Supporting Information

H_2S-donating trisulfide linkers confer unexpected biological behaviour to poly(ethylene glycol)-cholesteryl conjugates

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Experimental Details

Materials

Cholesteryl (((2-methoxyPEGamino)carbonyloxyethyl)trisulfanylethyl)carbamate (**T**), cholesteryl (((2-methoxyPEGamino)carbonyloxyethyl)disulfanylethyl)carbamate (**D**), cholesteryl-PEG-OMe carbamate (**C**) were synthesized as previously described[1], according to Scheme S1-S3. MeO-PEG-SSS-OMe (**P**) was synthesized using a different method for the generation of the trisulfide bridge as described below, according to Scheme S4. The fluorescent probe for H_2S detection in cells, SF4, was synthesized as described in literature.[2]

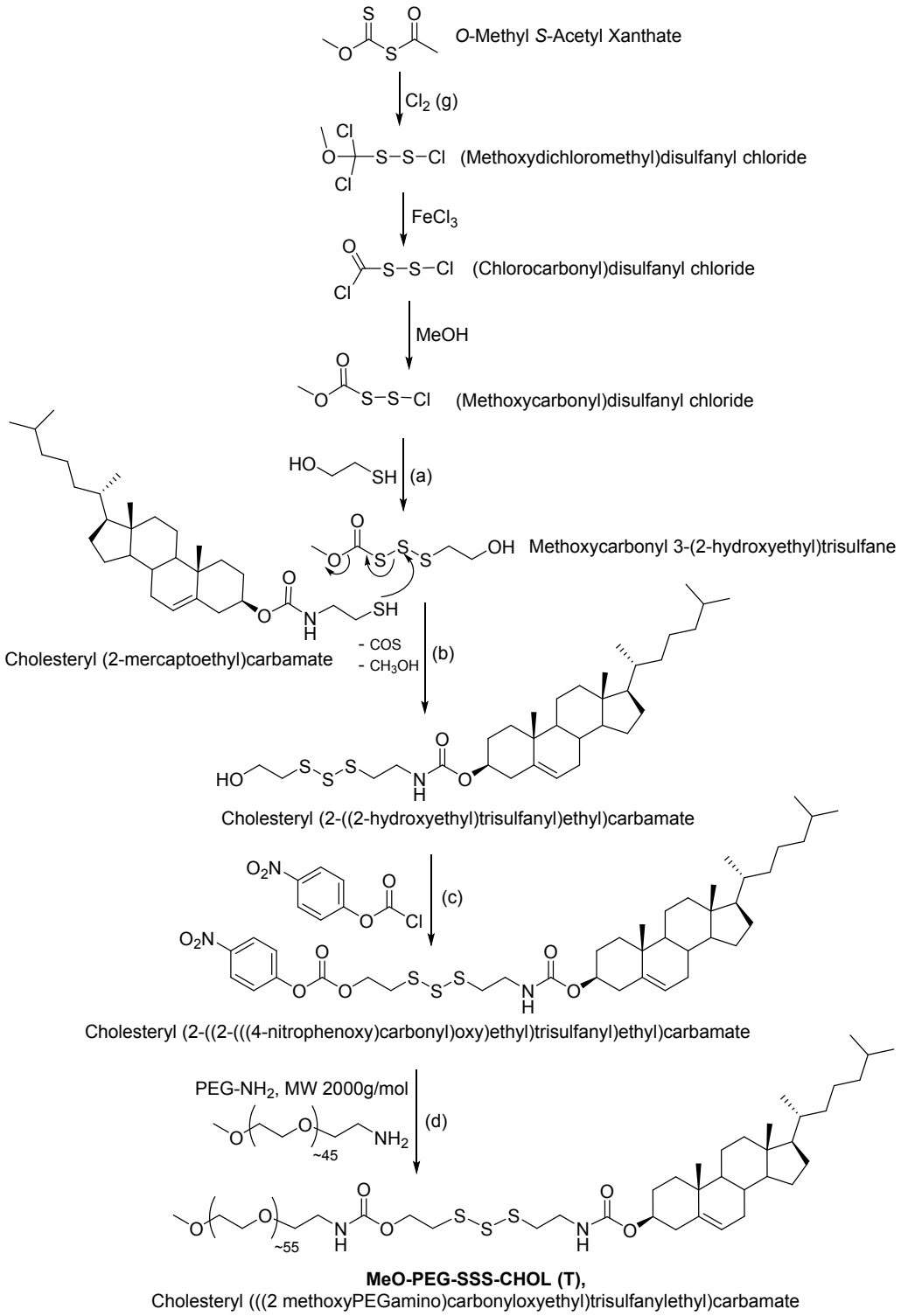
MeO-PEG-NH_2 MW 2000 g/mol (Linear Monofunctional PEG Amine NH_2) was purchased from Creative PEG Works. Sodium sulfide (Na_2S) anhydrous was purchased from Alfa Aesar and hydrochloric acid 32% was purchased from Ajax FineChem. Solvents (except anhydrous solvents) were purchased from Merck Millipore 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 silica gel (230-400 mesh) cartridges was used for purification of monomer and intermediates. TLC was performed on Merck Silica 60F254 plates. PBS 7.4 (phosphate buffered saline, pH 7.4) was reconstituted from Aldrich powder which, when dissolved in 1 L of deionized water, yielding 0.01 M phosphate buffered saline (NaCl 0.138 M, KCl 0.0027 M), pH 7.4 at 25 °C.

Characterization

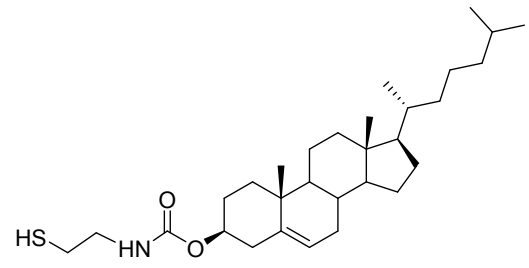
^1H (400 MHz) and ^{13}C NMR (100 MHz) Spectroscopy

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.

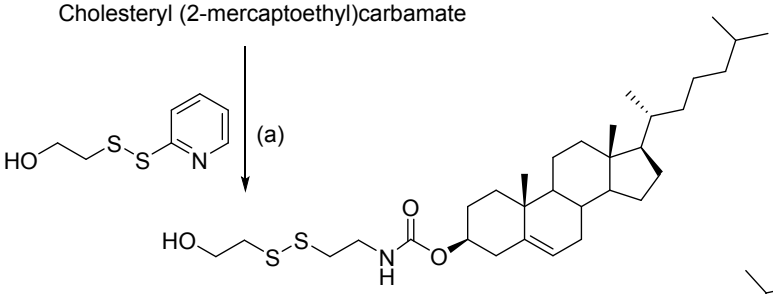
Scheme S1. Synthesis of MeO-PEG-SSS-CHOL (**T**)[1] conjugate, showing thiol mediated fragmentation reaction of methoxycarbonyl 3-(2-hydroxyethyl)trisulfane, to make trisulfide bridge (a) CH_2Cl_2, 0 °C, 1 hour (b) $CHCl_3$, *N*-methylmorpholine, 25 °C, ~ 1 hour (c) TEA, CH_2Cl_2, 25 °C, 4 hours (d) DIPEA, DMAP, CH_2Cl_2, 25 °C, 16 hours.



