Electronic Supplementary Material (ESI) for Biomaterials Science.

SUPPORTING INFORMATION

Trisulfide linked cholesteryl PEG conjugate attenuates intracellular ROS and collagen-1 production in a breast cancer co-culture model

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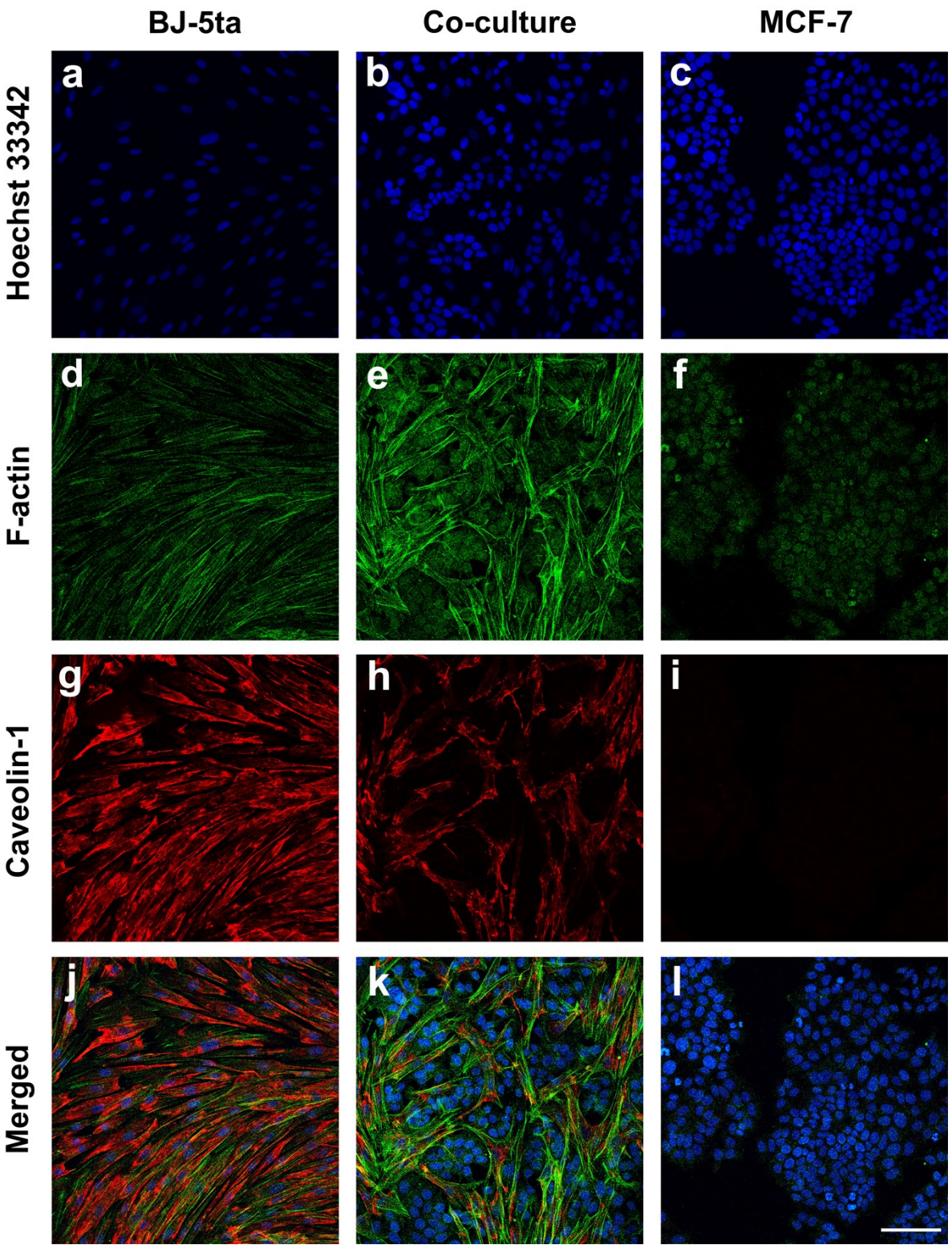


Figure S1. F-actin and caveolin-1 expression of BJ-5ta (***a***, ***d***, ***g, j***), MCF-7 (***c***, ***f***, ***i, l***) and their co-cultured equivalents (***b***, ***e***, ***h, k***). Cells were fixed and stained after five days plating. Data were taken from the Leica TCS-SP8 confocal lightning system using a 20X magnification objective. Picture with maximum intensity of a z-stack projection are shown. Scale bar: 100 µm.

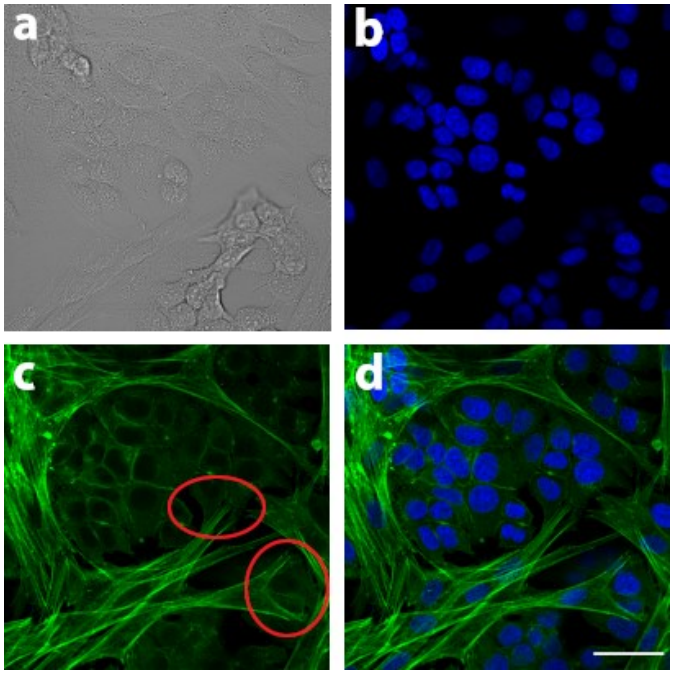


Figure S2. Pictures are taken from an SP8-TCS confocal system with a 20X magnification: (***a***) bright-field channel, (***b***) Hoechst 33342 channel, (***c***) AlexaFluor 488 phalloidin channel and (***d***) merged channel of Hoechst 33342 and Alexa 488. Cells are co-cultured at the ratio BJ-5ta: MCF-7 of 1:1 and are stained five days after plating. Notably, the red ovals highlight the points of contact between the co-cultured cells. Scale bar: 100 µm.

