (2-mercaptoethyl)carbamate. A solution of cholesteryl chloroformate (2.46 g, 5.50 mmol) in CH_2Cl_2 (10 mL) was added dropwise to a stirred solution of cysteamine (0.93g, 0.012 mol) in CH_2Cl_2 (30 mL) at 0°C. The resulting solution was stirred at room temperature for a further 5 hours. The formed cysteamine hydrochloride salt was filtered off, the CH_2Cl_2 removed under vacuum and the resulting residue purified using a Reveleris® Flash Chromatography System fitted with a GRACE® silica cartridge, with solvent system 1% MeOH in CH_2Cl_2. The product was afforded as a white solid (2.30 g, 86%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.37 (m, 1H, H6), 4.99 (m, 1H, NH), 4.50 (m, 1H, H3), 3.35 (m, 2H, C*H*$_2$-NH), 2.66 (m, 2H, S H-C*H*$_2$), 2.22-2.39 (m, 2H, H4), 1.78-2.06 (m, 5H, H2, H7, H8), 0.94 - 1.61 (m, 24H, H1, H9, H11, H12, H14-H17, H19, H20, H22-H25), 0.91 (d, *J* 6.5 Hz, 3H, H21), 0.87 (2 × d, *J* 6.5 Hz, 6H, H26, H27), 0.67 (s, 3H, H18) ppm. ^{13}C NMR (50 MHz, $CDCl_3$) δ:

11.8, 18.7, 19.3, 21.0, 22.5, 22.8, 23.8, 24.2, 25.0, 27.8, 28.1, 28.2, 31.8, 31.9, 35.8, 36.2, 36.5, 37.0, 38.5, 39.5, 39.7, 42.3, 43.9, 50.0, 56.1, 56.6, 74.5, 122.5, 139.7, 156.0 ppm.

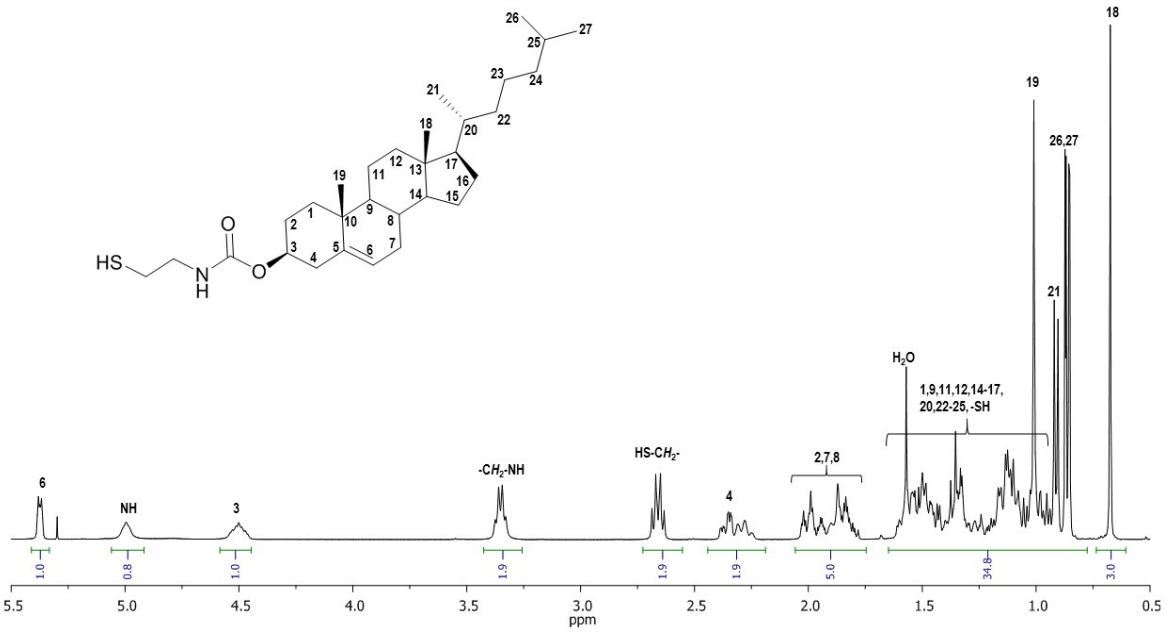
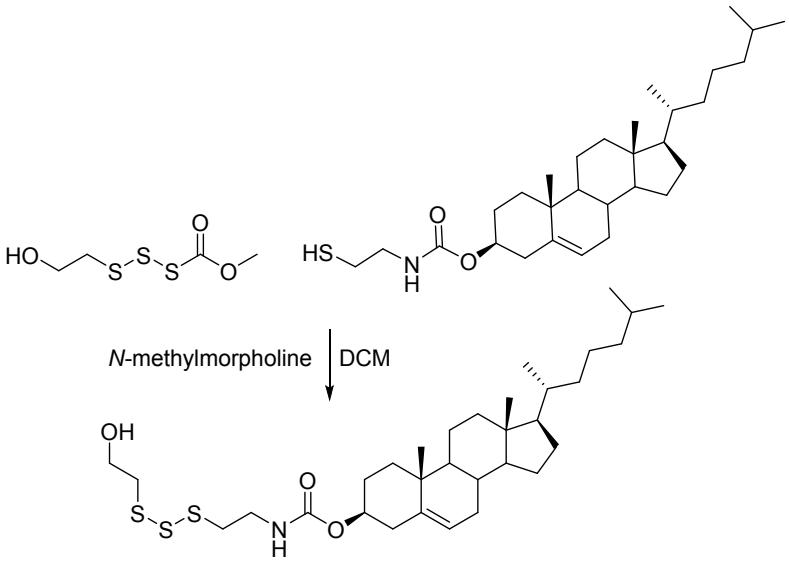


Figure S1: ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of Cholesteryl (2-mercaptoethyl)carbamate with main peak assignments.



Cholesteryl (2-((2-hydroxyethyl)trisulfanyl)ethyl)carbamate

Cholesteryl (2-((2-hydroxyethyl)trisulfanyl)ethyl)carbamate. Cholesteryl (2-mercapto ethyl)carbamate (400 mg, 8.16×10^{-4} mol) in 2 mL of $CHCl_3$ was added dropwise to a solution of methoxycarbonyl 3-(2-hydroxyethyl)trisulfane (150 mg, 7.48×10^{-4} mol) in 2 mL of $CHCl_3$. The mixture was stirred at 25 °C until all the starting material was consumed (evident either by TLC or ^{1}H NMR). The crude material was then concentrated and purified using a

Reveleris® Flash Chromatography System fitted with a GRACE® silica cartridge, with solvent system 1:9 ethyl acetate/cyclohexane → i1:1 ethyl acetate/cyclohexane. The product was isolated as a waxy, sticky white solid. (140 mg, 33%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.37 (m, 1H, H6), 5.08 (br m, 1H, NH), 4.50 (m, 1H, H3), 3.98 (t, 2H, *J* 5.8Hz, -C*H*$_2$-OH), 3.58 (m, 2H, C*H*$_2$-NH), 3.03 (t, *J* 5.8Hz, 2H, -CH_2-SSS-CH_2-), 3.08 (t, *J* 5.8Hz, 2H, -CH_2-SSS-CH_2-), 2.22-2.39 (m, 2H, H4), 1.78-2.06 (m, 5H, H2, H7, H8), 0.94 -1.61 (m, 24H, H1, H9, H11, H12, H14-H17, H19, H20, H22-H25), 0.91 (d, *J* 6.5 Hz, 3H, H21), 0.87 (2 × d, *J* 6.5 Hz, 6H, H26, H27), 0.67 (s, 3H, H18) ppm. ^{13}C NMR (50 MHz, $CDCl_3$) δ: 11.8, 18.7, 19.3, 21.0, 22.5, 22.8, 23.8, 24.2 , 28.0, 28.1, 28.2, 31.8, 31.9, 35.8, 36.2, 36.5, 36.9, 38.5, 38.5 (obscured), 39.2, 39.5, 39.7, 41.7, 42.3, 50.0, 56.1, 56.7, 59.8, 74.7, 122.6, 139.7, 156.0 ppm. HRMS (ESI): m/z calc'd for [M]+, (M = $C_{32}H_{55}NO_3S_3$: 597.3344), found 620.3242 ($[C_{32}H_{55}NO_3S_3]$+Na)$^+$ and 1217.6570 (2$[C_{32}H_{55}NO_3S_3]$+Na)$^+$