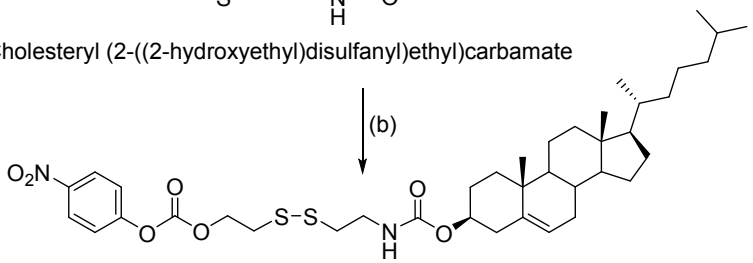
Scheme S2. Synthesis of MeO-PEG-SS-CHOL (**D**) conjugate: (a) THF, 0°C, 4 hours (b) TEA, CH_2Cl_2, 25 °C, 4 hours (c) DIPEA, DMAP, CH_2Cl_2, 25 °C, 16 hours.[1]



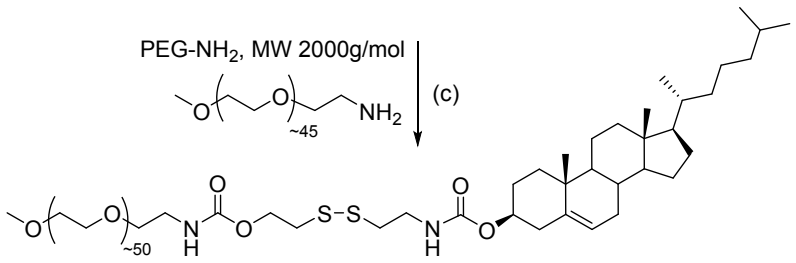
Cholesteryl (2-mercaptoethyl)carbamate



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

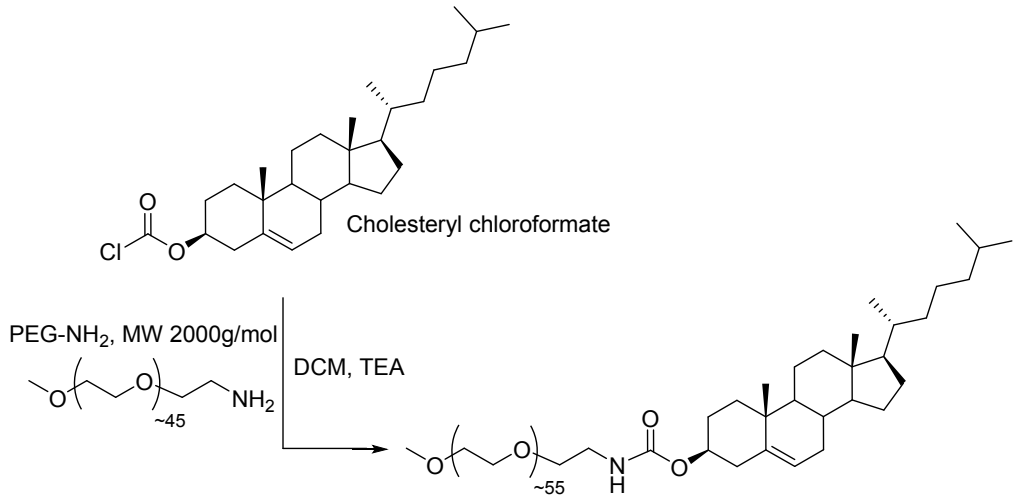


Cholesteryl (2-((2-(((4-nitrophenoxy)carbonyl)oxy)ethyl)disulfanyl)ethyl)carbamate



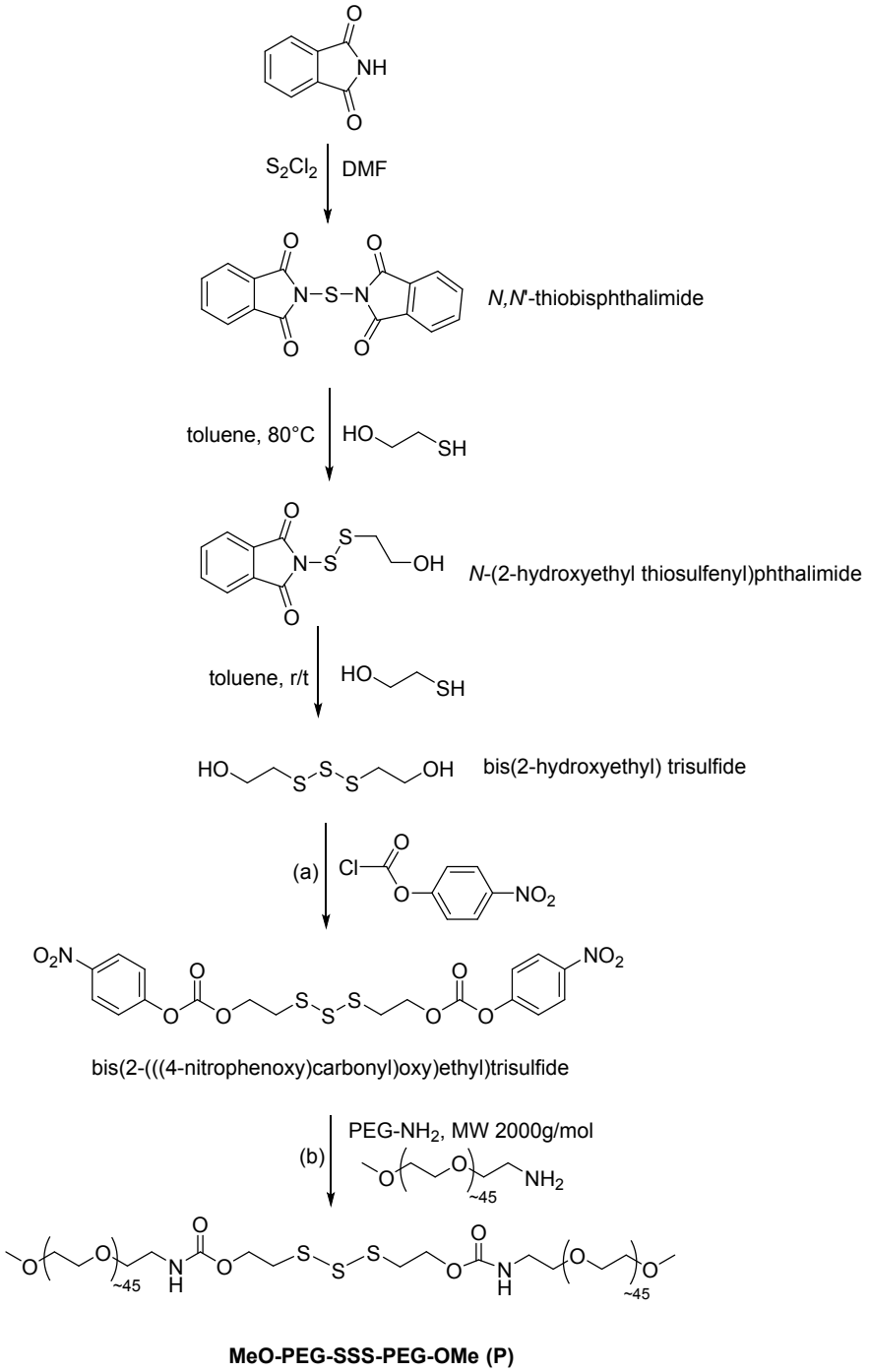
MeO-PEG-SS-CHOL (D),
Cholesteryl (((2 methoxyPEGamino)carbonyloxyethyl)diisulfanylethyl)carbamate

