Electronic Supplementary Material (ESI) for Medicinal Chemistry Communications.

Polymeric Micelles for Hydrogen Sulfide Delivery

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Supporting information

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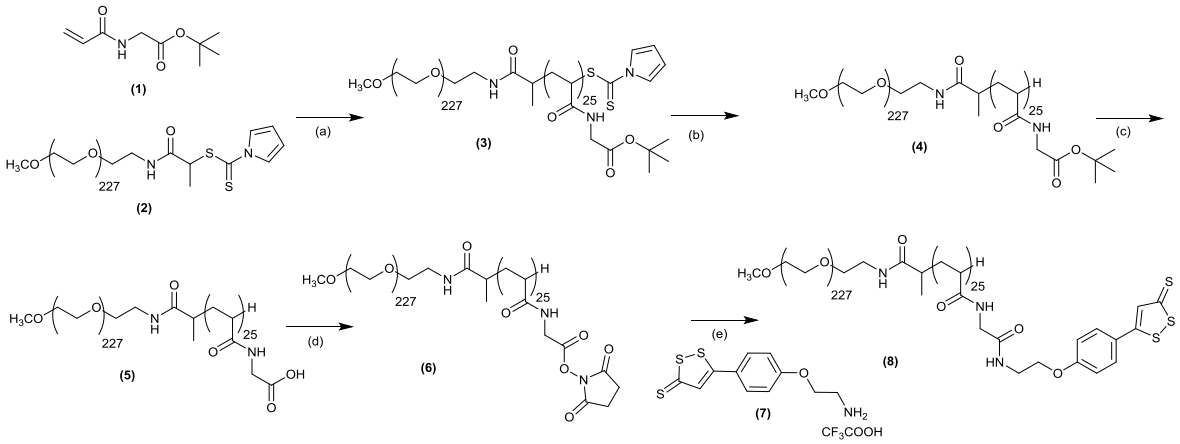
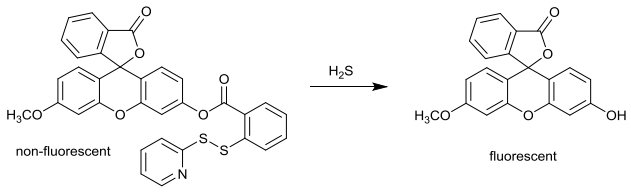
Scheme S1. Synthetic scheme for the PEG-PADT block copolymer (**a**) AIBN, dioxane 70°C (**b**) Ethylpiperidine hypophosphite, AIBN, dioxane, 95°C (**c**) TFA/H_2O 9:1 (**d**) NHS, DCC, DMAP, DMF (**e**) Et_3N, DMF.



Scheme S2. Structure of the H_2S-detection dye, WSP-1, used for measuring the released H_2S in RAW Blue murine macrophages.



Scheme S3. Synthetic scheme for the coumarin-labeled PEG-PADT block copolymer. (**a**) AIBN, dioxane 70°C. (**b**) Ethylpiperidine hypophosphite, AIBN, dioxane, 95°C. (**c**) TFA/H_2O 9:1. (**d**) NHS, DCC, DMAP, DMF. (**e**) Et_3N, DMF.