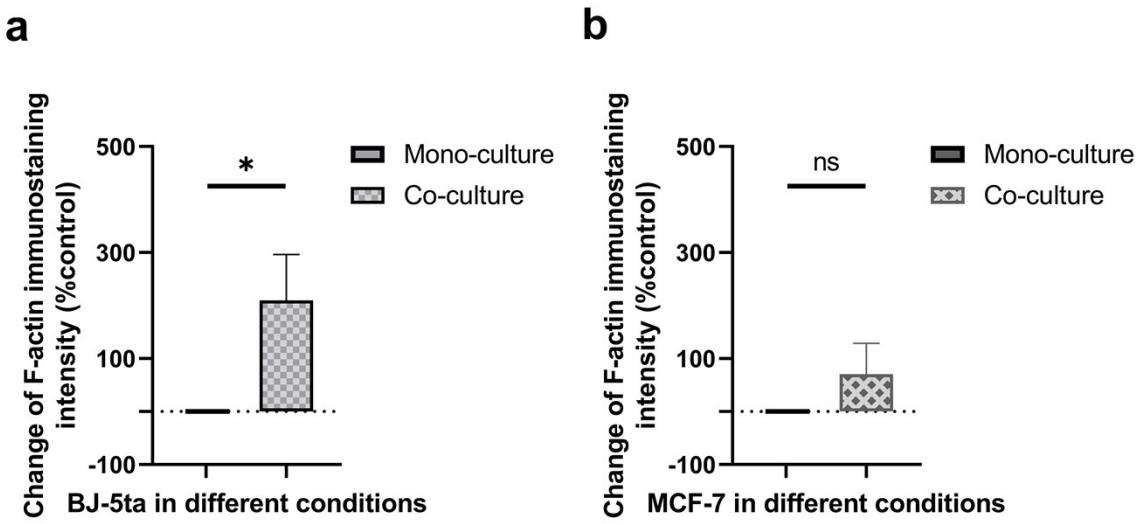


Figure S3. Percentage of changes in F-actin immunostaining intensity in co-cultured fibroblasts (***a***) and in co-cultured MCF-7 cells (***b***) at a seeding ratio 1:1 compared to the corresponding monoculture section (n=4-5). Paired *t*-test was used for statistical analysis. (*): $p < 0.05$; *ns: not significant*; e*rror bar: SEM.* Notably, F-actin immunostaining intensity was obtained from a FiJi software as average intensity per area.

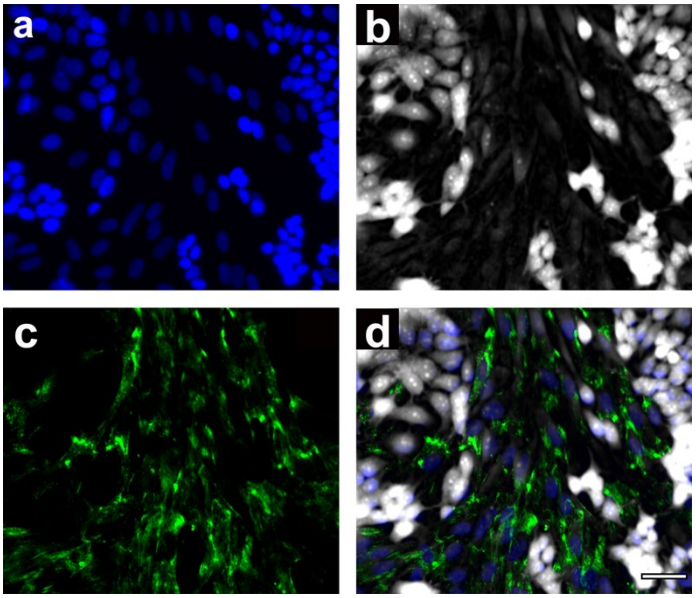


Figure S4. Hoechst 33342 channel (a), digital phase contrast channel (b), caveolin-1 channel (c) and their merged channel (d) of co-cultured BJ-5ta and MCF-7 at a seeding ratio of 1:1. Notably, cells with lower intensity (the dimmer areas) in the digital phase contrast channel correspond to fibroblast sections which correlate with caveolin-1 visualisation as a marker for fibroblasts. Images were obtained from an Operetta High content imaging system with a 20X magnification objective. Scale bar 50 µm.

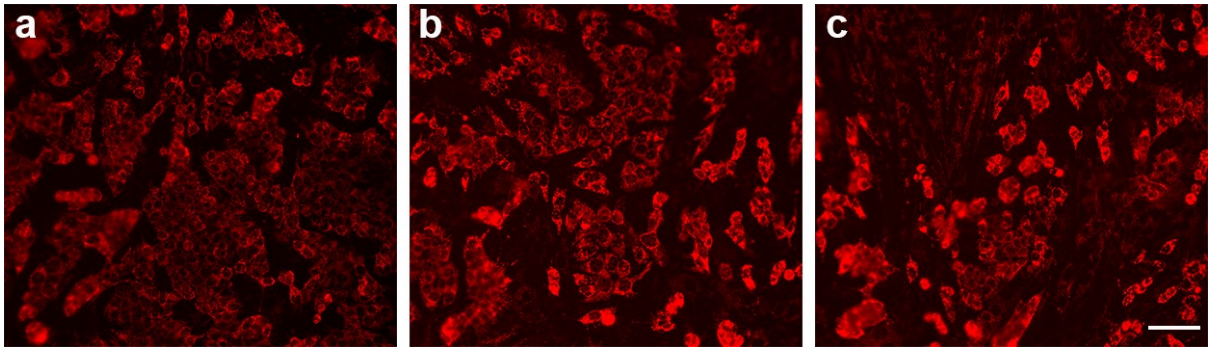


Figure S5. Cellrox Deep Red channels for ROS detection of co-cultured cells at different BJ-5ta:MCF-7 seeding ratios, 1:1 (***a***), 3:1 (***b***), and 5:1 (***c***). Cells were loaded with Cellrox deep red after five days of plating. Notably, the dimmer areas in each image stand for the fibroblast region with lower intensity compared to the corresponding value in MCF-7 cells. Images were obtained from an Operetta High content imaging system with a 20X magnification objective. Scale bar 100 µm.

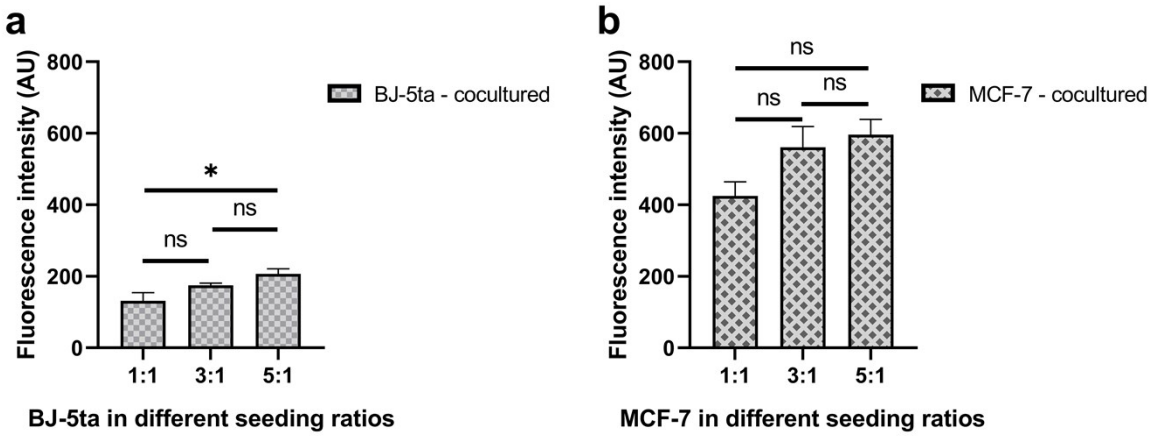


Figure S6. Average fluorescence intensity in co-cultured BJ-5ta and MCF-7 cells after ROS staining at day five of plating at varied seeding ratio of 1:1; 3:1 and 5:1 (*n=3*); Matched one-way ANOVA with Fisher's LSD test was used for statistical analysis; (*), *p < 0.05; ns: not significant; error bar: SEM.*