Scheme S3. Synthesis of MeO-PEG-CHOL (**C**) conjugate: TEA, CH_2Cl_2, 0 °C → 25 °C, 16 hours.[1]



MeO-PEG-CHOL (C)
Cholesteryl poly(ethylene glycol)carbamate

Scheme S4. Synthesis of MeO-PEG-SSS-PEG-OMe conjugate (**P**): (a) S_2Cl_2, 20 hours, 28 °C (b) toluene, 80 °C, 1 hour (c) toluene, room temp, 16 hours (d) TEA, CH_2Cl_2, 25 °C, 16 hours (c) DIPEA, DMAP, CH_2Cl_2, 25 °C, 16 hours.



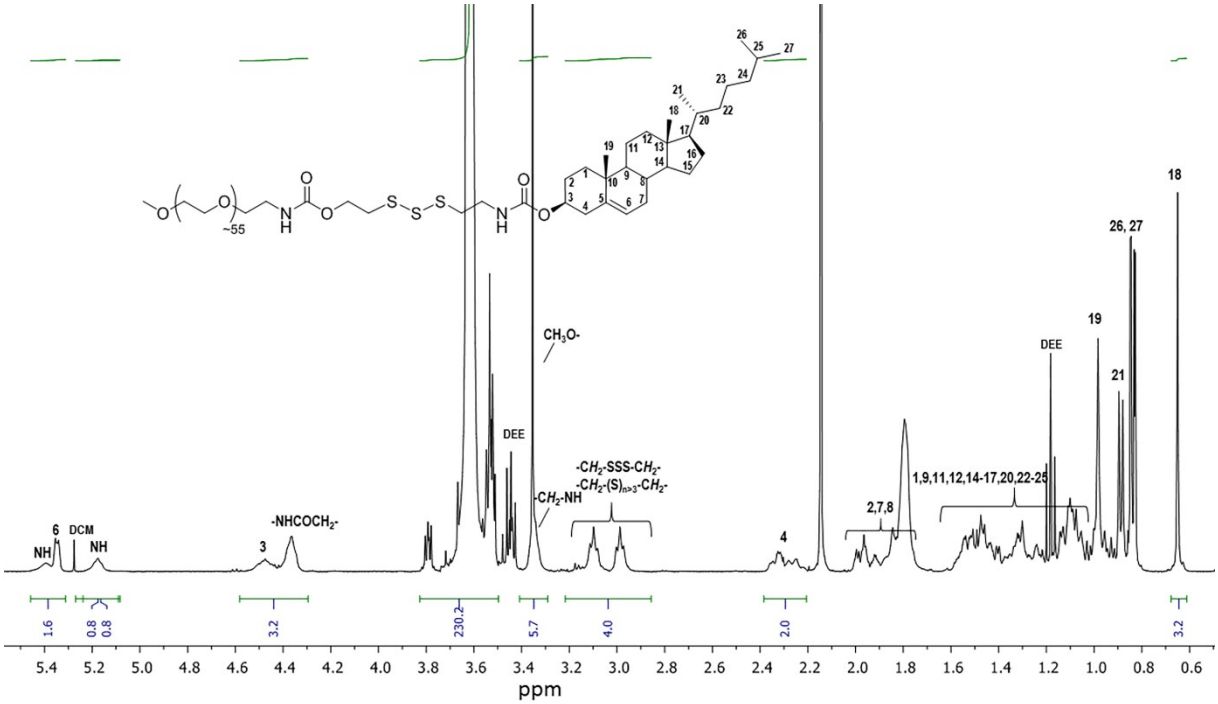


Figure S1: ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of cholesteryl (((2-methoxyPEGamino) carbonyloxyethyl)trisulfanylethyl)carbamate, MeO-PEG-SSS-CHOL (**T**), with main peak assignments. DEE = diethyl ether, DCM = dichloromethane.

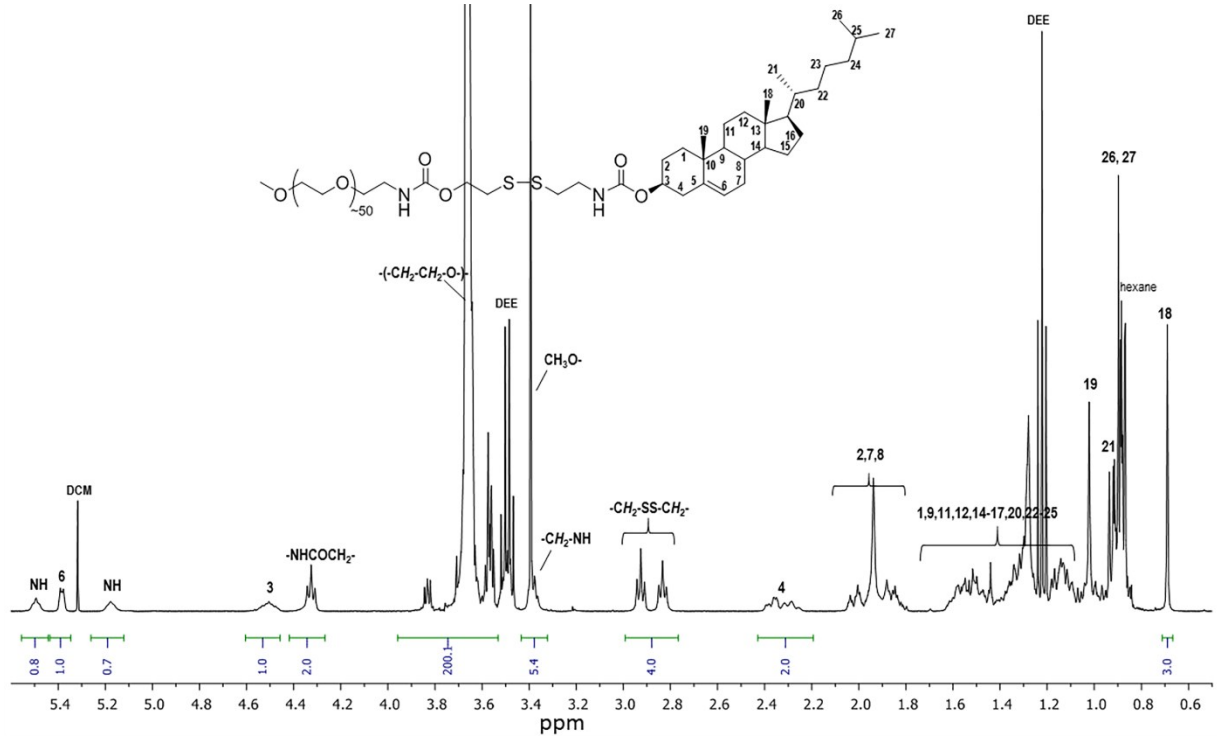


Figure S2: ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of cholesteryl (((2-methoxyPEGamino)carbonyloxyethyl)disulfanylethyl)carbamate, MeO-PEG-SS-CHOL (**D**), with main peak assignments. DEE = diethyl ether, DCM = dichloromethane