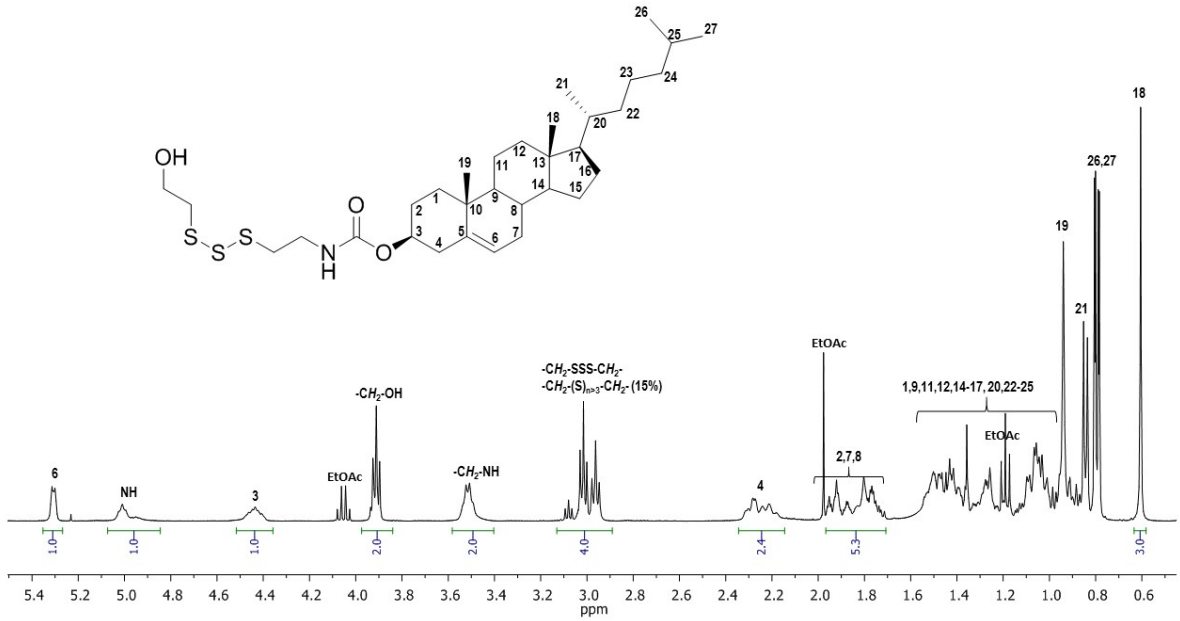
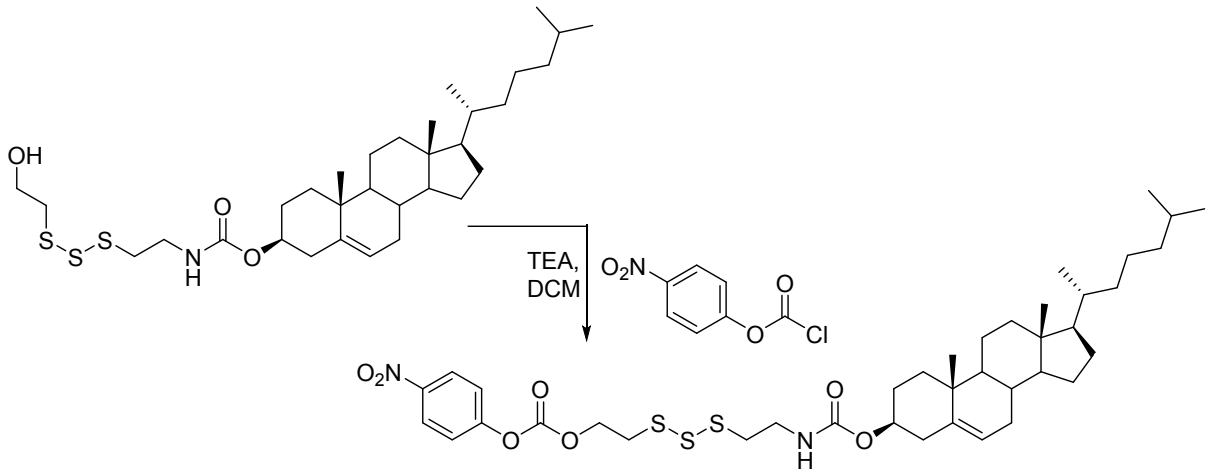


Figure S2: ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of cholesteryl (2-((2-hydroxyethyl) trisulfanyl)ethyl)carbamate with main peak assignments.



Cholesteryl (2-((2-(((4-nitrophenoxy)carbonyl)oxy)ethyl)trisulfanyl)ethyl)carbamate

Cholesteryl (2-((2-(((4-nitrophenoxy)carbonyl)oxy)ethyl)trisulfanyl)ethyl)carbamate. 4-Nitrophenyl chloroformate (57 mg, 2.80×10^{-4} mol) in 2 mL of CH_2Cl_2 was added dropwise to a solution of cholesteryl (2-((2-hydroxyethyl)trisulfanyl)ethyl)carbamate (140 mg, 2.34×10^{-4} mol) and triethylamine (50 μL, 3.51×10^{-4}) in 2 mL of CH_2Cl_2. The mixture was stirred at 25 °C for 4 hours. The crude material was then concentrated and purified using a Reveleris® Flash Chromatography System fitted with a GRACE® silica cartridge, with solvent system CH_2Cl_2/cyclohexane → 100% CH_2Cl_2. The product was isolated as a waxy, sticky white solid. (70 mg, 39%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.29 (d, 2H, Ar-H, *J* 9.3Hz), 7.40 (d, 2H, Ar-H, *J* 9.3Hz), 5.37 (m, 1H, H6), 5.02 (br m, 1H, NH), 4.62 (t, *J* 6.6 Hz, 2H, ArROCOC*H*$_2$-), 4.50 (m, 1H, H3), 3.58 (m, 2H, C*H*$_2$-NH), 3.24 (t, *J* 6.6 Hz, 2H, -CH_2SSS-), 3.04 (m, 2H, -CH_2SSS-), 2.22-2.39 (m, 2H, H4), 1.78-2.06 (m, 5H, H2, H7, H8), 0.94 -1.61 (m, 24H, H1, H9, H11, H12, H14-H17, H19, H20, H22-H25), 0.91 (d, *J* 6.5 Hz, 3H, H21), 0.87 (2 × d, *J* 6.5 Hz, 6H, H26, H27), 0.67 (s, 3H, H18) ppm. ^{13}C NMR (50 MHz, $CDCl_3$) δ: 155.9, 155.4, 152.3, 145.5, 139.7, 125.3, 122.6, 121.7, 74.6, 66.5, 56.7, 56.1, 50.0, 42.3, 39.7, 39.5, 39.2, 38.6, 38.5, 37.0, 36.5, 36.4, 36.2, 35.8, 31.9, 31.9, 28.2, 28.1, 28.0, 24.3, 23.8, 22.8, 22.5, 21.0, 19.3, 18.7, 11.8 ppm.