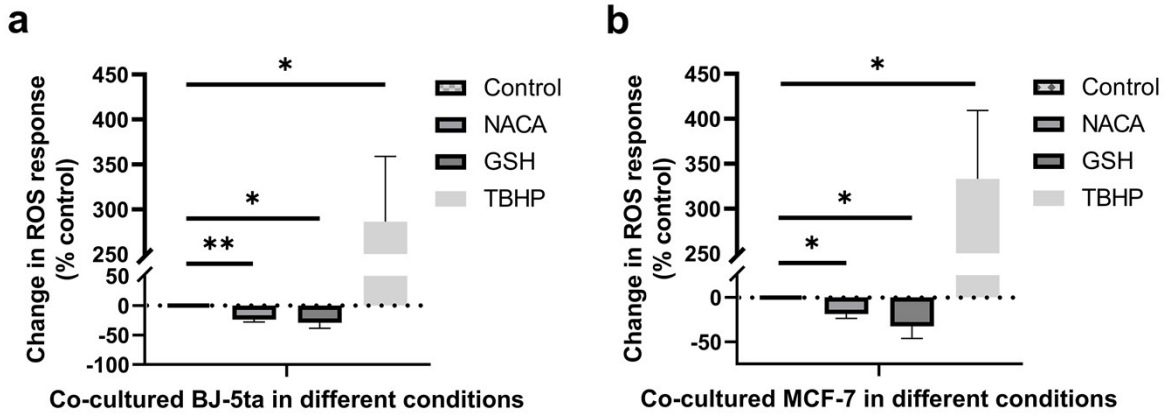


Figure S7. Relative ROS production of co-cultured BJ-5ta (***a***) and co-cultured MCF-7 (***b***) at a seeding ratio of 1:1 of treated groups with *N*-acetyl cysteine amide (NACA, 5.0 mM) or glutathione (GSH, 2.5 mM) or TBHP (100 µM) compared to the corresponding cells in the co-cultured control group (*n=3*). ROS were stained by Cellrox deep red and were detected using an Operetta High content imaging system with a 20X magnification objective (Perkin Elmer). *t*-test was used for statistical analysis; (*), $p < 0.05$; (**), $p < 0.01$; e*rror bar: SEM*.

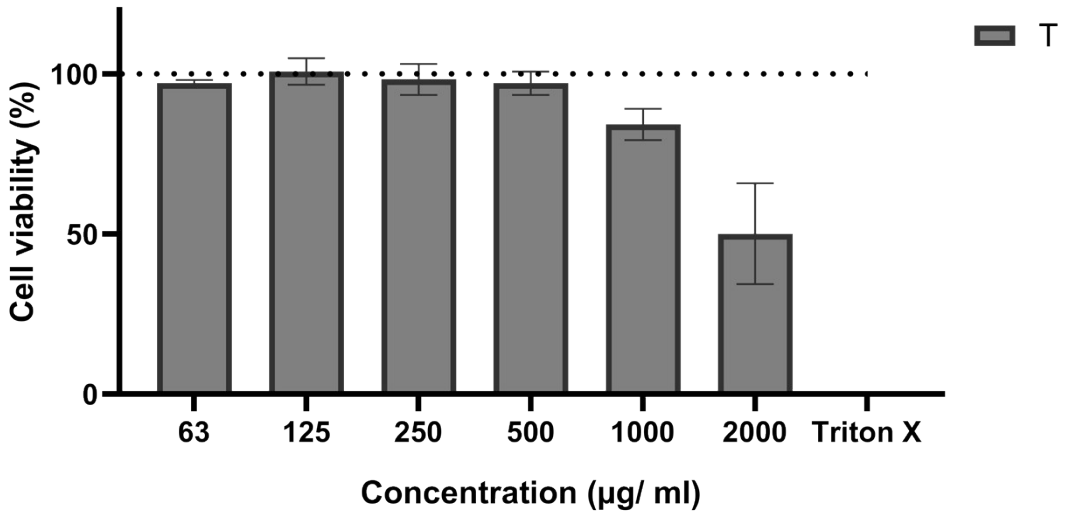


Figure S8. Cell viability (%) of co-cultured cells in groups treated with **T**, compared to control group. Cell viability was achieved after 24 hour treatment using AlamarBlue assay. Data were presented for four independent experiments (*n=4*).

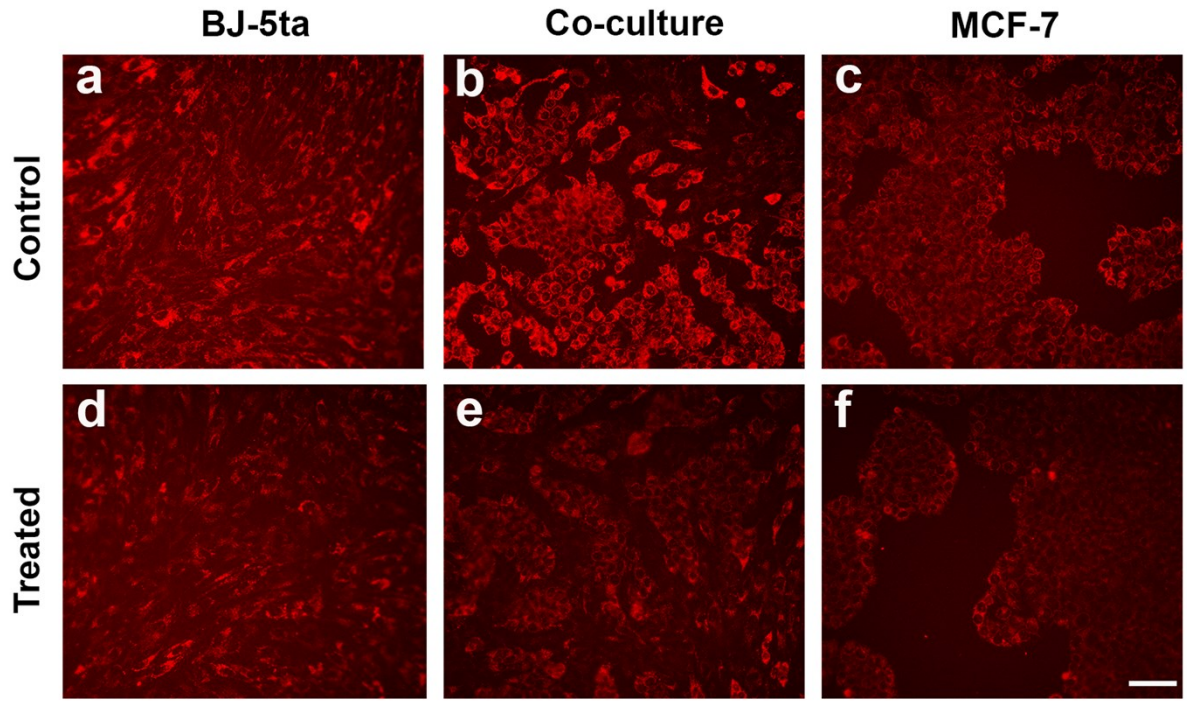


Figure S9. ROS expression of different cultures exposed to compound **T** for 24 hours. Upper panels are control groups, and lower panels are treated groups of BJ-5ta monoculture (***a***, ***d***), MCF-7 mono-culture (***c***, ***f***) and the co-cultured cells (***b***, ***e***). Cellrox Deep red was employed for ROS staining. Images were taken from an Operetta High content Imaging system with a 20X magnification objective and represent four separate experiments. Scale bar: 100µm.