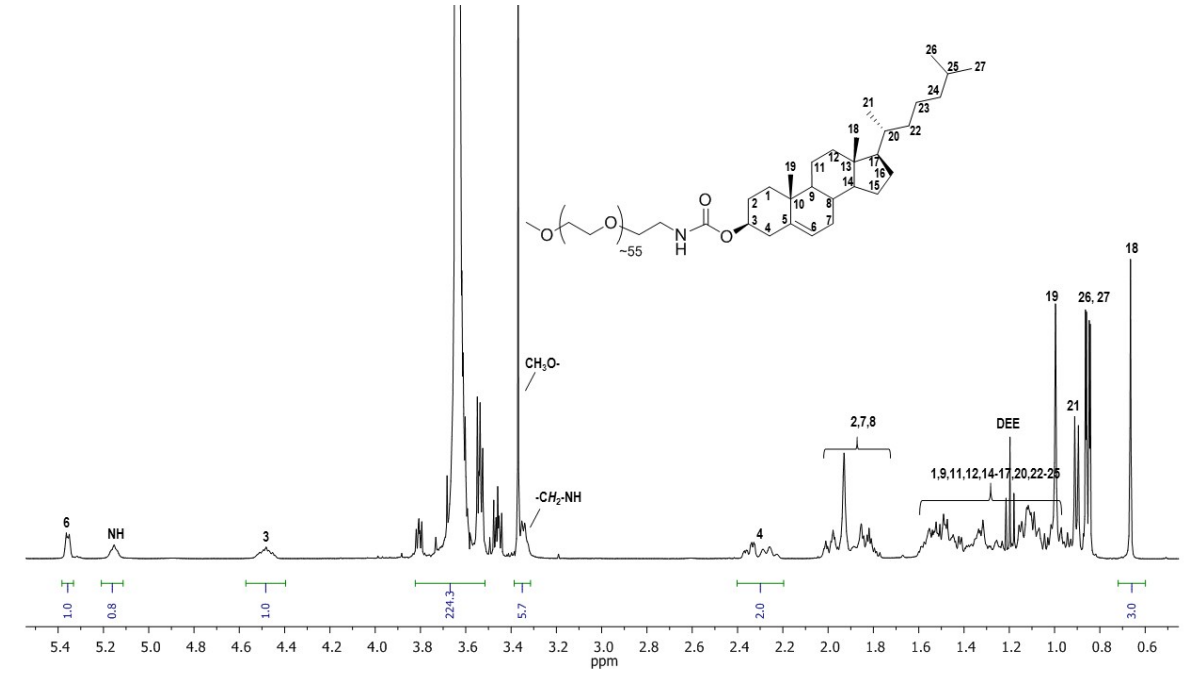


Figure S3: ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of cholesteryl poly(ethylene glycol)carbamate, MeO-PEG-CHOL (**C**), with main peak assignments.

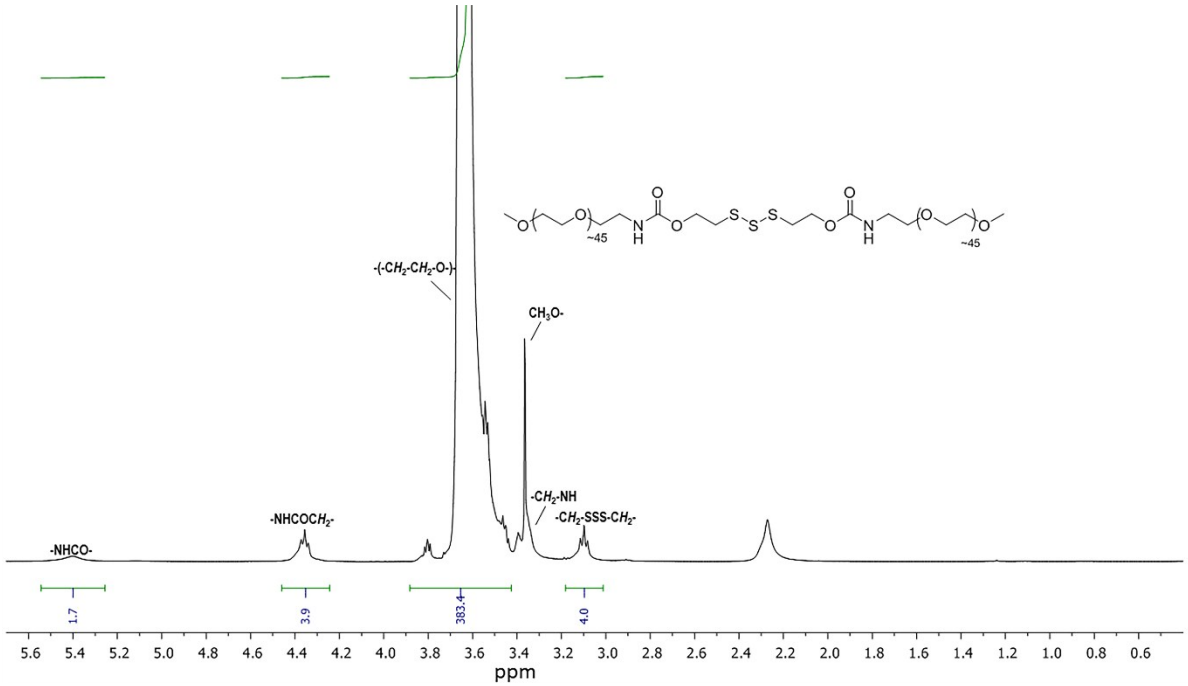


Figure S4. ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of MeO-PEG-SSS-PEG-OMe (**P**), with main peak assignments.