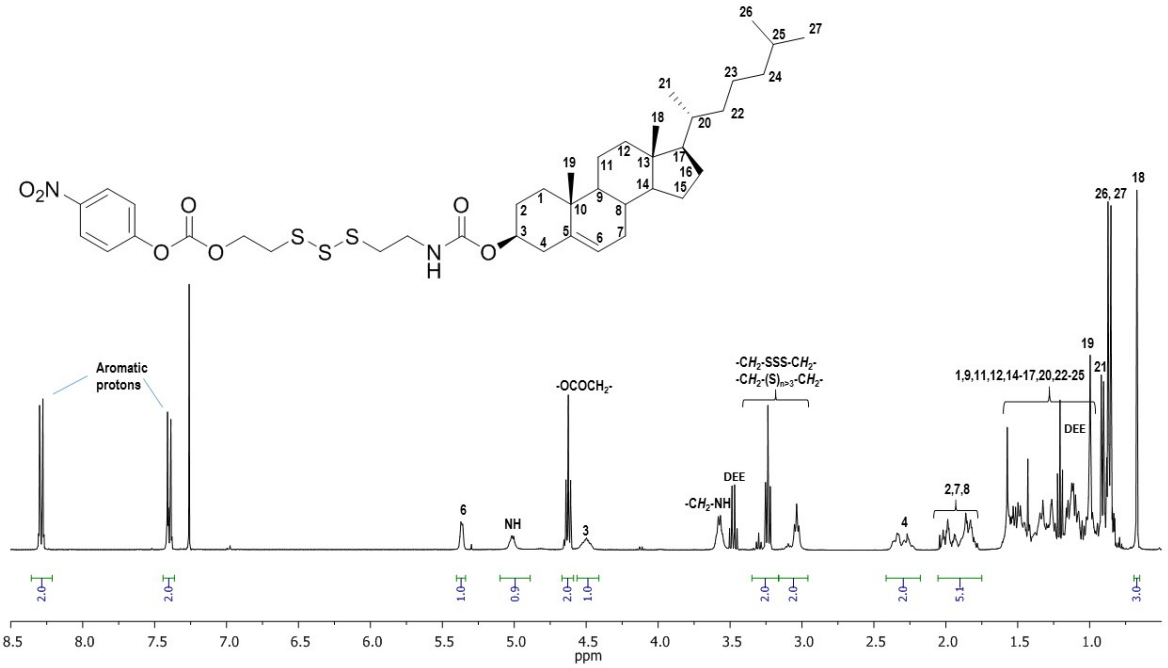
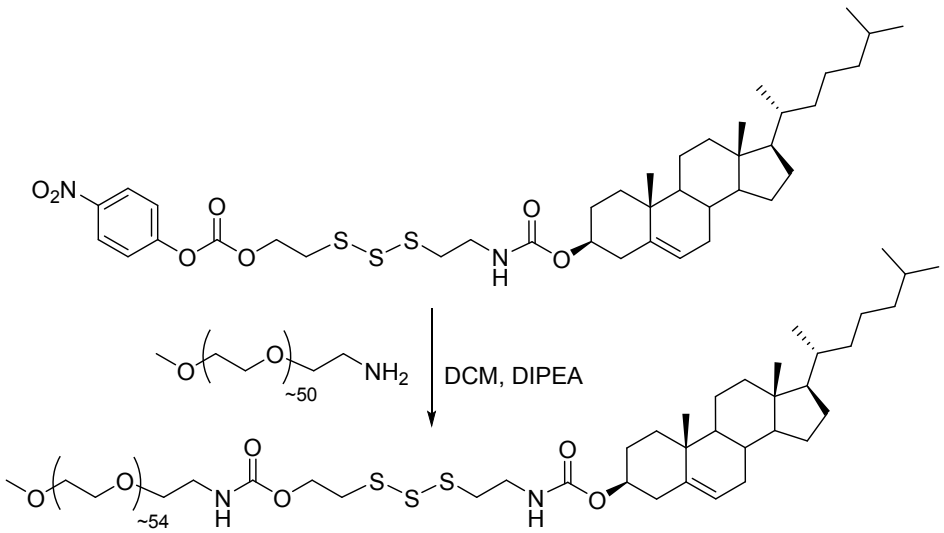


Figure S3: ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of cholesteryl (2-((2-(((4-nitrophenoxy) carbonyl)oxy)ethyl)trisulfanyl)ethyl)carbamate with main peak assignments.



Cholesteryl (((2-methoxyPEGamino)carbonyloxyethyl)trisulfanylethyl)carbamate, mPEG-SSS-CHOL

Cholesteryl (((2-methoxyPEGamino)carbonyloxyethyl)trisulfanylethyl)carbamate, mPEG-SSS-CHOL. mPEG-Amine (MW 2000 g mol^{-1}, 74 mg, 3.67 × 10^{-5} mol), *N,N*-diisopropylethylamine (DIPEA, 5.7 mg, 8.0 µL, 4.40 × 10^{-5} mol) and 4-(dimethylamino)pyridine (DMAP, ~0.5 mg) were dissolved in CH_2Cl_2 and added dropwise to a solution of cholesteryl (2-((2-(((4-nitrophenoxy)carbonyl)oxy)ethyl)trisulfanyl)ethyl) carbamate (56 mg, 7.33 × 10^{-5} mol) in 2 mL of dry CH_2Cl_2. The reaction was left to stir at room

temperature for 16 hours. The solution was then concentrated using a stream of nitrogen. The product polymer was recovered by multiple precipitations into diethyl ether/petroleum ether. To remove all remaining traces of unreacted (2-((2-(((4-nitrophenoxy) carbonyl)oxy)ethyl)trisulfanyl)ethyl) the polymer solution was transferred to dialysis tubing (Cellu Sep, nominal MWCO 1000 g/mol^{-1}) and dialysed against acetone with exchanges of solvent. The final product mPEG(2000)NHCOO-$(CH_2)_2$-SSS-$(CH_2)_2$-CHOL, (Average MW = 3050 g/mol) was analysed by ^{1}H NMR: (400 MHz, $CDCl_3$) δ 5.45 (m, 1H, NHCO), 5.37 (m, 1H, H6), 5.02 (br m, 1H, NH), 4.50 (m, 1H, H3), 4.30 (3.58 (m, 2H, CH_2-NH), 2.90-3.30 (m, 2H, 2 × t, *J* 6.5Hz, 4H, -CH_2-SSS-CH_2-), 2.22-2.39 (m, 2H, H4), 1.78-2.06 (m, 5H, H2, H7, H8), 0.94 -1.61 (m, 24H, H1, H9, H11, H12, H14-H17, H19, H20, H22-H25), 0.91 (d, *J* 6.5 Hz, 3H, H21), 0.87 (2 × d, *J* 6.5 Hz, 6H, H26, H27), 0.67 (s, 3H, H18) ppm.

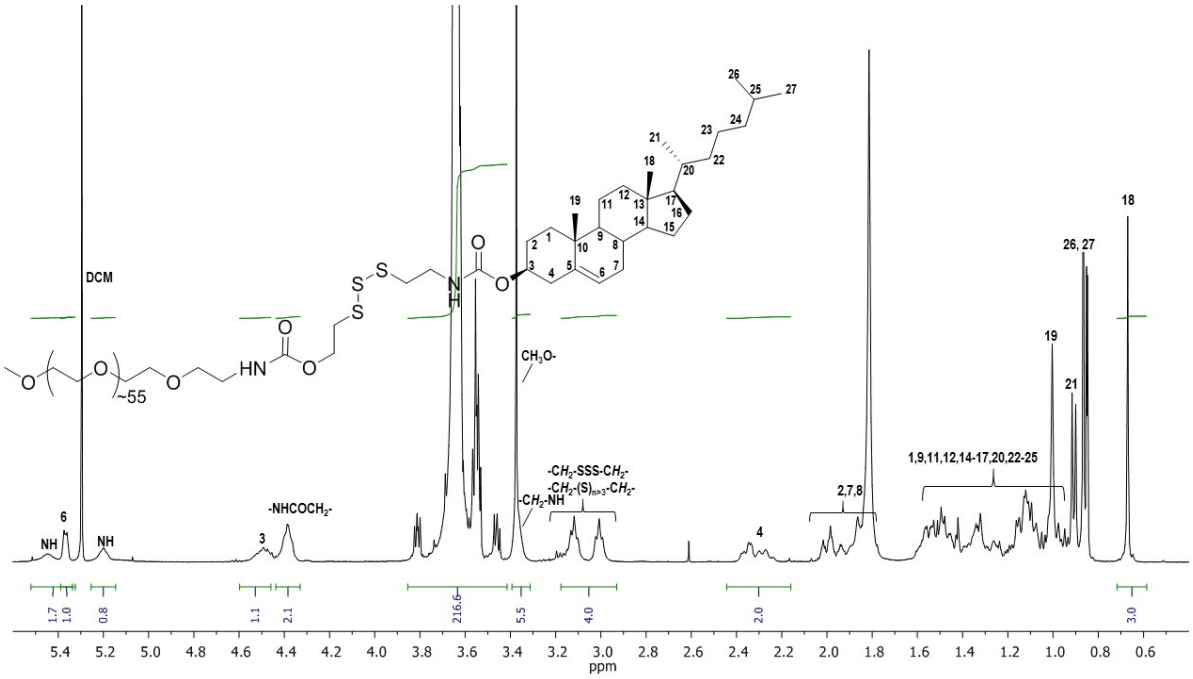
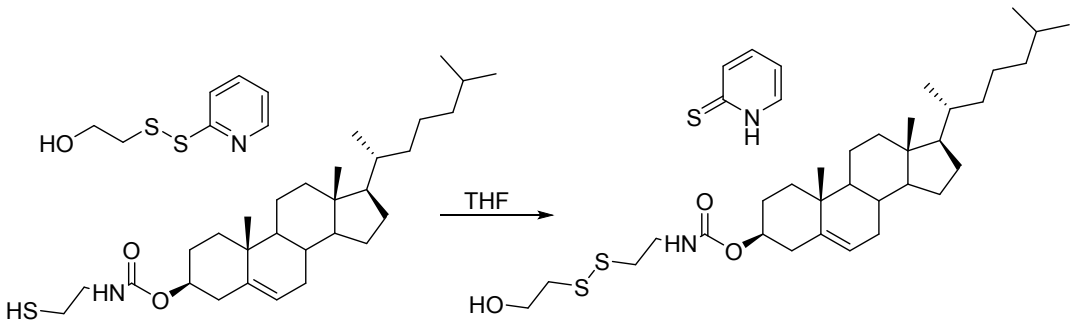