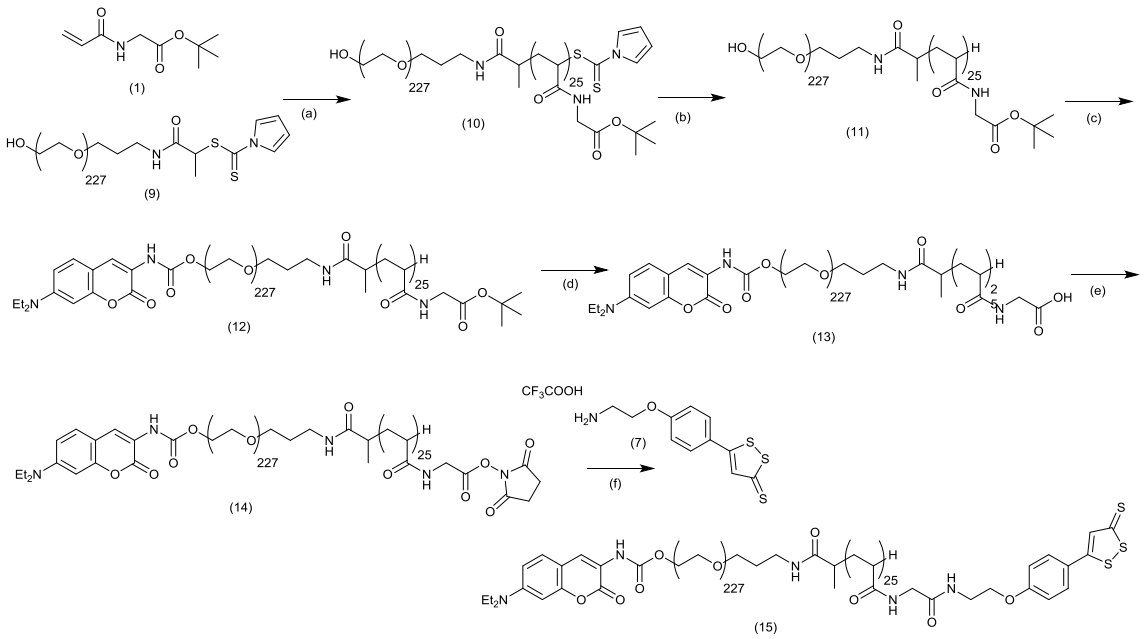


Table S1. M_n and M_w/M_n as determined by GPC using a PEG standard.

Polymer	M_n	M_w/M_n
PEG-Gly$(OtBu)_{25}$-H	11948	1.05
HO-PEG-Gly$(OtBu)_{25}$-H	12992	1.03

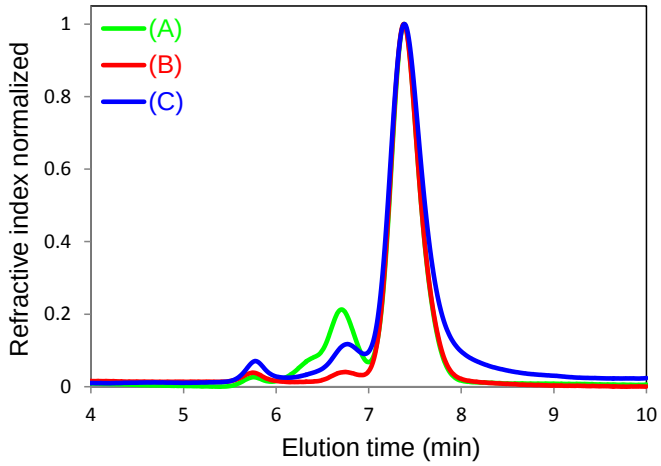
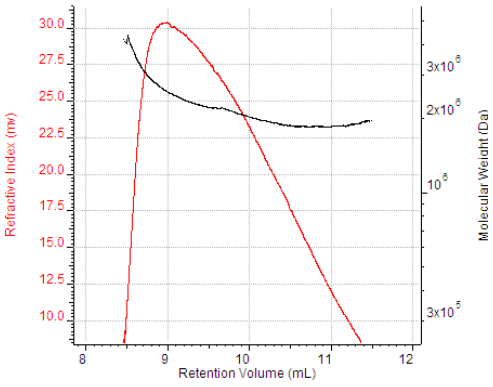


Figure S1. GPC elution profiles of (**A**) PEG-Gly(OtBu)$_{25}$-pyrrole, (**B**) PEG-Gly(OtBu)$_{25}$-H and (**C**) PEG-PADT.



Mw	2.29x 10^6
Mn	2.23x 10^6
Mw/Mn	1.03

Figure S2. Molecular weight distribution of the ADT micelles as measured by SEC-LALS. Eluent: Phosphate buffered saline (1/15 M, pH 7.4). Flow rate: 0.5 mL/min.

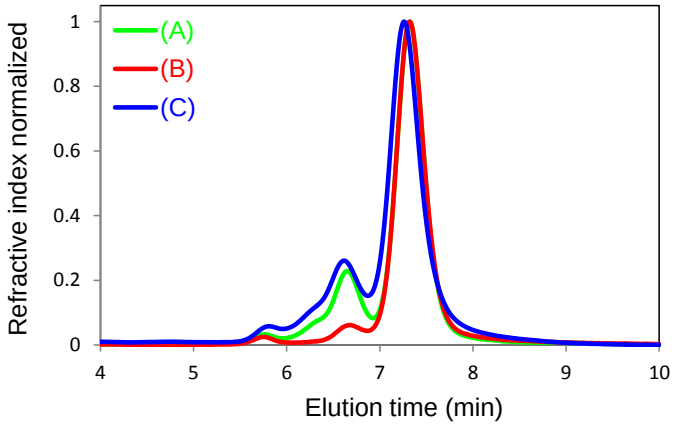


Figure S3. GPC elution profiles of (**A**) HO-PEG-Gly(OtBu)$_{25}$-pyrrole (**B**) Coumarin-PEG-Gly(OtBu)$_{25}$ -H and (**C**) Coumarin-PEG-PADT.