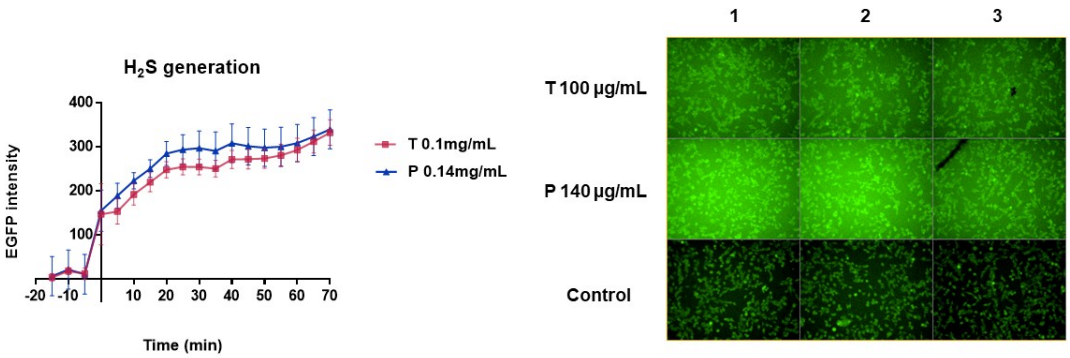


Figure S5. Representative fluorescent images using SF4 for H_2S detection in cells. Left image shows the average fluorescence result over time. Right images are the individual images of cells taken at 70-minute time point.

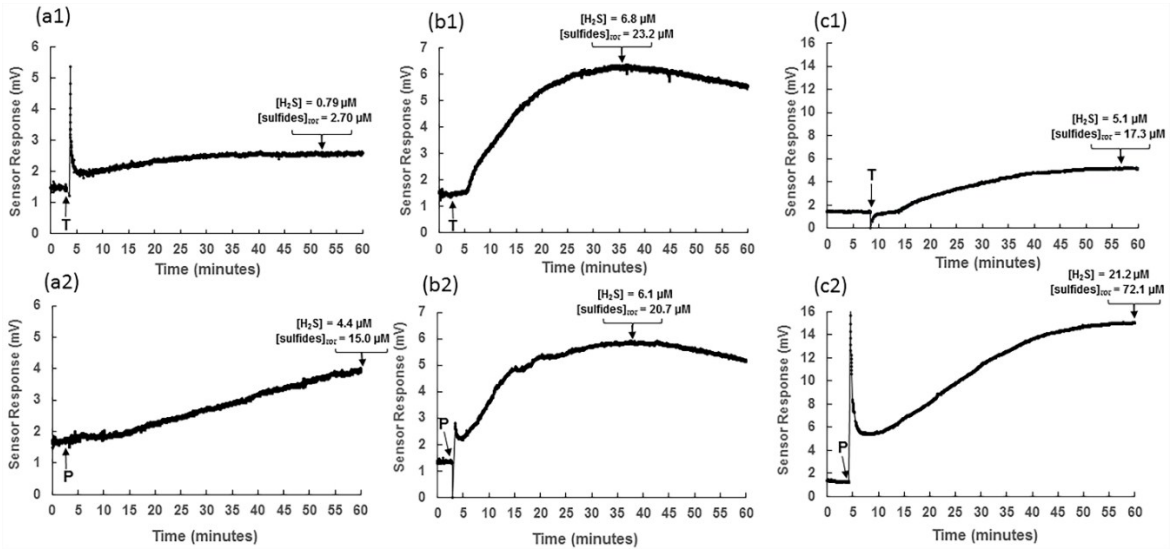


Figure S6. H_2S detection from HEK 293 cells (Human embryonic kidney cells, ATCC) using an amperometric microsensor (Unisense). (a1) H_2S release from ~1.0×10^7 cells (500 µL volume) exposed to **T**; (a2) H_2S release from ~1.0×10^7 cells (500 µL) exposed to **P**; (b1) H_2S release from ~3.1×10^7 cells (650 µL) exposed to **T**; (b2) H_2S release from ~3.1×10^7 cells (650 µL) exposed to **P**; (c1) H_2S release from ~7.5×10^7 cells (600 µL) exposed to **T**; (c2) H_2S release from ~7.5×10^7 cells (600 µL) exposed to **P**. Cells were grown in Dulbecco's Modified Eagle Media, DMEM, with 10% Fetal Bovine Serum, FBS. Counted cells were dispersed in fresh media and then two individual groups of equal volume were dispensed into separate vials. The sensor was immersed in each cell population, a signal was measured (~ 2 min) and then an aliquot of **T** or **P** was added, as indicated by first arrow: 50 µL × 5mg/ml solution in water, 8.2×10^{-8} moles of **T** and 50 µL × 7.2 mg/ml solution in water, 8.2×10^{-8} moles of **P**. Each graph shows peaking [H_2S] and peaking $[sulphides]_{tot}$ as derived from calibration plot.

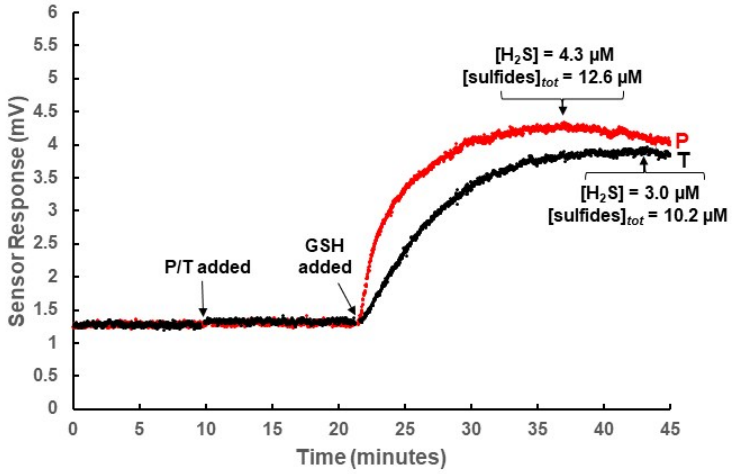


Figure S7. H_2S release curve for **P** (red) and **T** (black) in cell culture medium (Dulbecco's Modified Eagle Media, DMEM) with 10% Fetal Bovine Serum, FBS), as measured using amperometric microsensor (Unisense). Note: The first 10 minutes is measured in cell culture media only, then **P** (50 µL of 7.2 mg/ml solution in water added, 8.2 × 10-8 moles) or **T** (50 µL aliquot of 5mg/ml solution in water is added, 8.2 × 10^{-8} moles) is added. Glutathione (GSH) is then added where indicated (20 µL of a 250 mM solution in water, 5.0 × 10-7 moles). The values for peaking [H_2S] and peaking $[sulphides]_{tot}$ shown are derived from calibration plot.

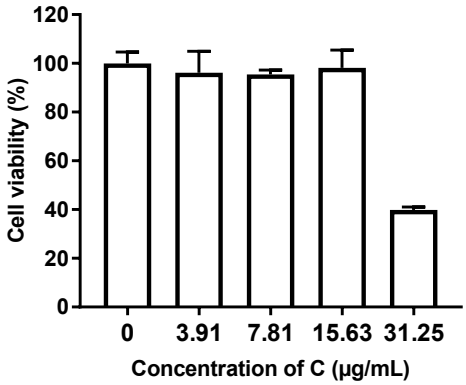


Figure S8. Cytotoxicity evaluation of OMe-PEG-CHOL (**C**) (concentrations ⩽ 31.25 µg/mL) against HEK-293 cells determined by Alamar Blue assay after 24 h of exposure.