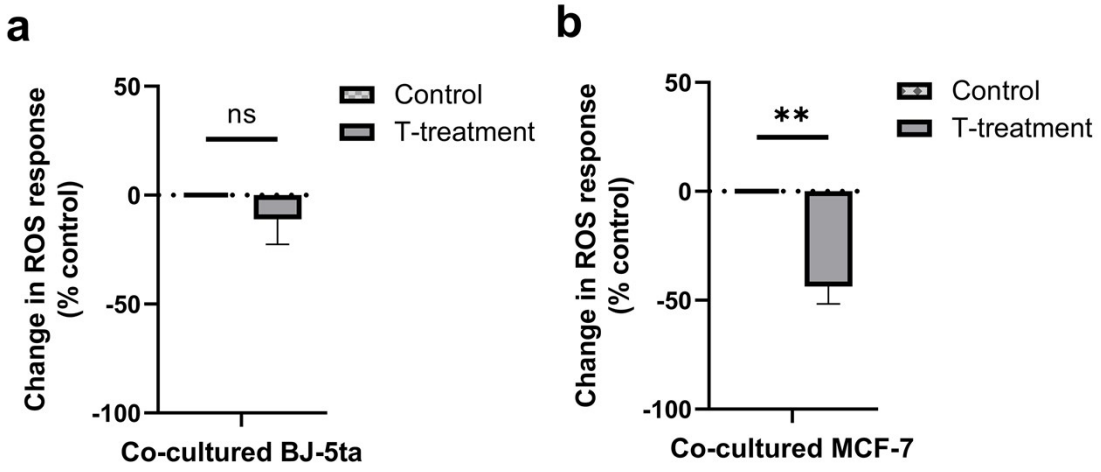


Figure S10. Relative ROS production of co-cultured BJ-5ta (**a**) and co-cultured MCF-7 (**b**) at a seeding ratio of 1:1 of treated groups with **T** (0.5 mg/mL) compared to the corresponding cells in the co-cultured control group (*n=3*). ROS were stained by Cellrox deep red and were detected using an Operetta High content imaging system (Perkin Elmer). *t*-test was used for statistical analysis; (*), $p < 0.05$; (**), $p < 0.01$; *error bar: SEM*.

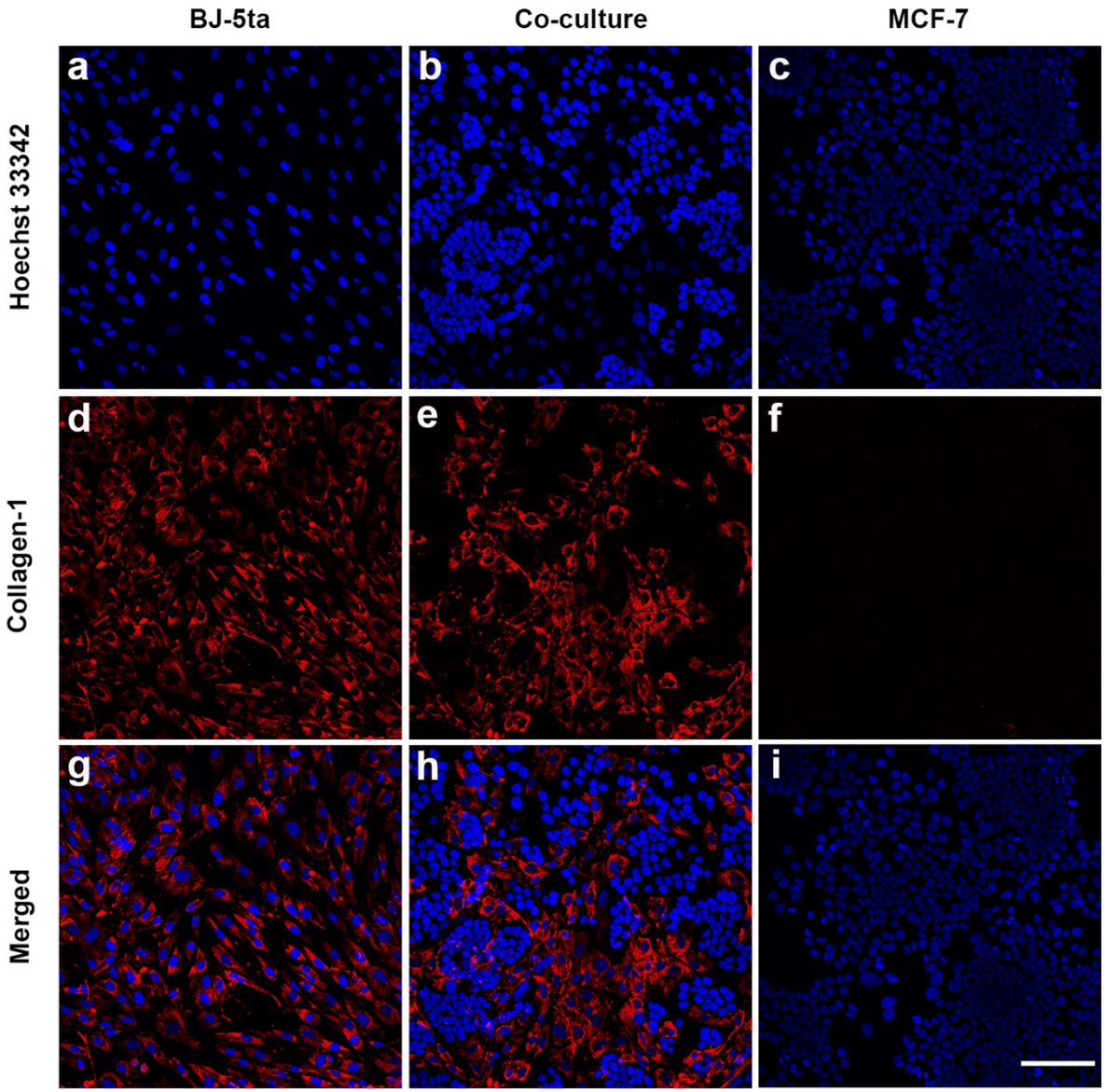


Figure S11. Collagen-1 expression from BJ-5ta (***a***, ***d***, ***g***), MCF-7 (***c***, ***f***, ***i***) and their co-cultures (***b***, ***e***, ***h***). Cells were fixed and stained with collagen-1 antibody after five days of plating. Data were obtained using the Leica TCS-SP8 confocal system with a 20X magnification objective. Notably, picture with maximum intensity of a z-stack projection has been illustrated. Slices were analysed for average collagen-1 intensity per area using FiJi software. Scale bar 100 µm.