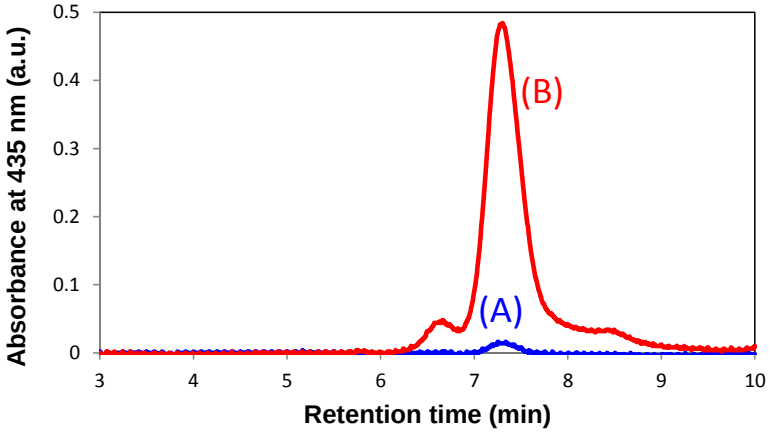


Figure S4. GPC elution profiles showing the UV absorption at 435 nm for (**A**) HO-PEG-Gly(OtBu)$_{25}$-H and (**B**) Coumarin-PEG-Gly(OtBu)$_{25}$-H

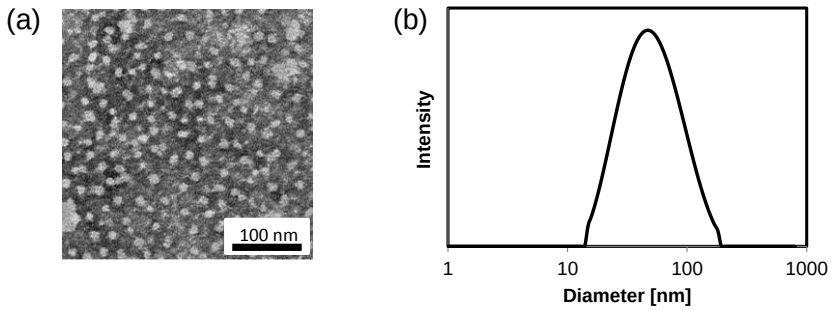


Figure S5. Characterization of coumarin-labeled ADT micelles by (**a**) transmission electron microscopy (negative staining with 3wt% Preyssler-type phosphotungstaste solution), (**b**) dynamic light scattering.

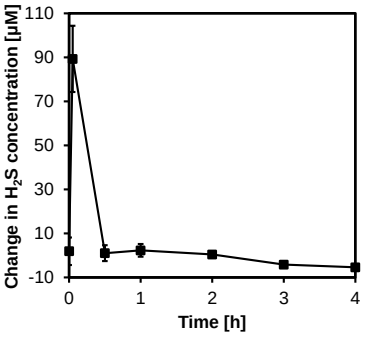


Figure S6. H_2S release from Na_2S in the presence of RAW Blue macrophages. The H_2S concentration in the culture medium was determined using a fluorescent dye WSP-1. Concentration of Na_2S: 100 μM.

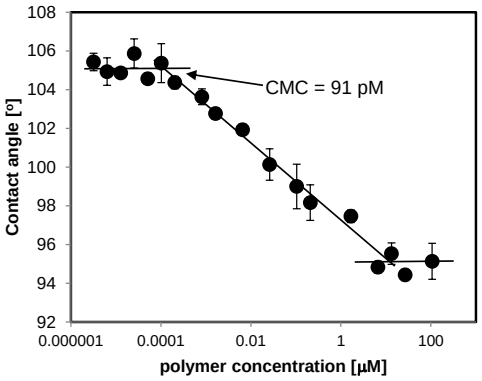


Figure S7. Critical micelle concentration (CMC) of the ADT micelles as determined by surface tension method.

Instrumententation

^{1}H NMR. Spectra were acquired on a Bruker DPX400 NMR spectrometer at room temperature with the residual undeuterated solvent signal as reference (2.50 d_6-DMSO and 7.26 $CDCl_3$).