Figure S4: ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of cholesteryl (((2-methoxyPEGamino) carbonyloxyethyl)trisulfanylethyl)carbamate, mPEG-SSS-CHOL with main peak assignments.



Cholesteryl (2-((2-hydroxyethyl)disulfanyl)ethyl)carbamate

Cholesteryl (2-((2-hydroxyethyl)disulfanyl)ethyl)carbamate. 2-(Pyridin-2-yldisulfanyl) ethan-1-ol (*aka,* hydroxyethyl pyridyldisulfide) was synthesized according to a published procedure, *Biomacromolecules,* 2008, 9, 1934–1944. To a solution of hydroxyethyl pyridyl disulfide (150 mg, 8.0×10^{-4} mol) in tetrahydrofuran (THF, 3 mL), maintained at 0°C, was added cholesteryl (2-mercaptoethyl)carbamate (0.392 mg, 8.0×10^{-4} mol) in THF (5 mL). The reaction was left to stir at room temperature for a further 4 hours. The crude material was then concentrated and purified using a Reveleris® Flash Chromatography System fitted with a GRACE® silica cartridge, with the solvent system 2:8 ethyl acetate/cyclohexane → 6:4 ethyl acetate/cyclohexane. The product was isolated as a white solid. (334 mg, 74%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.37 (m, 1H, H6), 4.98 (br m, 1H, NH), 4.50 (m, 1H, H3), 3.89 (t, 2H, *J* 5.8Hz, -C*H*$_2$-OH), 3.51 (m, 2H, C*H*$_2$-NH), 2.90 (t, *J* 6.6 Hz, 2H, -CH_2SS-), 2.80 (m, 2H, -CH_2SS-), 2.22-2.39 (m, 2H, H4), 1.78-2.06 (m, 5H, H2, H7, H8), 0.94 -1.61 (m, 24H, H1, H9, H11, H12, H14-H17, H19, H20, H22-H25), 0.91 (d, *J* 6.5 Hz, 3H, H21), 0.87 (2 × d, *J* 6.5 Hz, 6H, H26, H27), 0.67 (s, 3H, H18) ppm. ^{13}C NMR (50 MHz, $CDCl_3$) δ: 156.1, 139.7, 122.6, 74.7, 60.2, 56.7, 56.1, 50.0, 42.3, 41.7, 39.7, 39.6, 39.5, 38.5, 38.0, 36.9, 36.5, 36.2, 35.8, 31.9, 31.9, 28.2, 28.1, 28.0, 24.3, 23.8, 22.8, 22.5, 21.0, 19.3, 18.7, 11.8 ppm. HRMS(ESI): M = $C_{32}H_{55}NO_3S_2$: 565.3623, found 566.3692, (m/z calc'd for (M+H)+, $[C_{32}H_{55}NO_3S_2]+H)^+$ = 566.3696; found 1131.7315 (m/z calc'd for $(2M+H)^+$, $(2[C_{32}H_{55}NO_3S_2]+H)^+$ = 1131.7319; found 1153.7129 (m/z calc'd for (2M+Na)+, $(2[C_{32}H_{55}NO_3S_3]+Na)^+$ = 1153.7139.

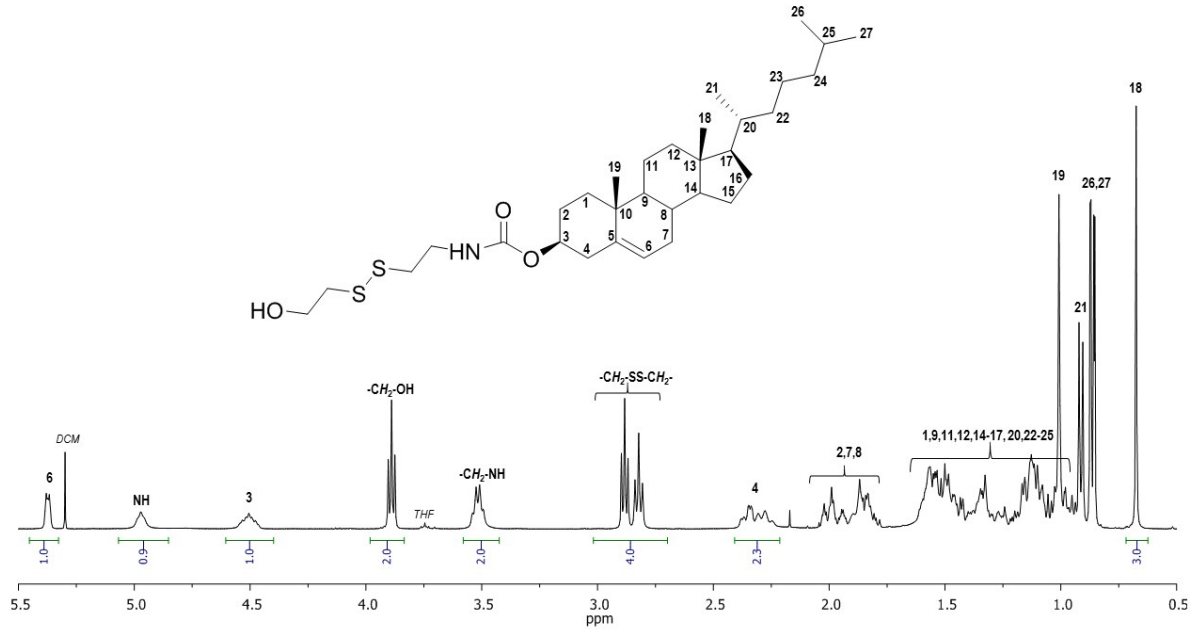
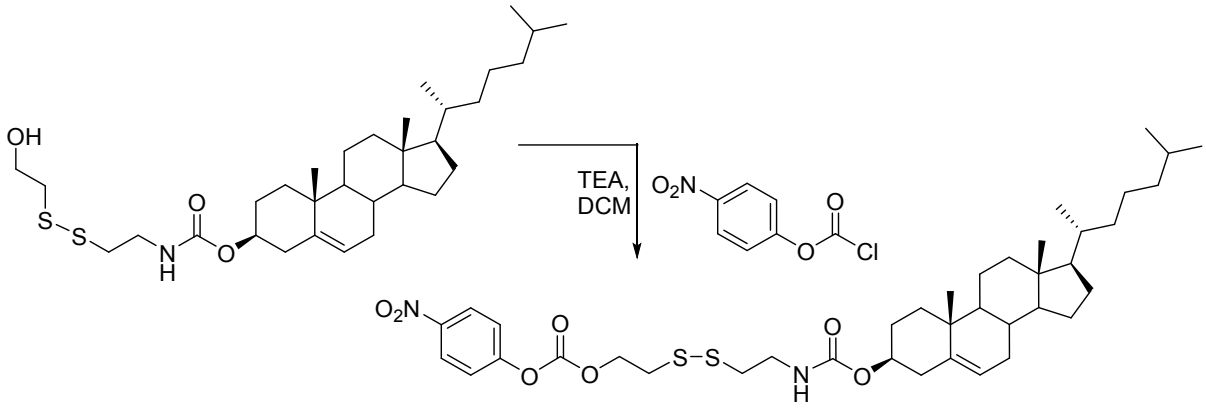


Figure S5: ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of cholesteryl (2-((2-hydroxyethyl) disulfanyl)ethyl)carbamate with main peak assignments.