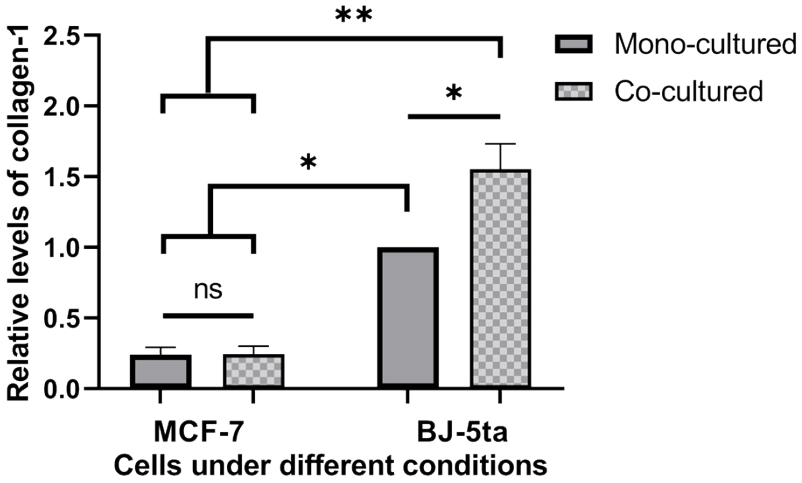


Figure S12. Relative levels of collagen-1 production in MCF-7 and BJ-5ta cells under different conditions compared to the monoculture BJ-5ta section (n=4). Matched two-way ANOVA with Tukey's multiple comparison *post-hoc* test was used for statistical analysis; *(*) $p < 0.05$; (**) $p < 0.01$; ns: not significant; error bar: SEM.* Notably, collagen-1 immunostaining intensity was obtained from a FiJi software as average intensity per area.

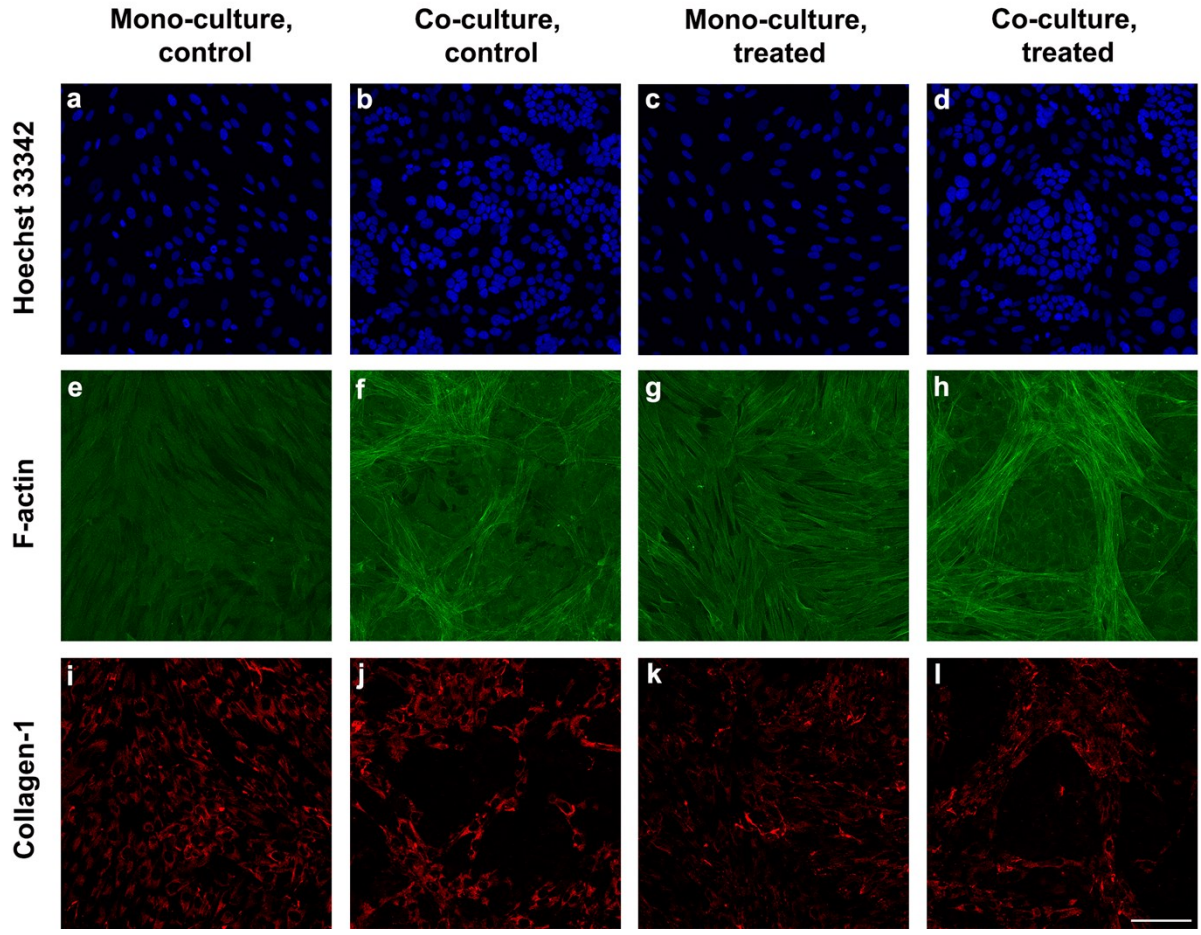


Figure S13. Hoechst 33342, F-actin and Collagen-1 channels of mono-culutre of fibroblasts (***a***, ***c***, ***e***, ***g***, ***i***, ***k***) or when co-cultured with MCF-7 at a seeding ratio of 1:1 (***b***, ***d***, ***f***, ***h***, ***j***, ***l***) of control groups (***a***, ***b***, ***e***, ***f***, ***i***, ***j***) and **T** treated groups (***c***, ***d***, ***g***, ***h***, ***k***, ***l***). Cells were treated with **T** at 0.5 mg/mL for one day before immunostaining. Pictures were taken from a SP8-TCS confocal microscope with a 20X magnification. Notably, slices with maximum intensity of a z-stack projection have been illustrated. Scale bar: 100 µm.