High resolution mass spectometory. ESI-TOF MS analyses were performed on a Bruker microTOFII mass spectrometer.

Gel permeation chromatography (GPC). Elution profiles of the polymers (10 mg/mL) were collected on a Shodex KD-803 column at 50°C equipped with a Tosoh differential refractometer and photodiode array detector. Dimethylformamide (DMF) containing 100 mM LiCl (flow rate of 1.0 mL/min) was used as the eluent. The polydispersity index (PDI) of the polymers was calculated based on the elution time of polyethylene glycol standard polymers.

Dynamic light scattering (DLS). Hydrodynamic diameter of the micelles was obtained on an Otsuka ELSZ machine. The mean diameter (Z-average) and polydispersity index ($PDI=\mu_2/\Gamma^2$) were calculated by the cumulant method.

Transmission electron microscopy (TEM). Carbon coated 250 mesh copper grids were prepared under glow discharge. The micelle solution was placed onto the grid and then dried by blotting the side of the grid with a filter paper. The grids were negatively stained with 3wt% Preyssler-type phosphotungstate solution. Images were acquired on a HITACHI H-7650 TEM.

Size exclusion chromatography-low angle light scattering (SEC-LALS). The ADT micelles were dispersed in phosphate buffered saline (1/15 M, pH 7.4) at 2.17 mg/mL and filtered through a cellulose acetate syringe filter (pore size: 0.2 μm). The sample (100 μL) was analyzed on a Tosoh chromatography system connected to a Malvern Viscotek 305 TDA. A Superose 6 10/300 GL (GE Healthcare Life Sciences) was used as a SEC column. Phosphate buffered saline (1/15 M, pH 7.4) was used as an eluent at a flow rate of 0.5 mL/min. The average molecular weight (M_w and M_n) and polydispersity index (M_w/M_n) were determined using OmniSEC software.

H_2S electrode sensor. H_2S was measured on a four-channel free radical analyzer equipped with an ISO-H2S-2 hydrogen sulfide sensor (diameter: 2 mm) and a temperature sensor. The sensors were placed in a four-port closed chamber at 37°C.

Confocal laser scanning fluorescence microscope (CLSFM). Fluorescent images were acquired on an Olympus FluoView FV1000-D confocal microscope equipped with 405, 473, 559 and 635 nm lasers.

UV/VIS and fluorescence spectrometry. Spectra were obtained on a Tecan infinite M200 well plate reader using transparent or black polystyrene well plates.

CMC measurement The micelle solution in milliQ water at different concentrations (2 µL) was dropped onto Parafilm and the contact angle was measured on a Kyowa Interface Science Drop Master DM 300 to determine the critical micelle concentration.

Materials

Chemistry

CF_3COOH (TFA), 1-ethylpiperidine hypophosphite (EPHP), *N*-hydroxysuccinimide (NHS), polyethylene glycol (PEG) standard polymers (20,000, 12,000, 6,000, 4,000 and 2,000 Da), CH_3O-PEG_{114}-OH (M_n = 5,000) and sodium sulfide nonahydrate ($Na_2S{\cdot}9H_2O$) were purchased from Sigma-Aldrich. Sodium hydrogensulfate ($NaHSO_4$), anhydrous sodium sulfate (Na_2SO_4), potassium hydrogen carbonate ($KHCO_3$), anhydrous potassium carbonate (K_2CO_3), *N*,*N*′-dicyclohexylcarbodiimide (DCC), anhydrous dimethylformamide (DMF), anhydrous dimethylsulfoxide (DMSO), diethylether (Et_2O), acryloyl choride, 2,2'-azobis(2-methylpropionitrile) (AIBN) and phosphate buffer powder (1/15 mol/L, pH 7.4) were purchased from Wako Pure Chemical Industry. 4-(Dimethylamino)pyridine (DMAP), *tert*-butyl aminoacetate hydrochloride were purchased from Tokyo Chemical Industry. Sunbright HO-PEG_{227}-$CH_2CH_2CH_2$-NH_2, (M_p =11076) HO-PEG_{114}-$CH_2CH_2CH_2$-NH_2, (M_p =4704) and CH_3O-PEG_{227}-CH_2CH_2-NH_2 (M_p =9988) were purchased from NOF Corporation. Ethyl acetate (EtOAc), hexane, methanol (MeOH), toluene, dichloromethane (CH_2Cl_2), 1,4-dioxane and molecular sieves 3A were purchased from Nacalai Tesque. 7-Diethylaminocoumarin-3-carbonyl azide was from Invitrogen. Deuterated solvents for NMR ($CDCl_3$, d_6-DMSO) were purchased from Cambridge Isotope Laboratories.