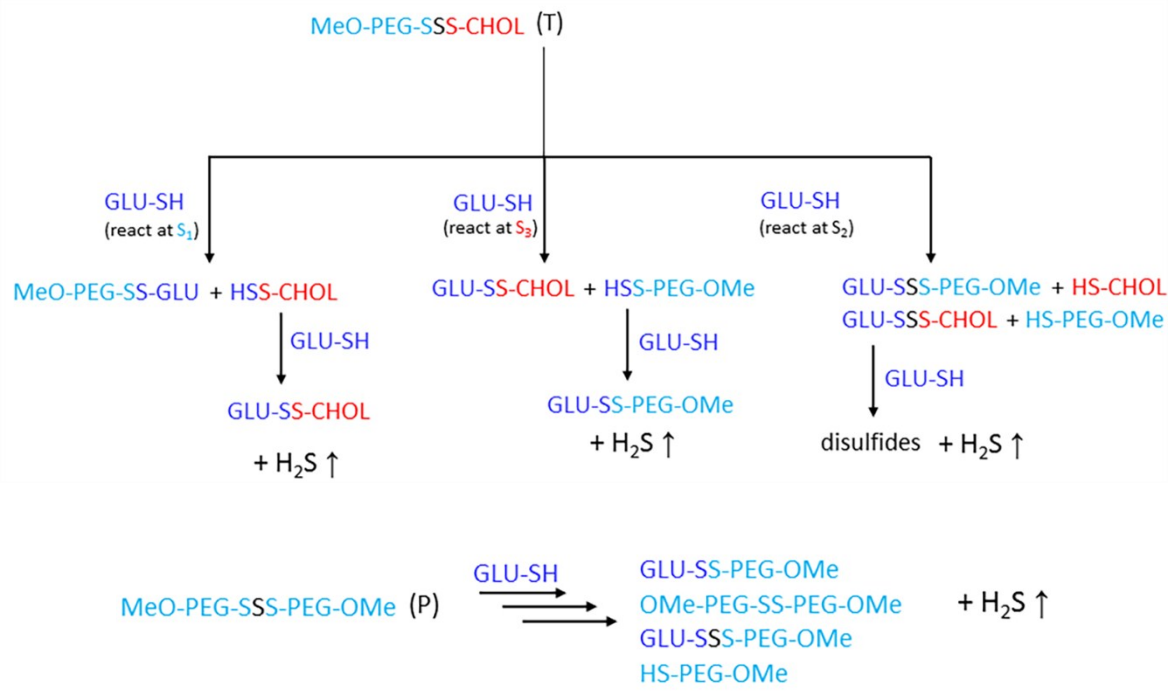


Figure S9. Potential products of thiol-mediated cleavage of MeO-PEG-SSS-CHOL (**T**) and MeO-PEG-SSS-CHOL (**P**).

Synthetic Protocols

N,N'-Thiobisphthalimide*[3]*: Phthalimide (7.36 g, 0.05 mol) was dissolved in anhydrous dimethylformamide (DMF, 40 mL dried over Molecular Sieves Type 4Å) and heated to 28 °C under a nitrogen atmosphere. To the resulting solution, sulfur monochloride (6.75 g, 0.05 mol) was added in several portions and then the yellow mixture was left to stir under nitrogen at 28 °C for 20 hours. In the first 30 minutes a precipitate should appear. The suspension was then filtered and the cream solid dried under high vacuum (6.70 g, 82 % yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.79 (m, 2H, ArH), 7.95 (m, 2H, ArH) ppm.

N-(2-hydroxyethyl thiosulfenyl)phthalimide:[4,5] A mixture of *N,N'*-thiobisphthalimide (1.85 g, 5.7 mmol) and mercaptoethanol (0.40 g, 0.36 mL, 5.12 mmol, 0.9 equivs.) were heated in toluene (30 mL) at 80 °C for 1 hour. The reaction progress was monitored using thin layer chromatography using ethyl acetate/hexane/dichloromethane (EA/HX/DCM) mixture. The product appears as a spot with Rf 0.43. The reaction was cooled to room temperature and the filtered. The white solid was washed 3× with DCM and the combined filtrate and washings were evaporated to dryness. The resulting crude solid (from filtrate and washings) was then purified using a Reveleris Flash Chromatography System fitted with a silica cartridge, using solvent system 10 % → 60 % EA in Hexane gradient mixture. The product was afforded as a

white solid (0.92 g, 70 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.23 (t, 2H, *J* 5.9 Hz, -C*H*$_2$CH$_2$-OH), 4.10 (t, 2H, *J* 5.9 Hz, -CH$_2$C*H*$_2$-OH), 7.80 (m, 2H, ArH), 7.94 (m, 2H, ArH).

Bis(2-hydroxyethyl)trisulfide.[6] To a suspension of *N*-(2-hydroxyethyl thiosulfenyl)phthalimide (0.45 g, 1.8 mmol) in toluene (10 mL), was added dropwise mercaptoethanol (0.14 g, 0.13 mL, 1.8 mmol). The reaction was left to stir at room temperature for 16 hours. The reaction progress was monitored using thin layer chromatography using ethyl acetate/hexane/dichloromethane (EA/HX/DCM) mixture. The product appears as a spot with Rf 0.20, as the starting material disappears (Rf 0.43). The reaction was cooled to room temperature and the filtered. The white solid was washed 3× with DCM and the combined filtrate and washings were evaporated to dryness. The resulting crude solid (from filtrate and washings) was then purified using a Reveleris® Flash Chromatography System fitted with a silica cartridge, using solvent system EA/HX/DCM → EA/DCM. The product was afforded as a clear oil. (280 mg, 80 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.09 (t, 2H, *J* 5.9 Hz, -C*H*$_2$CH$_2$-OH), 3.99 (t, 2H, *J* 5.9 Hz, -CH$_2$C*H*$_2$-OH) ppm. ^{13}C NMR (50 MHz, $CDCl_3$) δ: 41.9, 59.7 ppm.

Bis(2-(((4-nitrophenoxy)carbonyl)oxy)ethyl)trisulfide. A solution of bis(2-hydroxyethyl)trisulfide (210 mg, 1.13 × 10^{-3} mol) and triethylamine (470 µL, 3.39 × 10^{-3}) in 1.5 mL of CH_2Cl_2 was added slowly and dropwise to a solution of 4-nitrophenyl chloroformate (547 mg, 2.71 × 10^{-3} mol) in CH_2Cl_2 (1.5 mL). The mixture was stirred at 25 °C overnight. The crude material was then concentrated and purified by Flash Chromatography using 50/50 EA in hexane solvent system. The product was afforded as a clear liquid (270 mg, 46 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.25 (t, 2H, *J* 6.5 Hz, -SSS-C*H*$_2$CH$_2$-OCO-), 4.63 (t, 2H, *J* 6.5 Hz, -SSS-CH$_2$C*H*$_2$-OCO-), 7.39 (m, 2H, ArH), 8.28 (m, 2H, ArH) ppm. ^{13}C NMR (50 MHz, $CDCl_3$) δ: 36.5, 66.5, 121.7, 125.3, 145.6, 152.3, 155.5 ppm.