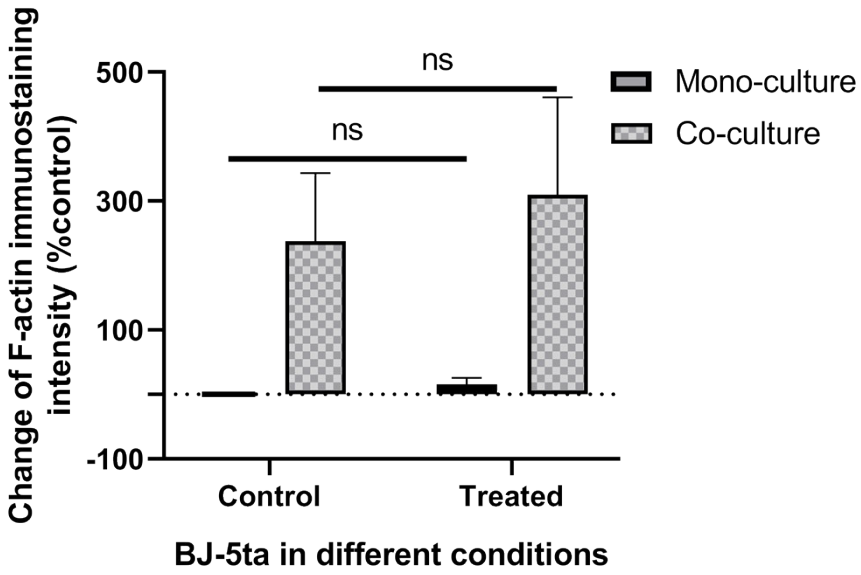


Figure S14. Percentage of changes in average F-actin immunostaining intensity of fibroblast cells when mono-cultured or co-cultured with cancer cells. The cells in treated groups were exposed to **T** at 0.5 mg/mL for one day before staining (n=4). Matched two-way ANOVA with Fisher's LSD test was used for statistical analysis; *ns: not significant; error bar: SEM.* Notably, images were analysed for average F-actin immunostaining intensity per area, using FiJi software.

Materials

MeO-PEG-NH_2 MW 2000 g/mol (Linear Monofunctional PEG Amine NH_2) was purchased from Creative PEG Works. 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.

Cholesteryl(2-mercapto ethyl)carbamate was synthesised as previously published.[1]

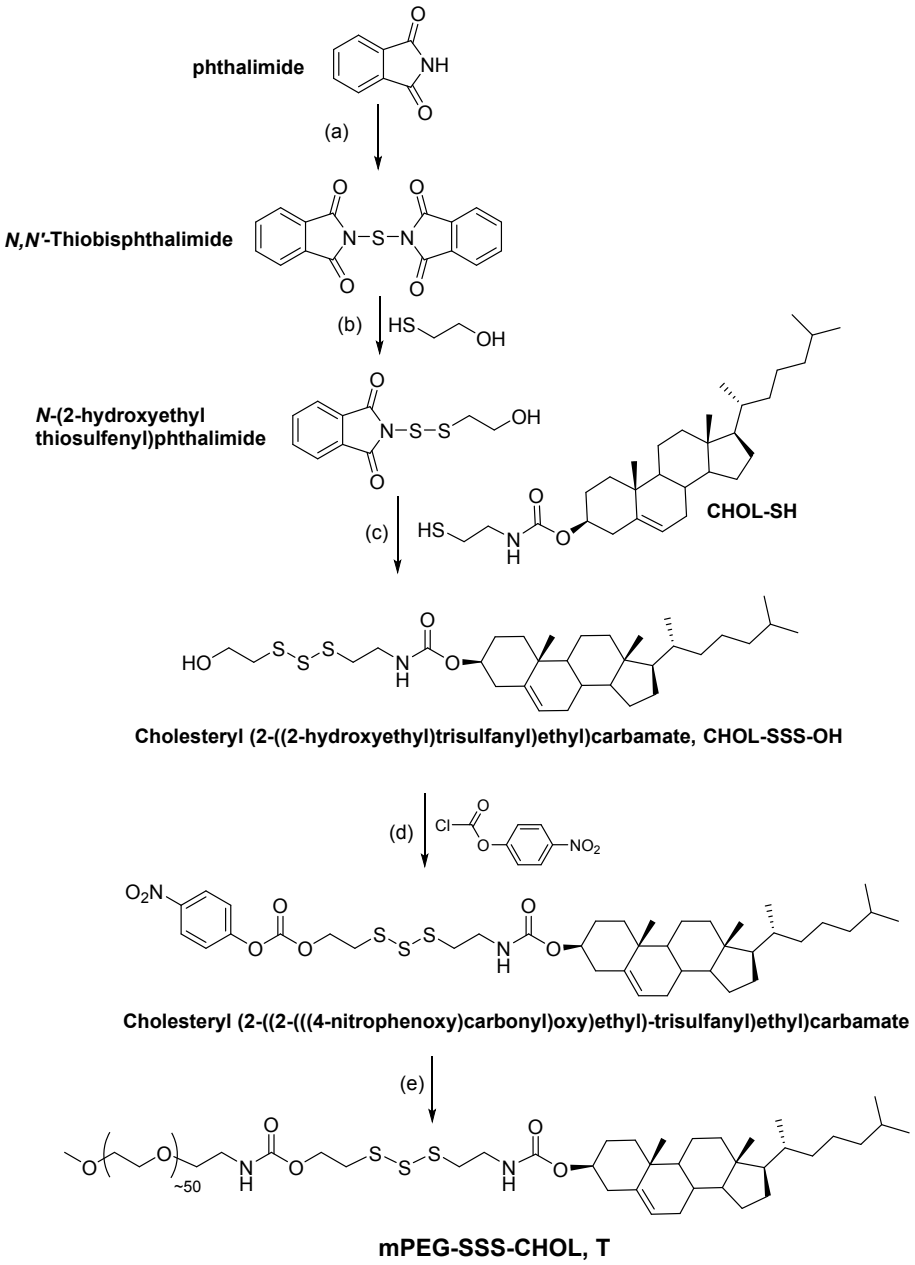
MeO-PEG-SSS-OMe (**P**) was synthesised as described below, according to Scheme S2 (and as previously described).[2]

Characterization

^{1}H (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.

Synthetic Protocols



Scheme S1. Synthesis of MeO-PEG-SSS-CHOL (**T**) conjugate: (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 (e) DIPEA, DMAP, CH_2Cl_2, 25°C, 16 hours.

Synthesis of cholesteryl trisulfide PEG polymer (T)

The PEG-cholesteryl trisulfide was synthesized as shown in Scheme S1. The procedure includes five main steps with the following products:

N,N'-Thiobisphthalimide: This reaction was based on work by Kalnins.[3] Briefly, phthalimide (14.72 g, 0.10 mole) was dissolved in anhydrous dimethylformamide (DMF, 80 mL dried over Molecular Sieves Type 4Å) and heated to 28 °C under a nitrogen atmosphere. To the resulting

solution, sulfur monochloride (13.50 g, 0.10 mol) was added in several portions, and then the yellow mixture was left to stir under nitrogen at 28 °C for 20 hours. The obtained suspension was then filtered and the cream solid dried under high vacuum (9.0 g, 61 % yield). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.79 (m, 2H, ArH), 7.95 (m, 2H, ArH) ppm. ^{13}C-NMR (50 MHz, $CDCl_3$) 166.2, 135.3, 131.7, 124.8.