Biology

Thiazolyl Blue tetrazolium bromide (MTT) was purchased from Tokyo Chemical Industry. LPS-free water was purchased from Otsuka Pharmaceutical Factory. DMEM GlutaMax, fetal bovine serum (FBS) and penicillin-streptomycin were purchased from Invitrogen. Passive Lysis Buffer was purchased from Promega. WSP-1 was purchased from Cayman Chemical. RAW Blue cells, QUANTI-Blue and Zeocin were purchased from Invivogen. Mouse TNF-α DuoSet Economy Pack was purchased from R&D Systems. Slide-A-Lyzer G2 (MWCO 2 kDa) was purchased from Thermo Scientific. Triple-wells glass-based dishes and 96-well microplates were purchased from Iwaki. Water was freed from salt using MilliQ water system.

Synthesis

General

All reactions were done under an inert argon atmosphere. CH_3OH and CH_2Cl_2 were dried over molecular sieves. 1,4-dioxane was distilled from CaH_2 under reduced pressure at 40°C and kept over molecular sieves and Xpell pellets to avoid the built up of peroxides. Triethylamine (Et_3N) was distilled from ninhydrin and CaH_2 before use. AIBN was recrystallized from methanol and stored at -20°C. For column chromatography SiO_2 was used. TLC was done on silica gel 60 F254 TLC plates. Molecular sieves were heated at 180°C under reduced pressure for at least 16 h.

Synthesis of PEG-PADT block copolymer (Scheme S1).

Glycine *tert*-butylester acrylamide (1). 2.73 g (16.3 mmol) of *tert*-butyl aminoacetate hydrochloride and 4.54 mL (32.6 mmol, 2 eq) of Et_3N were dissolved in 150 mL CH_2Cl_2 and cooled at 0°C. To the cooled solution was added drop wise in about 20 min a solution of 1.34 mL (16.3 mmol, 1 eq) of acryloyl chloride in 10 mL CH_2Cl_2. After stirring for 21 h the mixture was concentrated under reduced pressure. The residue was dissolved in 100 mL EtOAc/50 mL 1 M $NaHSO_4$ (aq) and the layers separated. The organic phase was extracted with 1 M $NaHSO_4$ (aq) (2 x 50 ml), 5% $KHCO_3$ (aq) (3 x 50 mL) and brine (100 mL). After drying over Na_2SO_4 the solution was concentrated to yield 2.0 g (11.0 mmol, 67%) of a white solid. ^{1}H NMR ($CDCl_3$): δ = 6.31 (dd, 1H, *J*=1.6 and 17.2 Hz, CH=C$\underline{H}_{trans}$), 6.15 (dd, 1H, C$\underline{H}$=CH_2 *J*=10.2 and 17.2 Hz), 6.12 (bs, 1H, NH), 5.67 (dd, 1H,CH=C$\underline{H}_{cis}$ *J*=1.6 and 10.2 Hz), 4.02 (d, 2H, C$\underline{H}_2$-NH), 1.48 (s, 9H, $C(CH_3)_3$). MS (m/z, M+Na^+, ESI-TOF-MS): calculated for $C_9H_{15}NO_3$ 208.0944, found 208.0939.

CH_3O-PEG_{227}-pyrrole carbodithioate (**2**) was prepared as reported[1].

CH_3O-PEG_{227}-Gly(OtBu)$_{25}$-pyrrole (**3**). 186 mg (1.0 mmol) of (**1**), 407 mg (0.040 mmol) of (**2**) and 0.66 mg (0.0040 mmol) of AIBN were dissolved in 1,4-dioxane with a final volume of 1 mL. The mixture was freed from oxygen by five freeze/thaw cycles with argon. The mixture was heated at 70°C for 24 h before being quenched by cooling in liquid nitrogen and exposing to air. After warming to room temperature the clear yellow solution was diluted with 2 mL CH_2Cl_2 and added to 200 mL Et_2O. The suspension was filtered, the residue washed with Et_2O (2 x 50 mL) and dried under reduced pressure to yield 562 mg (95%) of a yellow powder. ^{1}H NMR ($CDCl_3$): δ = 8.1-7.4 (bs, NH + 2 x $CH_{pyrrole}$), 6.27 (s, 2 x $CH_{pyrrole}$), 4.7-3.9 (bs, CH_2 glycine), 3.9-3.0 (bs, CH_2CH_2O PEG), 3.38 (s, OCH_3 PEG), 2.6-1.9 (bs, CH backbone), 1.9 -1.0 (bs, $C(CH_3)_3$, + CH_2 backbone). The number of Gly(OtBu) units per PEG chain was calculated from the integral value of the signals of $C(CH_3)_3$ and CH_2 backbone