MeO-PEG-SSS-OMe, P. mPEG-Amine (MW 2000 g mol^{-1}, 697 mg, 3.48 × 10^{-4} mol), *N,N*-diisopropylethylamine (DIPEA, 59 mg, 80 µL, 4.53 × 10^{-4} mol) and 4-(dimethylamino)pyridine (DMAP, ~1.0 mg) were dissolved in CH_2Cl_2 (4 mL) and added dropwise to a solution of bis(2-(((4-nitrophenoxy)carbonyl)oxy)ethyl)trisulfide (90 mg, 1.74 × 10^{-4} mol) in 4 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 and then purified of unreacted reagents by precipitation into diethyl ether/petroleum ether (bp 40-60 °C) 70/30 v/v. The polymer was then dissolved into water, transferred to dialysis tubing (Cellu Sep, nominal MWCO 1000 g/mol^{-1}) and dialysed against water with 6 exchanges of solvent over three days

after which time the product was freeze dried to remove water. The final product, MeO-PEG-SSS-PEG-OMe (Average MW = 4,400 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, C*H*$_2$-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.

Methods

CMC determination[7]

990 µL of 13 different polymer solutions of decreasing concentration (1.0 to 0.000001 mg/mL) were added individually to 13 vials. These are prepared by diluting a 5.0 mg/mL polymer stock solution with MilliQ water. A pyrene stock solution (c = 5 × 10^{-5} M) was prepared in THF and 10 µL aliquots were added to the 13 vials containing the polymer solutions. The final pyrene concentration in each vial was 5 × 10^{-7} M. Vials were shaken gently for two hours at room temperature, shielded from the light with aluminium foil. The fluorescence spectra were recorded using an excitation wavelength of 336 nm and emission spectra were recorded ranging from 350 to 450 nm. From the pyrene emission spectra, the intensities (peak height) of the 393 nm peak (I_1) and 384 nm peak (I_3) were used to calculate the I_3/I_1 ratio.

A CMC value was determined from plots of Log C vs Pyrene I_3/I_1 ratio by fitting the curves to a Boltzmann sigmoid-type equation, given by:

$$y = \frac{A_1 - A_2}{1 + e^{(x - x_0)/\Delta x}} + A_2$$

Where y corresponds to the pyrene I_3/I_1 ratio value, the independent variable (x) is the concentration of polymer, A_1 and A_2 are the upper and lower limits of the sigmoid respectively, x_0 is the centre of the sigmoid and Δx is directly related to the independent variable (x) range where there is an abrupt change of the dependent (y) variable. A CMC value (CMC_1) was taken to be, x_0 the centre of the sigmoid, as determined using KaleidaGraph curve fitting software for the series of data points.

Table S1: Boltzmann sigmoid-type equation fitting parameters of pyrene 1:3 ratio data for **T**, **D** and **C** conjugates: *n* is the number of points used in the fit, r^2 and χ^2 have their usual meanings, as derived using KaleidaGraph curve fitting software.

Conjugate	*n*	x_0	Δx	r^2	χ^2	$(xCMC)_1$ (μg/mL)	$(xCMC)_2$ (μg/mL)
C	13	4.10	0.242	0.9996	6.71×10^{-4}	12.6	38.5
D	13	4.83	0.289	0.9993	1.12×10^{-3}	67.0	253
T	14	4.95	0.324	0.999	2.67×10^{-3}	90.0	399

Determination of hydrogen sulfide release profile using an amperometric sensor

The H_2S-generating capability of polymers **T** and **P** was examined using amperometry with an H_2S selective micro-sensor manufactured by Unisense. The working concept behind the sensor has been published by Jeroschewski *et al*.[8]

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. recorded sensor response (mV).

Considerations for H_2S release measurement using amperometric sensor

H_2S is a weak acid and when it is dissolved in water ionization equilibriums are established with its two anions, hydrosulfide HS^- and sulfide S^{-2}, according to equations (1) and (2):

$$H_2S + H_2O \underset{}{\overset{K_1}{\rightleftharpoons}} HS^- + H_3O^+ \quad (1)$$

$$HS^- + H_2O \underset{}{\overset{K_2}{\rightleftharpoons}} S^{-2} + H_3O^+ \quad (2)$$

The proportions of molecular H_2S, HS^- and S^{-2} which are established in water are therefore determined by the equilibrium constants (K_1 and K_2) for the first and second ionizations of the sulfide species according to (3) and (4):

$$K_1 = \frac{[HS^-][H^+]}{[H_2S]} \quad (3) \qquad K_2 = \frac{[S^{-2}][H^+]}{[HS^-]} \quad (4)$$

The total sulfides generated $[Sulfides]_{tot}$ represents all sulfides generated from a donor according to (5):

$$[Sulfides]_{tot} = [H_2S] + [HS^-] + [S^{-2}] \quad (5)$$

A high pK_2 (~14 at 25°C)[9] and low K_2 value (~10^{-14}) for equilibrium (2) results in a negligible percentage of [S^{-2}] existing in the relevant pH range for most studies (i.e., when pH < 9 and [H^+] > 10^{-9}). Therefore, the second equilibrium can be neglected in our H_2S calculations.[10]
The total concentration of dissolved sulfides in solution is thus simplified to (6):

$$[Sulfides]_{tot} = [H_2S] + [HS^-] \quad (6)$$

As described in more depth in previous work[11], the amperometric sensor only detects partial pressure of H_2S and since the test is carried out at a pH of 7.4, this only accounts for one component of the total sulfides generated, $[Sulfides]_{tot}$. At pH 7.4, the $[Sulfides]_{tot}$ generated = $[H_2S]_{measured}$ / 0.296 (or = $[H_2S]_{measured}$ × 3.40). Throughout our amperometric studies we therefore also calculate a value of $[Sulfides]_{tot}$ to represent the true H_2S-generating capability of the conjugates.

Glutathione-mediated H_2S release from the conjugates T and P in cell media (DMEM with 10% v/v FBS) as measured using the amperometric sensor.

Polymeric solutions of **P** and **T** were prepared in Milli Q water to a final polymer concentration of 7.2 mg/mL and 5 mg/mL respectively. The sensor tip was immersed into a vial containing cell culture media only (500 μL) for the first 10 minutes, then **P** (50 μL of 7.2 mg/mL stock solution, 8.2×10^{-8} moles) or **T** (50 μL aliquot of 5mg/mL stock solution, 8.2×10^{-8} moles) is added. As with the calibration, in order to minimize exposure to air and loss of H_2S to the environment, Parafilm was used to cover the mixture during the measurements. During the measurements the mixture was stirred with a magnetic stirrer bar. H_2S release was monitored for a period of 10 - 12 minutes. Glutathione (GSH) was then injected into the stirring solution using a micro-syringe (20 μL of a 250 mM solution in water, 5.0×10^{-7} moles) and then H_2S release was monitored for another period of 20 - 25 minutes. Each release curve generated over time shows peaking [H_2S], as derived from calibration plot, and the corresponding peaking $[\text{sulfides}]_{tot}$, as calculated according to previous considerations.