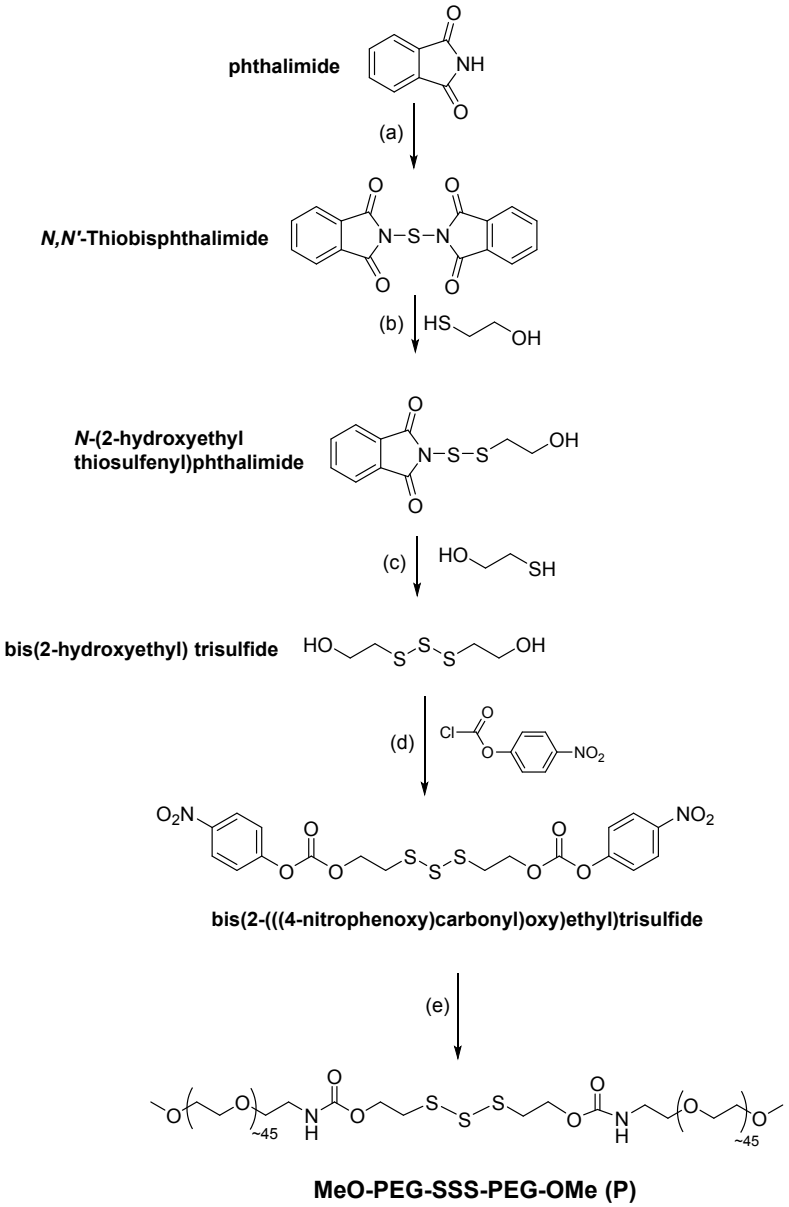
N-(2-hydroxyethyl thiosulfenyl)phthalimide: A mixture of *N,N'*-thiobisphthalimide (3 g, 9.3 mmoles) and mercaptoethanol (0.65 g, 0.58 mL, 8.3 mmol, 0.9 equivalents) was heated in toluene (50 mL) at 80 °C for 1 hour.[4] The reaction progress was monitored using thin-layer chromatography using ethyl acetate/ hexane/dichloromethane (EA/HEX/DCM) mixture. The product appears as a spot with R_f 0.42. The reaction was cooled to room temperature and then filtered. The white solid was washed three times with DCM, and the combined filtrate and washings were evaporated to dryness. The resulting crude solid was then purified using a Reveleris® Flash Chromatography System fitted with a silica cartridge, using a solvent system of 10 % → 70 % EA in HEX gradient mixture. The product was afforded as a white solid (1.20 g, 52 %). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 3.23 (t, 2H, *J* 5.8 Hz, -C***H*****$_2$**CH_2-OH), 4.10 (t, 2H, *J* 5.8 Hz, -CH_2C***H*****$_2$**-OH), 7.80 (m, 2H, ArH), 7.94 (m, 2H, ArH) ppm. ^{13}C-NMR (50 MHz, $CDCl_3$) 167.9, 135.0, 132.4, 124.3, 60.9, 43.6 ppm.

Cholesteryl (2-((2-hydroxyethyl)trisulfanyl)ethyl)carbamate. This reaction followed the method of Sullivan and Boustany.[5] *N*-(2-Hydroxyethyl thiosulfenyl)phthalimide (0.71 g, 2.76 × 10^{-3} mol) and cholesteryl(2-mercapto ethyl)carbamate (1.35 g, 2.76 × 10^{-3} mole) were mixed in toluene (35 mL) for 16 hours at room temperature. Cholesteryl (2-mercapto ethyl)carbamate has been synthesized as described in our previous work.[1] The reaction progress was monitored using thin-layer chromatography using EA/HEX/DCM mixture. The product appears as a spot with R_f 0.40. The reaction was cooled to room temperature and then filtered. The obtained white solid was washed three times with DCM, and the combined filtrate and washings were evaporated to dryness. The resulting crude solid was purified using a Reveleris® Flash Chromatography System fitted with a silica cartridge, using a solvent system (DEE/HEX/DCM) mixture. The product was afforded as a waxy, sticky white solid (1.40 g, 80 %). Analysis, as reported previously.[1]

Cholesteryl (2-((2-(((4-nitrophenoxy)carbonyl)oxy)ethyl)-trisulfanyl)ethyl)carbamate*:* 4-Nitrophenyl chloroformate (0.40 g, 2.01 × 10^{-3} mol) in 10 mL of anhydrous CH_2Cl_2 was added dropwise to a solution of cholesteryl (2-((2-hydroxyethyl)trisulfanyl)ethyl)carbamate (1000 mg, 1.67 × 10^{-3} mol) and triethylamine (420 µL, 3.01 × 10^{-3} mol) in 10 mL of anhydrous CH_2Cl_2. The mixture was stirred at 25 °C overnight. The crude material was then concentrated and purified using a Reveleris® Flash Chromatography System fitted with a silica cartridge, with gradient solvent system, 30 % DCM/HEX → 100% DCM. The product was isolated as an off white, waxy solid (0.70 g, 55%). ^{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.