and the CH_2CH_2O protons and was about 25 units. GPC data for this polymer are reported in Figure S1.

CH_3O-PEG$_{227}$-Gly(OtBu)$_{25}$-H (**4**). 306 mg (0.021 mmol) of (**3**), 188 mg (1.05 mmol, 50 eq) of 1-ethylpiperidine hypophosphite and 1.7 mg (0.010 mmol, 0.05 eq) of AIBN were dissolved in 850 μL dioxane and freed from oxygen by five freeze/thaw cycles with argon. The mixture was heated at 95°C during which the yellow solution became colorless[2]. After freezing in liquid nitrogen and exposing to air the solution was diluted with 50 mL CH_2Cl_2 and washed with water (2 x 50 mL). The CH_2Cl_2 solution was dried over Na_2SO_4, concentrated to a small volume and added to 200 mL Et_2O. The suspension was filtered, the residue washed with Et_2O (2 x 50 mL) and dried under reduced pressure to yield 299 mg (100%) of a white powder. ^{1}H NMR ($CDCl_3$): δ = 8.1-7.4 (bs, NH), 4.7-3.9 (bs, CH_2C=O glycine), 3.9-3.0 (bs, CH_2CH_2O PEG), 3.38 (s, OCH_3 PEG), 2.6-1.9 (bs, CH backbone), 1.9-1.0 (bs, $C(CH_3)_3$ + CH_2 backbone). Signals due to pyrrole group had disappeared. GPC data are reported in Figure S1 and Table S1.

CH_3O-PEG$_{227}$-Gly(OH)$_{25}$-H (**5**). 161 mg (0.011 mmol) of (**4**) was dissolved in 2 mL TFA/H_2O 9:1 and stirred for 21 h and concentrated in a flow of N_2 and dried under high vacuum. The foamy solid was dissolved in 8 mL milliQ water and lyophilized to yield 146 mg (100%) of a powder.

CH_3O-PEG$_{227}$-Gly(NHS)$_{25}$-H (**6**). 75 mg (0.14 mmol COOH groups) of (**5**) and 23 mg (0.20 mmol, 1.4 eq) of NHS were dissolved in 3 mL DMF. To the solution was added 1.7 mg (0.014 mmol, 0.1 eq) of DMAP in 159 μL DMF and 35 mg (0.17 mmol, 1.2 eq) of DCC in 686 μL DMF. The solution was stirred for 21 h during which a white precipitate formed. The mixture was filtered over a plug of glass wool and added to 200 mL Et_2O. The white solid that precipitated was filtered off and dried under reduced pressure to yield 90 mg (100%). ^{1}H NMR (d_6-DMSO): δ = 8.5-8.0 (bs, NH), 4.5-3.8 (bs, CH_2 glycine), 3.7-3.4 (bs, CH_2CH_2O, PEG), 3.38 (s, OCH_3), 2.9-2.7 (bs, 2 x CH_2 NHS), 2.3-1.8 (bs, CH backbone), 1.8-1.0 (bs, CH_2 backbone).

(5-(4-(2-aminoethoxy)phenyl)-3H-1,2-dithiole-3-thione) TFA salt (**7**) was prepared as reported[2].

PEG-PADT (**8**). 25 mg (0.039 mmol NHS groups) of (**6**) was dissolved in 2 mL DMF and to the clear solution was added 15.7 mg (0.041 mmol, 1.1 eq) of (**7**) in 500 μL DMF and 5.7 μL (0.041 mmol, 1.1 eq) triethylamine in 100 μL DMF. After 23 h the mixture was loaded onto a Sephadex LH20 size exclusion and eluted with DMF. Fractions were analyzed by TLC (SiO_2, MeOH) and those fractions containing polymer (R_f=0) were combined. After concentrating to about 3 mL the polymer was precipitated by adding 50 mL Et_2O. The solid was filtered and dried under reduced pressure to yield 23 mg (74%) of an orange solid. ^{1}H NMR (d_6-DMSO):