Cell culture. HEK 293 (Human embryonic kidney cells, ATCC) were grown in Dulbecco's Modified Eagle Media (DMEM, high glucose, Sigma-Aldrich) supplemented with 10% (v/v) Fetal Bovine Serum (FBS,). Cells were cultured at 37 ºC in a humidified incubator with 5% atmospheric CO_2.

Cell-mediated H_2S release from the conjugates T and P as measured using the amperometric sensor

HEK293 (cultured as described above and maintained in T75 flasks) were exposed to trypsin for detachment, isolated as a plug, re-dispersed in complete media and counted. Two individual groups of equal volume and cell concentration were dispensed in separate vials. The sensor was immersed in each cell population, a signal was measured (for approx. 2 minutes) and then a **T** or **P** aliquot was added: 50 μL, 5 mg/mL stock solution, 8.2×10^{-8} moles for T, and 50 μL, 7.2 mg/mL stock solution, 8.2×10^{-8} moles for P. Each release curve generated over time shows peaking [H_2S], as derived from calibration plot, and the corresponding peaking $[\text{sulfides}]_{tot}$, as calculated according to previous considerations.

Alamar Blue Assay

All assay plates were coated with poly-L-lysine solution (mol wt 150,000-300,000, 0.01%, sterile-filtered, BioReagent). HEK cells (cultured as described above) were seeded in a 96-well plate (1.5×10^4 cells/well) in the cell medium overnight and subsequently incubated with serial dilutions of polymer samples (1000, 500, 250, 125, 63 and 31 μg/mL for **T**, **C** and **D**) and

(1440, 720, 360, 180, 90 and 45 μg/mL for **P**) made up in cell culture medium containing 1% (v/v) antibiotic (penicillin-streptomycin solution), for 24 h at 37 °C and with 5% CO_2. Cell culture medium cell containing 1% (v/v) antibiotic (penicillin-streptomycin solution) was used as a control. Each polymer was tested in triplicate at each concentration to obtain representative cell viability values. After exposure of cells to polymer samples the media was removed and cells were incubated with 10% v/v AlamarBlue™ Cell Viability Reagent (ThermoFisher) in cell culture media containing 1% (v/v) antibiotic, for 4 h at 37 °C and 5% CO_2. A microplate reader (CLARIOstar, BMG LABTECH) was used to read the fluorescence at 560 nm excitation and 590 nm emission. Background values (10% AlamarBlue in cell culture medium with 1% antibiotic, with no cells) were subtracted from each well and the average fluorescence intensity of the triplicates was calculated.

Cell based high-content SF4 time course study.

Solutions of polymers were made to a final concentration of 1 mg/mL for **T** and 1.4 mg/mL for **P**. The solutions were then diluted 1:10 in PBS 7.4 prior to addition to the cells (final concentration 0.1 mg/mL for **T** and 0.14 mg/mL for **P**). 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).

HEK293 cells (1.5×10^4 cells/well) were seeded 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 90μL HBSS) for 10 minutes 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 4 minutes. After baseline fluorescence was determined for 8 minutes, the polymeric solution (10μL) was added into wells, then images were taken every 4 minutes for 1 hr at 37°C. After that, images were taken every 1 hr for 23 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 ± SD from three independent experiments.

ROS Detection

HEK 293 cells (1.5×10^4 cells/well) were exposed to materials for 1, 2 and 4 h in 96-well plates. Cell culture medium was used as a control. The CellROX™ Deep Red Reagent was added to the cells at a final concentration of 10 μM followed by incubation for 40 minutes at 37 °C. The medium was then removed and the cells were washed three times with PBS. Fluorescence imaging was performed using a high-content PerkinElmer Operetta with an Olympus LUCPlanFLN 20x (NA 0.45) objective. The fluorescence was visualized using the mCherry filter set (excitation 640 nm, emission 665 nm). Background values (PBS only) were subtracted from each well and the average fluorescent intensity of the triplicates was calculated.

MitoSOX (mitochondrial superoxide indicator) test

HEK293 cells (4.0×10^4 cells/well) were seeded in 8 well chamber and cultured overnight. Cells were washed with HBSS, then loaded with 2.5 μM MitoSOX™ Red Mitochondrial Superoxide Indicator, in 300 μL HBSS for 30 minutes at 37 °C and the plates were kept protected from light. Cells were washed gently three times with warm HBSS buffer, then stained with Hoechst 33342 stain for 1 minutes, followed by imaging.

PI staining experiment

HEK293 cells (3.0×10^4 cells/well) were seeded in 8 well chamber and cultured overnight. Cells were washed with HBSS, then loaded with **C**, **D**, **T** and **P** combinations (**T** 500 μg/mL; **C** 31.25 μg/mL; **D** 125 μg/mL; **P** 720 μg/mL) for 24h at 37 °C. After that, PI was added into the wells at a final concentration of 1μM for 30 minutes, and then imaging was started.

Ratiometric imaging

HEK293 cells (1.5×10^4 cells/well) were seeded in 8 well chamber coated with poly-D-lysine and cultured overnight. Prior to imaging, old media was removed and replaced with serum-free Opti-MEM (Life Technologies), with cells washed in between using Opti-MEM. Laurdan dye was added to each well to a final concentration of 5 μM and allowed to equilibrate with the cell membranes for at least 30 minutes. Cells were transferred to a Leica SP8 inverted confocal fluorescence microscope housed in a humidified, 37 °C, 5% CO_2 environment. Appropriate areas of each well, containing at least 10 viable cells, were identified. **C**, **D**, **T** and **P** (**T** 500 μg/mL; **C** 31.25 μg/mL; **D** 125 μg/mL; **P** 720 μg/mL) and combinations thereof were added to respective wells. Imaging was undertaken at 0 minutes（i.e. before adding the material）and 20 minute time points with a 40 × oil objective. Laurdan dye integrated into cell membranes was excited along the 405 nm laser line and emission read at 430~470 nm (representing the

lipid membrane at the gel/liquid ordered phase) or 480~550 nm (representing the lipid membrane at the liquid disordered phase). To calibrate dye background levels, a well containing dye only was excited on the 405 nm laser line using 0.5 and 2 × laser power. Images were false-coloured and any adjustments made using LAS X software (Leica).

Determination of generalised polarization (GP). The acquisition of GP images was performed using the ImageJ (National Institute of Health) software and custom-written macro by Owen et al.[12], with modifications. GP values were then calculated for each pixel of a cell membrane according to the following equation:

$$GP = \frac{I_{400-460} - I_{470-530}}{I_{400-460} + I_{470-530}},$$

where I represents the intensity of pixels in the areas of interest in the image acquired in the ordered (430~470 nm) and disordered (480~550 nm) spectral channels. The GP shift was observed by subtraction of the GP distribution peak maximum of each sample with 20 minutes of incubation time from the GP value of the image taken at the beginning of the experiment (at 0 minutes).

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