Cholesteryl (((2-methoxyPEGamino)carbonyloxyethyl)trisulfanylethyl)carbamate (mPEG-SSS-CHOL, T): mPEG-Amine (MW 2000 g mol^{-1}, 0.92 mg, 4.60×10^{-4} mol), *N,N*-diisopropylethylamine (DIPEA, 0.14 g, 192 µL, 1.10×10^{-3} mol) and 4-(dimethylamino)pyridine (DMAP, ~1.0 mg) were dissolved in CH_2Cl_2 (10 mL) and added dropwise to a solution of cholesteryl (2-((2-(((4-nitrophenoxy)carbonyl)oxy)ethyl)trisulfanyl)ethyl) carbamate (0.70 g, 9.20×10^{-4} mol) in 10 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 (4×) precipitations into diethyl ether/petroleum ether (7:3). 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 extensively against water. The final product mPEG(2000)NHCOO-$(CH_2)_2$-SSS-$(CH_2)_2$-CHOL (**T**), (average MW = 3,100 g/mol) was isolated as a white fluffy solid after removal of the water using freeze-drier. The final product was analysed by ^{1}H-NMR: (400 MHz, $CDCl_3$) δ 5.40 (m, 1H, NHCO), 5.37 (m, 1H, H6), 5.20 (br m, 1H, NH), 4.49 (m, 1H, H3), 4.38 (m, 2H, -COO-CH_2-), 3.60-3.80 (m, 5H, CH_3O- and -CH_2-NH^b-), 2.90-3.20 (m, 2H, 2 × t, *J* 6.5Hz, 4H, -CH_2-SSS-CH_2-), 2.20-2.40 (m, 2H, H4), 1.75-2.08 (m, 5H, H2, H7, H8), 0.93 -1.63 (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.



Scheme S2. 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 (e) DIPEA, DMAP, CH_2Cl_2, 25°C, 16 hours.

Synthesis of MeO-PEG-SSS-PEG-OMe (P)

The PEG trisulfide **P** was synthesized as shown in Scheme S2 and as previously described.[2] The procedure includes five main steps with the synthesis of the first two products described in the synthetic protocol for **T**:

Bis(2-hydroxyethyl)trisulfide: 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 (R_f 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.

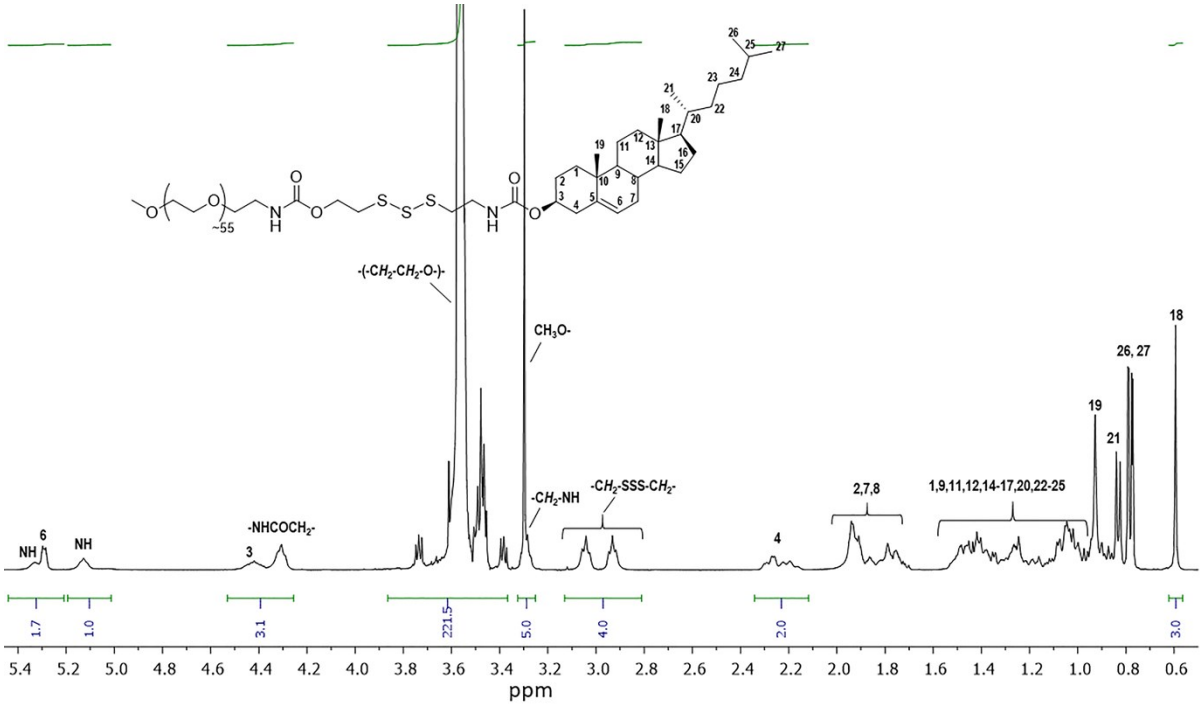


Figure S15. ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) of MeO-PEG-SSS-CHOL (**T**), with main peak assignments.

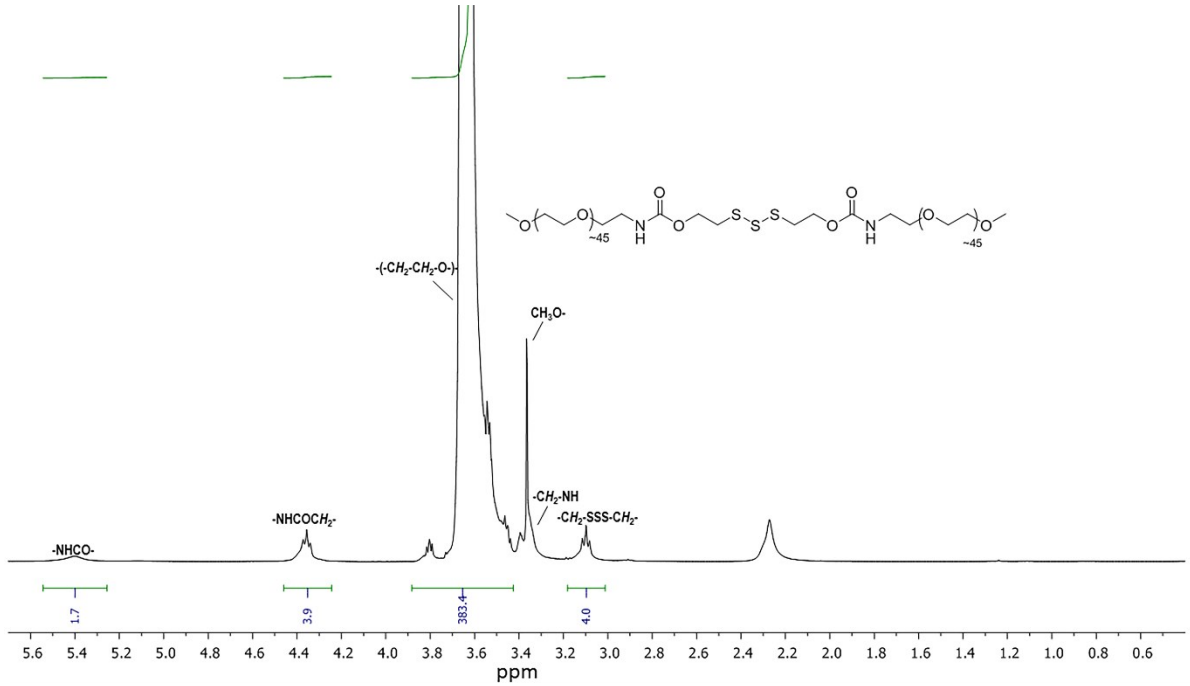


Figure S16. ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) of MeO-PEG-SSS-PEG-OMe (**P**), with main peak assignments.

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