Cholesteryl (2-((2-(((4-nitrophenoxy)carbonyl)oxy)ethyl)disulfanyl)ethyl)carbamate

Cholesteryl (2-((2-(((4-nitrophenoxy)carbonyl)oxy)ethyl)disulfanyl)ethyl)carbamate. 4-Nitrophenyl chloroformate (47 mg, 2.33×10^{-4} mol) in 2 mL of CH_2Cl_2 was added dropwise to a solution of cholesteryl (2-((2-hydroxyethyl)disulfanyl)ethyl)carbamate (120 mg, 2.12×10^{-4} mol) and triethylamine (60 µL, 43 mg, 4.25×10^{-4}) in 2 mL of CH_2Cl_2. The mixture was stirred at 25°C for 4 hours. The crude material was then concentrated and purified using a Reveleris® Flash Chromatography System fitted with a GRACE® silica cartridge, with solvent system CH_2Cl_2/cyclohexane → 100% CH_2Cl_2. The product was isolated as a waxy, sticky white solid. (106 mg, 68%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.29 (d, 2H, Ar-H, *J* 9.3Hz),7.40

(d, 2H, Ar-H, *J* 9.3Hz), 5.37 (m, 1H, H6), 4.96 (br m, 1H, NH), 4.55 (t, *J* 6.6 Hz, 2H, ArOCOCH_2-), 4.49 (m, 1H, H3), 3.56 (m, 2H, CH_2-NH), 3.03 (t, *J* 6.6 Hz, 2H, -CH_2SS-), 2.86 (m, 2H, -CH_2SS-), 2.22-2.39 (m, 2H, H4), 1.78-2.06 (m, 5H, H2, H7, H8), 0.94 -1.61 (m, 24H, H1, H9, H11, H12, H14-H17, H19, H20, H22-H25), 0.91 (d, *J* 6.5 Hz, 3H, H21), 0.87 (2 × d, *J* 6.5 Hz, 6H, H26, H27), 0.67 (s, 3H, H18) ppm. ^{13}C NMR (50 MHz, $CDCl_3$) δ: 156.0, 155.5, 152.4, 145.5, 139.7, 125.4, 122.6, 121.9, 74.8, 66.9, 56.7, 56.1, 50.0, 42.3, 39.7, 39.6, 39.5, 38.5, 38.5 obs, 36.9, 36.5, 36.2, 35.8, 31.9, 31.8, 28.2, 28.1, 28.0, 24.3, 23.8, 22.8, 22.5, 21.0, 19.3, 18.7, 11.8 ppm.

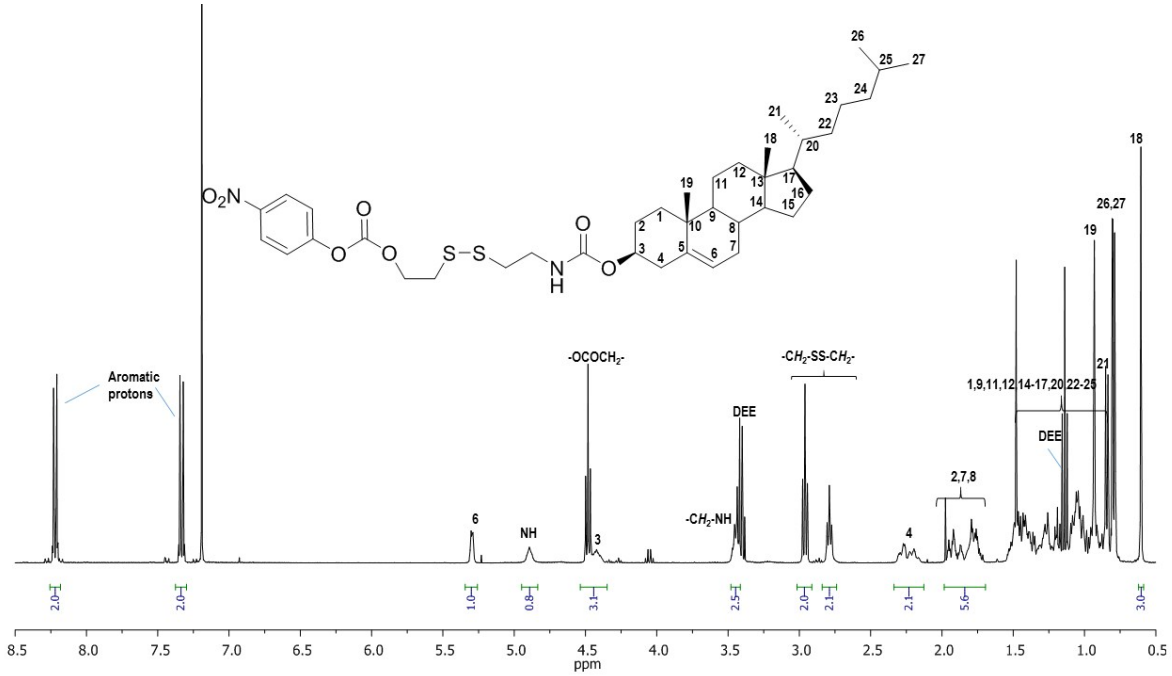
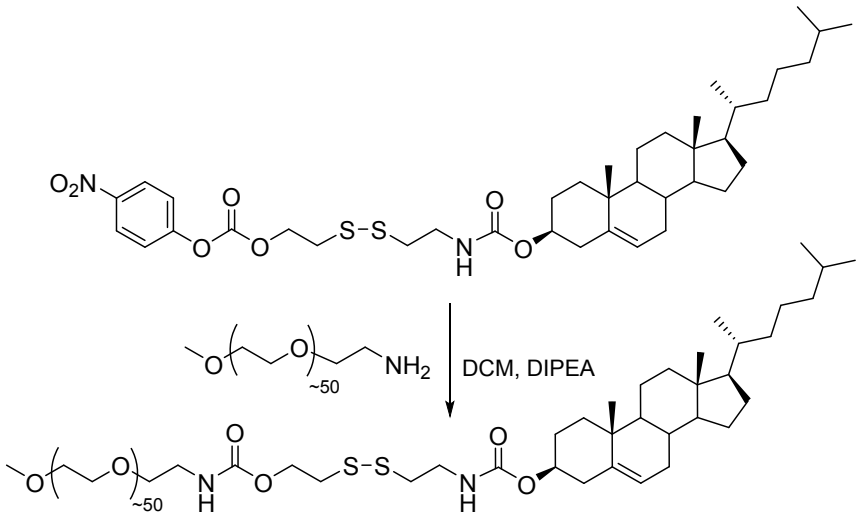


Figure S6: ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of cholesteryl (2-((2-(((4-nitrophenoxy) carbonyl)oxy)ethyl)disulfanyl)ethyl)carbamate with main peak assignments.