δ = 8.5-7.5 (bs, NH), 7.9-7.3 (bs, 2 x CH_{aromat} ADT + CH=C ADT), 7.2-6.7 (bs, 2 x CH_{aromat} ADT), 4.4-3.7 (bs, CH_2 glycine + $CH_2C\underline{H}_2O$-ADT), 3.7-3.4 (bs, CH_2CH_2O, PEG + $C\underline{H}_2CH_2O$-ADT), 3.38 (s, OCH_3), 2.3-1.8 (bs, CH backbone), 1.8-1.0 (bs, CH_2 backbone). The degree of functionalization was 75% by comparison of the integral values of the ADT and PEG signals. GPC data are reported in Figure S1.

Synthesis of Coumarin-PEG-PADT block copolymer block copolymer (Scheme S3).

HO-PEG$_{227}$-pyrrole RAFT (**9**) was prepared as reported[2].

HO-PEG$_{227}$-Gly(OtBu)$_{25}$-pyrrole (**10**). 299 mg (0.03 mmol) of (**9**), 139 mg (0.75 mmol) of (**1**) and 0.49 mg (0.003 mmol) of AIBN were reacted and worked-up as described for (**3**). Yield 392 mg (90%). NMR was the same as for (**3**) without showing the methoxy signal. The number of Gly(OtBu) units per PEG chain was about 25 units. GPC data are reported in Figure S2.

HO-PEG$_{227}$-Gly(OtBu)$_{25}$-H (**11**). 303 mg (0.021 mmol) of (**10**), 186 mg (1.04 mmol) of 1-ethylpiperidine hypophosphite and 1.7 mg (0.010 mmol) of AIBN was reacted in the same way as described for (**4**). Yield 254 mg (84%). NMR was the same as for (**4**) except for the absence of the methoxy signal. GPC charts are reported in Figure S3 and Table S1.

Coumarin-PEG$_{227}$-Gly(OtBu)-H (**12**). 104 mg (0.007 mmol) of (**11**) was dissolved in 160 mL toluene and dried by azeotropic distillation using a Dean-Stark trap. After 60 mL toluene/water had been collected the solution was cooled down to room temperature. To the clear solution was added 7 mg (0.023 mmol, 3.4 eq) of 7-diethylaminocoumarin-3-carbonyl azide and the solution while protected from light heated at 80°C for 21 h. After cooling to room temperature the solution was concentrated under reduced pressure and the residue purified by size exclusion chromatography using Sephadex LH-20 and DMF as the eluent. Fractions were analyzed by TLC (SiO_2, MeOH) and fractions containing no free dye were pooled and concentrated under reduced pressure to yield 98 mg (93%) of a yellow powder. GPC charts are reported in Figures S3 and S4 and show successful conjugation of the fluorescent dye.

Coumarine-PEG$_{227}$-Gly(OH)-H (**13**). This polymer was deprotected as described for (**5**) by dissolving 58 mg (**12**) in 1 mL TFA/H_2O 9:1 and reacting for 24 h. Yield 52 mg (99%).

Coumarin-PEG$_{227}$-Gly(NHS)$_{25}$-H (**14**). To a solution of 42 mg (0.08 mmol COOH groups) of (**13**) and 13 mg (0.11 mmol, 1. 5 eq) of NHS in 2.5 mL DMF was added a solution of 24 mg (0.15 mmol, 1.4 eq) of DCC in 485 μL DMF and 0.98 mg (0.008 mmol, 0.1 eq) of DMAP in 89 uL DMF. Reaction and work-up was done in the same way as that described for (**6**) to yield 29 mg (58%) of white solid.

Coumarin-PEG-PADT (**15**). 11 mg (0.018 mmol NHS groups) of (**14**) and 8 mg (0.020 mmol,

1.1 eq) of (**7**) in 2 mL DMF was reacted with 2.7 μL (0.020 mmol, 1.1 eq) of Et_3N in 100 μL DMF in the same way as that described for (**8**) yield 9.5 mg (69%). NMR was the same as for (**8**) except for absence of the methoxy signal. The degree of functionalization was 77%. GPC charts are reported in Figure S3.