Cholesteryl (((2-methoxyPEGamino)carbonyloxyethyl)disulfanylethyl)carbamate, mPEG-SS-CHOL

Cholesteryl (((2-methoxyPEGamino)carbonyloxyethyl)disulfanylethyl)carbamate, mPEG-SS-CHOL. mPEG-Amine (MW 2000 g mol^{-1}, 130 mg, 6.50 × 10^{-5} mol), *N,N*-diisopropylethylamine (DIPEA, 9.1 mg, 12.0 μL, 7.06 × 10^{-5} mol) and 4-(dimethylamino)pyridine (DMAP, ~0.5 mg) were dissolved in CH_2Cl_2 and added dropwise to a solution of cholesteryl (2-((2-(((4-nitrophenoxy)carbonyl)oxy)ethyl)trisulfanyl)ethyl) carbamate (100 mg, 1.38 × 10^{-4} mol) in 3 mL of dry CH_2Cl_2. The reaction was left to stir at room temperature for 16 hours. The solution was then concentrated using a stream of nitrogen. The product polymer was recovered by multiple precipitations into diethyl ether/petroleum ether. To remove all remaining traces of unreacted (2-((2-(((4-nitrophenoxy)carbonyl)oxy)ethyl)disulfanyl)ethyl) the polymer solution was transferred to dialysis tubing (Cellu Sep, nominal MWCO 1000 g/mol^{-1}) and dialysed against acetone with exchanges of solvent. The final product mPEG(2000)NHCOO-$(CH_2)_2$-SS-$(CH_2)_2$ –CHOL (Average MW = 2,900 g/mol), was analysed by ^{1}H NMR: (400 MHz, $CDCl_3$) δ 5.50 (m, 1H, NHCO), 5.37 (m, 1H, H6), 5.15 (br m, 1H, NH), 4.50 (m, 1H, H3), 4.30 (t, *J* 6.5 Hz, 2H, -NHCOCH_2-), 3.40 - 3.80 (m, 200H, 50 × -OCH_2CH_2-), 3.37 (coincident s + m, 5H, OCH_3 + CH_2NH), 2.90 (t, *J* 6.6 Hz, 2H, -CH_2SS-), 2.81 (m, 2H, -CH_2SS-), 2.22-2.39 (m, 2H, H4), 1.78-2.06 (m, 5H, H2, H7, H8), 0.94 -1.61 (m, 24H, H1, H9, H11, H12, H14-H17, H19, H20, H22-H25), 0.91 (d, *J* 6.5 Hz, 3H, H21), 0.87 (2 × d, *J* 6.5 Hz, 6H, H26, H27), 0.67 (s, 3H, H18) ppm.

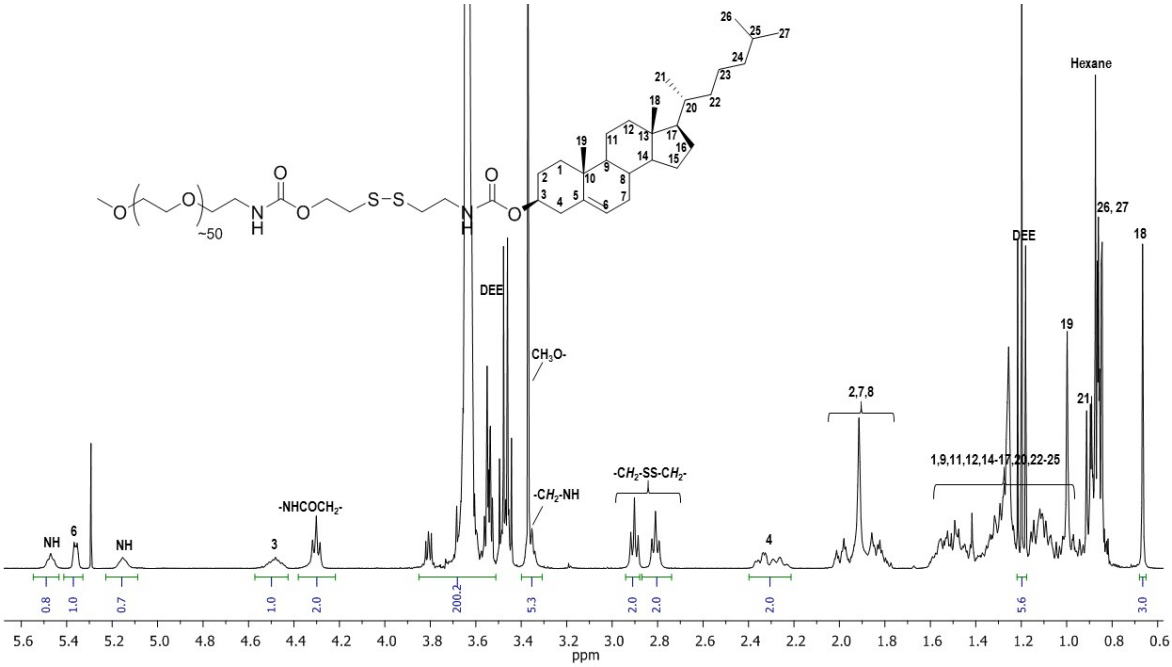
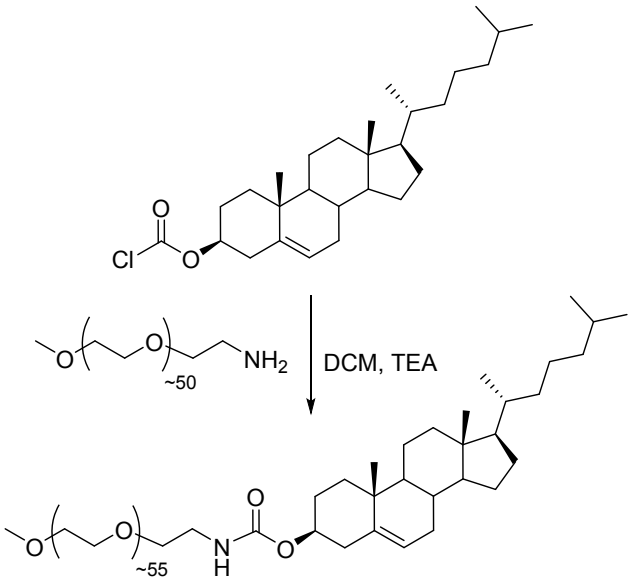


Figure S7: ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of Cholesteryl (((2-methoxyPEGamino)carbonyloxyethyl)disulfanylethyl)carbamate, mPEG-SS-CHOL with main peak assignments.



Cholesteryl poly(ethylene glycol)carbamate, mPEG-CHOL

Cholesteryl poly(ethylene glycol)carbamate, mPEG-CHOL. A solution of cholesteryl chloroformate (49.0 mg, 1.09×10^{-4} mol) in CH_2Cl_2 (1 mL) was added dropwise to a stirred solution of mPEG-Amine (MW 2000 g mol^{-1}, 218 mg, 1.09×10^{-4} mol) and triethylamine (17 mg, 1.63×10^{-4} mol) at 0°C. The reaction was left to stir at room temperature for 16 hours. The

solution was then concentrated using a stream of nitrogen. The product polymer was recovered by multiple precipitations into diethyl ether/petroleum ether. To remove all remaining traces of residual salts the polymer solution was transferred to dialysis tubing (Cellu Sep, nominal MWCO 1000 g/mol^{-1}) and dialysed against acetone with exchanges of solvent. The final product mPEG(2000)NHCOO-CHOL (Average MW = 2,900 g/mol) was analysed by ^{1}H NMR: (400 MHz, $CDCl_3$) δ 5.37 (m, 1H, H6), 5.15 (br m, 1H, NH), 4.50 (m, 1H, H3), 3.40 - 3.80 (m, 225H, 55 × -OCH_2CH_2-), 3.37 (coincident s + m, 5H, OCH_3 + CH_2NH), 2.22-2.39 (m, 2H, H4), 1.78-2.06 (m, 5H, H2, H7, H8), 0.94 -1.61 (m, 24H, H1, H9, H11, H12, H14-H17, H19, H20, H22-H25), 0.91 (d, *J* 6.5 Hz, 3H, H21), 0.87 (2 × d, *J* 6.5 Hz, 6H, H26, H27), 0.67 (s, 3H, H18) ppm.

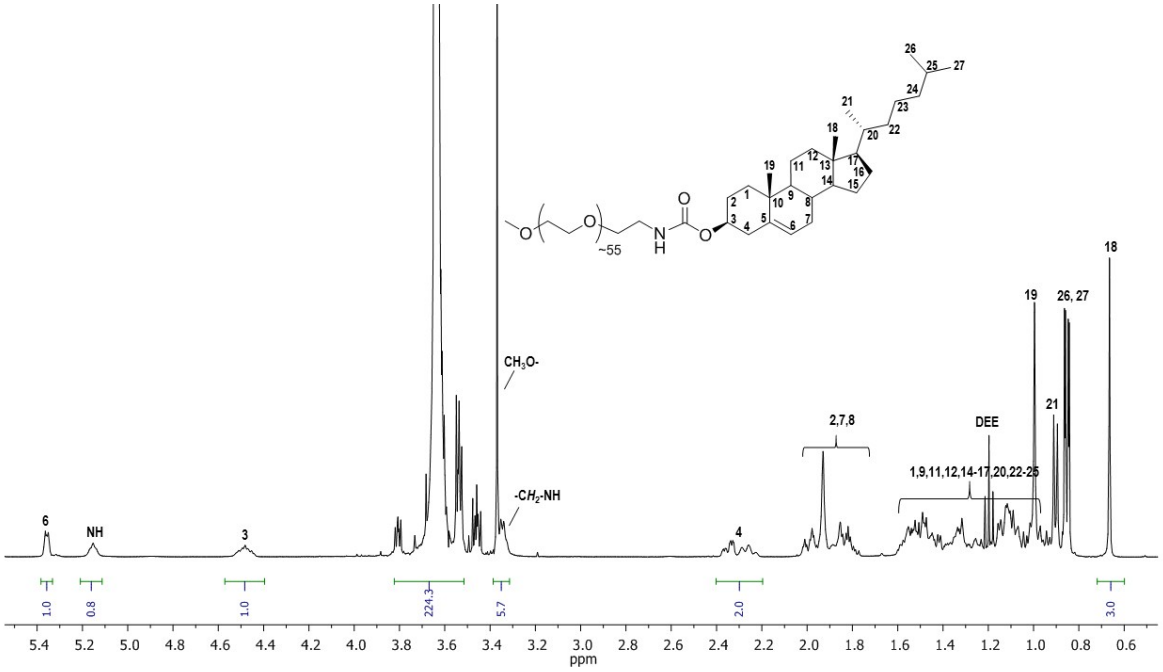


Figure S8: ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of Cholesteryl poly(ethylene glycol)carbamate, mPEG-CHOL with main peak assignments.

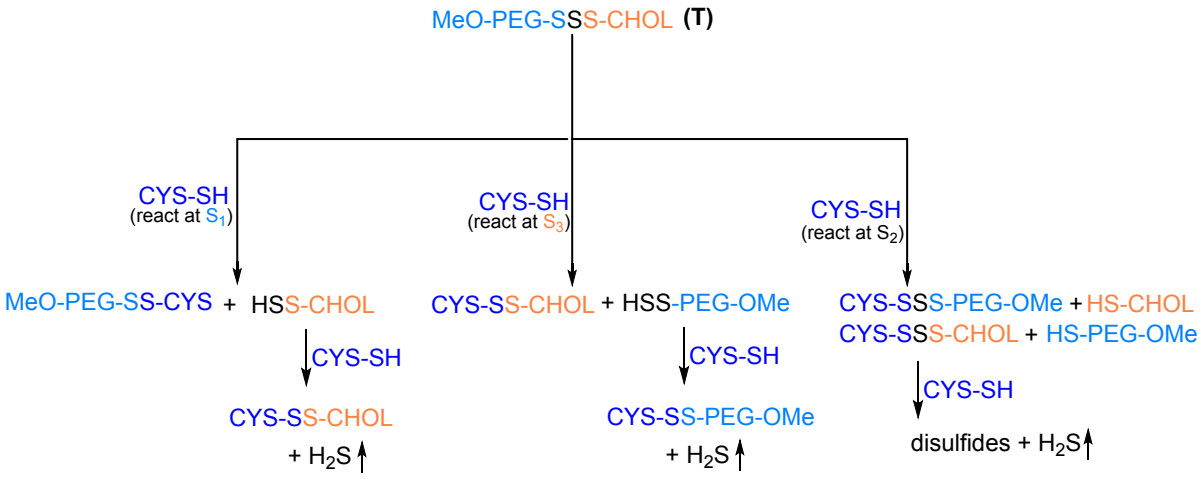


Figure S9. Potential products of thiol mediated cleavage of MeO-PEG-SSS-CHOL

Methods

Preparation of micelles. Micellar solutions of polymers were prepared by dissolving either mPEG(2000)-CHOL, mPEG(2000)-SS-CHOL or PEG(2000)-SSS-CHOL into acetone (1 mg/100 µL) and then injecting the acetone solution into PBS 7.4 under vigorous stirring at room temperature, to a final polymer concentration of 1 mg mL^{-1}. A stream of nitrogen was then blown over the stirring samples in order to remove the acetone (15 minutes). The resulting solutions were then passed through a 0.45 mm filter before analysis in a Zetasizer-Nano instrument (Malvern, UK). The analysis was performed at 25 °C for each sample and the mean diameter of 6 determinations was calculated. For micelles composed of mPEG(2000)-CHOL, the number-average hydrodynamic diameter, D_h, = 9.5 nm (0.19 PDI). For micelles composed of mPEG(2000)-SS-CHOL, D_h = 10.3 nm (0.16 PDI). For micelles composed of mPEG(2000)-SSS-CHOL, D_h = 11.8 nm (0.31 PDI).

Preparation of Nile Red encapsulated micelles. Nile Red was added to the polymers before addition of the polymeric-acetone solution to PBS 7.4. To facilitate this, a stock solution of Nile Red was prepared in acetone and a specific aliquot was then added to the polymer to give the final quantity of 0.1 mg Nile Red/mg of polymer using 100 µL of acetone per 1 mg of polymer. The resulting mixture of polymer and dye was then added into PBS 7.4 under vigorous stirring at room temperature, to a final polymer concentration of 1 mg/mL.

Nile Red fluorescence testing of micelles. A micellar solution was prepared as described above containing 2.2 mg of polymer and 0.22 mg of Nile Red in 2.2 mL of PBS 7.4. The solution was transferred to a quartz cuvette and the fluorescence measured (λ_{ex} = 530 nm; λ_{em}

= 540-800 nm). The solution was then transferred back to the original vial and a stirrer bar added. An 83 mM stock solution of L-cysteine was then prepared in degassed PBS 7.4. Whilst stirring, an aliquot of the L-cysteine solution (30 μL) was then added to the Nile Red-micellar solution which was then left to stir for a total of 60 minutes. The solution was transferred to a quartz cuvette and the fluorescence re-measured (λ_{ex} = 530 nm; λ_{em} = 540-800 nm). The change in fluorescence value was recorded. Data is tabulated in Table S1.

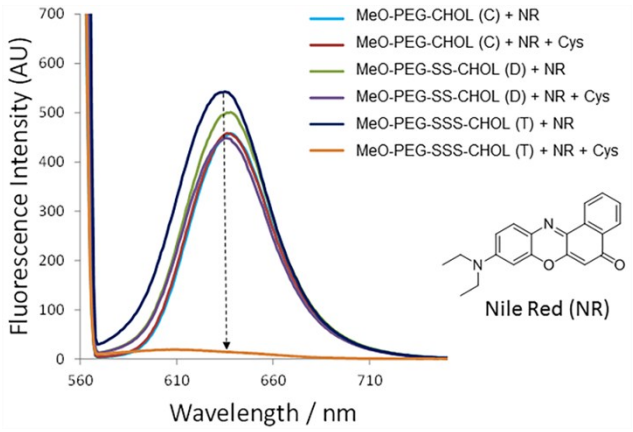


Figure S10. Overlaid fluorescence spectra of micellized conjugates encapsulated with Nile Red before and after L-cysteine addition.

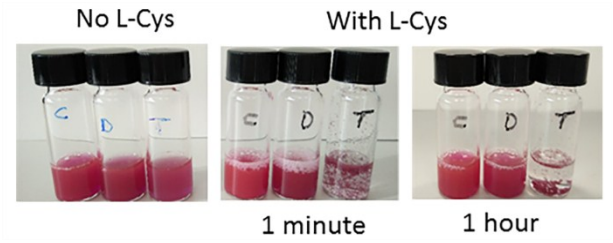


Figure S11. Photographs of Nile Red encapsulated micelles with and without L-cysteine added.

Table S1. Nile Red Encapsulated Micelles with and without addition of L-cysteine.