Micelle formation. A DMF solution of PEG-PADT or coumarin-PEG-PADT (50 mg/mL) was added drop wise to endotoxin-free water (1:9 v/v DMF/H_2O) under vigorous stirring at RT. After stirring for 30 min, the solution was transferred to a Slide-A-Lyzer G2 (MWCO 2 kDa) and dialyzed against milliQ water. Fluorescence intensity data as shown in Figure S5 show the presence of coumarin on the surface of the micelles after dialysis.

Cell experiments

Cell culture. RAW Blue cells were cultured in DMEM GlutaMAX supplemented with 10% heat-inactivated FBS, 50 U/mL-50 µg/mL penicillin-streptomycin and 200 mg/mL Zeocin in CO_2 incubator at 37^{o}C. Cells were passaged when reaching 70-80% confluency.

Preparation of cell lysate. RAW Blue cells at 80% confluency were scraped off and centrifuged at 500 rpm for 5 min. The cell pellet was washed with cold Dulbecco's Phosphate buffered saline (PBS) three times. The cell suspension was centrifuged at 500 rpm for 5 min, resuspended in Passive Lysis Buffer at 1×10^7 cells/mL and vortexed at RT for 10 min. The suspension was centrifuged and the clear supernatant was collected and stored at -20^{o}C.

Measurement of H_2S release by H_2S electrode sensor. Degassed PBS containing 10vol% FBS or 20vol% cell lysate (1 mL) was placed in a four-port closed chamber and incubated at 37°C. After the sensor signal became stable, 50 µL PEG-ADT or ADT micelle solutions was added (final concentration of ADT units: 25 µM) using a Hamilton syringe. Released H_2S was recorded on a four-channel free radical analyzer.

Measurement of H_2S concentration in cell culture medium by fluorescent dye. RAW Blue cells were seeded in a 96-well plate (5×10^4 cells/well) and cultured for 1 d. The medium was replaced with 100 µL/well of fresh medium and 1 µL/well of ADT in DMSO, 5 µL/well of PEG-ADT or ADT micelles in water were added (final concentration of ADT: 100 µM). At different time points, 5 µL of medium was withdrawn and diluted with 45 µL PBS. This sample solution was immediately mixed with 50 µL WSP-1/DMSO (100 µM). A serial dilution of Na_2S in PBS was used as a standard. Fluorescence intensity (λ_{ex}=465 nm, λ_{em}=515 nm) was measured on a Tecan well plate reader.

Cell viability assay. RAW Blue cells were seeded in a 96-well plate (5×10^4 cells/well) and cultured for 1 d. The medium was replaced with 100 µL/well of fresh medium and 1 µL/well of ADT in DMSO, 5 µL/well of PEG-ADT, ADT micelles and Na_2S in LPS-free water with different concentrations were added. Cells were cultured for 1 d in a CO_2 incubator at 37^{o}C. Thereafter, the medium was replaced with 100 µL/well fresh medium and 10 µL of MTT solution (5 mg/mL in PBS) was added to each well and then incubated for 2 h at 37^{o}C. Then 100 µL of 0.1 g/mL sodium dodecyl sulfate in 0.01M HCl (aq) was added to each well to lyse cells and solubilize formazan crystals, and the OD at 570 nm was measured.

Proinflammatory effects of H_2S donors. RAW Blue cells were seeded in a 96-well plate (5×10^4 cells/well) and cultured for 1 d. The medium was replaced with 100 µL/well of fresh medium and 1 µL/well of ADT in DMSO, 5 µL/well of PEG-ADT, ADT micelles and Na_2S in LPS-free water were added (final concentration of ADT: 50 µM). Cells were cultured for 3 h and thereafter 10 µL of 10 µg/mL gardiquimod in culture medium was added to each well. After 2 h of culture, 20 µL of medium was collected and TNF-α concentration was determined by ELISA. After 24 h of culture, the SEAP level in the medium was determined by Quanti-Blue assay. Statistical analysis was performed using the Student t-test.

Observation of intracellular distribution of the micelles. RAW Blue cells were seeded in a triple-wells glass-based dish (5×10^4 cells/well) and cultured for 1 d. The medium was replaced with 200 µL/well of fresh medium containing 1 mg/mL rhodamine-labeled dextran (10kDa) and 20 µL of coumarin-ADT micelles in water (5 mg/mL) were added. After 4 h of culture, cells were washed with PBS and 100 µL/well of fresh medium was added. Cells were observed with an Olympus FluoView FV1000-D confocal microscope.

References

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2. Hasegawa, U.; van der Vlies, A. J., *Bioconjugate Chem.,* **2014**, DOI: 10.1021/bc500150s.