Sample[a]	FI_0 no L-Cys[b]	FI_{1hr} with L-Cys[c]	% drop in FI
(T)	542	19	95
(D)	501	448	11
(C)	548	548	0

[a] Micellized conjugate incorporating Nile Red. [b] Initial Fluorescence Intensity of micellized conjugate incorporating Nile Red before addition of L-cysteine [c] Fluorescence Intensity of micellized conjugate incorporating Nile Red 1 hour after addition of L-cysteine

H_2S release fluorescence test using SF4 for micelles. A stock solution of SF4 was prepared fresh in DMF/DMSO (6.1 mM). An aliquot (110 μL) was then diluted into water with a final

volume of 5 mL and concentration of 0.134 mM. Separately, 1.5 mg of polymer was dissolved into 300 μL of THF and this was mixed with 1 mL of SF4 solution (0.134 mM) and 300 μL of PBS 7.4. The final volume was therefore 1.6 mL. The solution was transferred to a quartz cuvette and the fluorescence measured (λ_{ex} = 488 nm; λ_{em} = 490-700 nm), representing mPEG(2000)-SSS-CHOL + SF4 measurement. L-cysteine-mediated H_2S release from the polymer, mPEG(2000)-SSS-CHOL, was then was measured by adding 40 μL of L-cysteine solution (5 mL stock solution with a concentration of 33 mM in degassed PBS 7.4). The solution was left to mix for 2 minutes and then the fluorescence re-measured. Negative controls were also measured containing mPEG(2000)-SS-CHOL instead of the trisulfide polymer, as well as a sample containing 1 mL of SF4 solution (0.134 mM), 600 μL of PBS 7.4 and 40 μL of L-cysteine solution (33 mM). As a positive e control, a sample was measured containing Na_2S: A stock solution of Na_2S was initially prepared fresh in degassed PBS 7.4 (10 mM) and then an aliquot (10μL) of this was mixed with 1 mL of SF4 solution (0.134 mM) and 600 μL of PBS 7.4. Solution measured after 2 minutes.

Preparation of Liposomes. Liposomes composed of DOPC, cholesterol (CHOL), PEG2000-PL and PEG(2000)-$(S)_n$-CHOL in a 10:5:1:0.33 molar ratio were prepared. As an example, the following lipid components were individually weighed and added to a flask: DOPC (60 mg, 7.60×10^{-5} mol), PEG(2000)-PL (21 mg, 7.50×10^{-6} mol); CHOL (15 mg, 3.90×10^{-5} mol); PEG2000-$(S)_n$-CHOL (7.5 mg, 2.5×10^{-6} mol). To this was added chloroform (2 mL) and the mixture briefly vortexed to allow solubilisation and mixing of the components. The chloroform was then removed using a rotary evaporator followed by drying under a stream of nitrogen (1 hour). The resulting film was then solubilised in tetrahydrofuran (THF, 700μL) followed by hydration into PBS 7.4 buffer (3 mL). The mixture was then vortexed until milky in appearance, the THF removed using a rotary evaporator, followed by drying under a gentle stream of nitrogen whilst stirring (30 minutes). Liposomes were then subjected to freeze-thaw cycles (5×) using liquid nitrogen (for freezing) and an acetone bath (for thawing). After each thaw the mixture was quickly vortexed to disperse the components. The liposomes were then extruded through a 200 nm membrane for 31 passes, placing the uniform sized vesicles into a clean vial. DLS was conducted on diluted (1 in 3) and filtered samples to confirm the formation of stable liposomes. For liposomes containing 3% mPEG(2000)-CHOL, the number-average hydrodynamic diameter, D_h, = 102 nm (0.12 PDI). For liposomes containing 3% mPEG(2000)-SS-CHOL, D_h = 90 nm (0.13 PDI). For liposomes containing 3% PEG(2000)-SSS-CHOL, D_h = 93 nm (0.14 PDI).

Determination of hydrogen sulfide release profile using an amperometric sensor

The H_2S-generating capability of polymers was examined using amperometric approach using an H_2S selective micro-sensor manufactured by Unisense. The working concept behind the sensor has been published by P. Jeroschewski *et al*. (P. Jeroschewski, C. Steuckart, M. Kuhl, An amperometric microsensor for the determination of H_2S in aquatic environments. Anal. Chem. 1996, 68, 4351-4357.)

Calibration of the sensor was performed after the sensor signal had stabilized over a pre-polarization period (usually 2 hours or more). A 2.0 mM stock solution of Na_2S was prepared anaerobically by dissolving a known quantity of the salt into N_2-flushed, deionized water in a closed container. The acidic calibration buffer was prepared by adding aqueous HCl to PBS at pH 7.4 giving a pH value < 4 (e.g. a pH value of 3.8 was deemed acceptable for use). This solution was also deoxygenated for at least 10 minutes by bubbling with N_2 gas at a rate that was found to not cool the acidic buffer too significantly. The acidic buffer (20 mL) was transferred to a nitrogen-flushed bottle equipped with a stirrer and the bottle capped with a septum. The sensor was then immersed into the solution, through the septum. This was facilitated via a specialized opening on the septum which enabled the bottle to be capped after the sensor tip had been carefully passed through. Once the signal stabilized to a low, stable reading, this was taken as the zero [H_2S] value. Calibration points within the expected range of measurement were collected by injecting known amounts of Na_2S stock solution using a micro-syringe into the stirred calibration buffer solution. The current increased upon addition of the first aliquot and reached a plateau after several seconds. Further calibration values were obtained as subsequent aliquots were added, six in total, ranging from 10 – 160 μL. The recorded data was used to generate a linear calibration curve for [H_2S] vs. current (amps).

L-Cysteine-mediated H_2S release from the conjugates (micelles and liposomes) as measured using the calibrated sensor

All buffer solutions used for testing were deoxygenated by bubbling with N_2 gas through them prior to use. Micellar solutions of polymers were prepared as described earlier in PBS 7.4 to a final polymer concentration of 1 mg mL^{-1} and using 2.0 mg of PEG(2000)-$(S)_n$-CHOL in the composition. Analogously, liposome solutions were prepared as described composed of DOPC, CHOL, PEG2000-PL, PEG(2000)-$(S)_n$-CHOL and cholesterol (CHOL) in a 10:5:1:0.4 molar ratio, in a total volume of 1.8 mL of PBS 7.4 and incorporating 1.8 mg of PEG-SSS-

CHOL in the composition. Once prepared, the sensor tip was immersed into the liposome/micelle solution. As with the calibration, a septum was used for the recording experiments to minimise exposure to air on conducting the measurements. H_2S release was monitored for a period of time (5 -10 minutes). Then an aliquot of L-cysteine solution (83 mM in PBS 7.4, 35 μL for liposomes and 40 μL for micelles) was injected into the stirring solution using a micro-syringe. The times at which the tip was immersed, the experiment was started and the L-cysteine was added, were all noted.

Cell based high-content SF4 time course study.

Micelle solutions of polymers were prepared ~1 hour prior to cell experiments, as described previously. The final concentration of micelle solution was 1 mg/mL. The micelle solutions were then diluted 1:10 in PBS 7.4 prior to addition to the cells (final concentration 0.1 mg/mL). The solutions of SF4 were prepared fresh in DMF/DMSO (5 mM) and diluted to give a 5 μM solution in Hank's Balanced Salt Solution (HBSS).

Cell culture. HEK293 cells were grown in DMEM supplemented with 5% v/v FBS, and all assay plates were coated with poly-D-lysine (5 $\mu g/cm^2$) prior to use.

HEK293 cells were seeded in triplicate in black, optically clear 96-well plates and grown to 90% confluency. Cells were washed with HBSS, then loaded with 5 μM SF4 probe (in HBSS) for 10 min at 37°C. Fluorescence imaging was performed using a high-content PerkinElmer Operetta with an Olympus LUCPlanFLN 20x (NA 0.45) objective. SF4 fluorescence was visualized using the EGFP filter set (excitation 460-490, emission 500-550). Images were taken every 2 min. Baseline fluorescence was determined for 8 min followed by the addition of the polymeric solution, then images were taken every 2 min for 1 hr at 37°C. Data were automatically analyzed by determining the mean SF4 fluorescence per well using Harmony High Content Imaging and Analysis software (v3.5.2). SF4 fluorescence was PBS vehicle-subtracted and expressed relative to the baseline fluorescence for each experimental condition. Data are expressed as the mean ± S.E.M from three independent experiments.