Therapeutic Delivery of H₂S via COS: Small Molecule and Polymeric Donors with Benign Byproducts

Chadwick R. Powell, Jeffrey C. Foster, Benjamin Okyere, Michelle H. Theus, and John B. Matson

Department of Chemistry and Macromolecules Innovation Institute, Virginia Tech, Blacksburg, Virginia 24061, United States

Department of Biomedical Sciences and Pathobiology, Virginia–Maryland Regional College of Veterinary Medicine, Duck Pond Drive, Blacksburg, VA 24061, United States
Materials and Methods:

All reagents were obtained from commercial vendors and used as received unless otherwise stated. NMR spectra were measured on Agilent 400 MHz or Bruker 500 MHz spectrometers. $^1$H and $^{13}$C NMR chemical shifts are reported in ppm relative to internal solvent resonances. Yields refer to chromatographically and spectroscopically pure compounds unless otherwise stated. GC-MS experiments were performed on a Hewlett Packard 5890 Series II GC and a Hewlett Packard 5972 Series Mass Selective Detector, equipped with a Restek RTX 1 DB-5 capillary column (30 m, 1.25 mm diameter, 0.25 μm film thickness), 250 °C injection temperature, 250 °C transfer line, 1 mL/minute flow rate. Analytical LCMS was performed on an Agilent 1200 system with a Waters SQ Detector 2 quadrupole-time-of-flight mass spectrometer using an XBridge C18 column (5 μm particle size, 6 x 100 mm) eluting with a gradient of 0% ACN to 15% ACN in water, with each solvent containing 0.1% formic acid. UV absorbance was monitored at 220 nm with a Waters 2489 UV/Vis detector. UV-Vis absorbance spectra were recorded on a Spectramax M2 plate reader (Molecular Devices). Fluorescence spectra were recorded on a Cary Eclipse fluorescence spectrophotometer equipped with a PMT detector (600 V), excitation and emission slit widths of 5 nm. Size exclusion chromatography (SEC) was carried out in THF at 1 mL min$^{-1}$ at 30 °C on two Agilent PLgel 10 μm MIXED-B columns connected in series with a Wyatt Dawn Heleos 2 light scattering detector and a Wyatt Optilab Rex refractive index detector. No calibration standards were used, and dn/dc values were obtained by assuming 100% mass elution from the columns. High-resolution mass spectra were taken on an Agilent Technologies 6230 TOF LC/MS mass spectrometer.

Scheme S1. Synthesis of NTA1

2-[(Ethoxycarbonothioyl)thio]acetic acid (1)

2-[(Ethoxycarbonothioyl)thio]acetic acid was synthesized according to literature procedures.$^{1,2}$ Briefly, a 2-necked roundbottom flask was equipped with an N$_2$ inlet and a septum and then charged with ethanol (17 mL), NaOH (2.19 g, 54.7 mmol), water (42 mL), and a stir bar. Once
the NaOH was completely dissolved, the solution was bubbled with N\textsubscript{2} for 15 min and the flask was cooled to 0 °C on an ice bath. Under N\textsubscript{2} flow, CS\textsubscript{2} (4.00 mL, 65.6 mmol) was added dropwise via syringe with vigorous stirring at 0 °C. After complete addition, the ice bath was removed and the reaction mixture was stirred for 2 h under N\textsubscript{2} and allowed to return to rt. In a separate flask, bromoacetic acid (7.60 g, 54.7 mmol) was dissolved in deionized water (20 mL) and added to the pale yellow reaction mixture via addition funnel. Immediately the reaction mixture turned dark orange. The reaction mixture was allowed to stir for 14 h at rt. The reaction mixture was acidified by slow addition of concentrated HCl (5 mL) and extracted with CH\textsubscript{2}Cl\textsubscript{2} (3 x 50 mL). The organic layers were combined, washed once with brine (10 mL), dried over Na\textsubscript{2}SO\textsubscript{4}, and concentrated under vacuum. The resulting yellow solid was recrystallized from CH\textsubscript{2}Cl\textsubscript{2}/hexanes to afford the product as yellow crystals (7.41 g, 41.1 mmol, 75 % yield). \textsuperscript{1}H NMR (CDCl\textsubscript{3}): δ 4.66 (q, 2H), 3.98 (s, 2H), 1.43 (t, 3H). \textsuperscript{13}C NMR (CDCl\textsubscript{3}): δ 173.12, 71.10, 37.63, 13.84.

**Figure S1.** \textsuperscript{1}H NMR of xanthate 1.
Figure S2. $^{13}$C NMR of xanthate 1.

$N$-(Ethoxycarbonothioyl)-$N$-methylglycine (2)

$N$-(Ethoxycarbonothioyl)-$N$-methylglycine was synthesized according to literature procedures.$^1$ A roundbottom flask was charged with xanthate 1 (6.88 g, 38.1 mmol), sarcosine (3.40 g, 38.1 mmol), NaOH (3.05 g, 76.0 mmol), water (15 mL), and a stir bar to give a clear yellow solution. The reaction mixture was stirred at rt for 48 h and monitored by TLC eluting with 10 % MeOH in CH$_2$Cl$_2$ (UV visualization). Once the starting material had been completely consumed, the reaction mixture was acidified by dropwise addition of concentrated HCl, which immediately led to the formation of a white precipitate. HCl addition was continued until formation of the precipitate subsided (~ 10 mL HCl). The mixture was extracted with CH$_2$Cl$_2$ (3 x 30 mL). The organic layers were then combined and washed with 1 N HCl (2 x 15 mL) and brine (1 x 10 mL). The clear solution was then dried over Na$_2$SO$_4$ and concentrated under vacuum to afford the product as a white solid (6.62 g, 37.4 mmol, 98 % yield). The thiocarbamate product was used directly in the next step without further purification.
An oven-dried, 2-necked roundbottom flask was equipped with an N\textsubscript{2} inlet, a septum, and a stirbar and then charged with thiocarbamate \textbf{2} (5.2 g, 29.3 mmol) and dry CH\textsubscript{2}Cl\textsubscript{2} (37 mL) under N\textsubscript{2} flow. PBr\textsubscript{3} (2.80 mL, 29.3 mmol) was added dropwise via syringe at 0 °C to give a pale yellow solution. The reaction mixture was allowed to stir at 0 °C for 15 min before removing the ice bath and allowing the reaction mixture to warm to rt as it stirred for an additional 1 h. The reaction mixture was transferred to a separatory funnel and washed carefully with saturated sodium bicarbonate solution (3 x 15 mL) followed by brine (1 x 15 mL). The organic solution was then dried over Na\textsubscript{2}SO\textsubscript{4}, and concentrated under vacuum to obtain the crude product as a yellow oil. The crude product was purified on a silica column, eluting with 1:1 hexanes/EtOAc to give the pure product as a light yellow oil (2.04 g, 15.6 mmol, 53 % yield). \textsuperscript{1}H NMR (CDCl\textsubscript{3}): δ 4.18 (s, 2H), 3.08 (s, 3H). \textsuperscript{13}C NMR (CDCl\textsubscript{3}): δ 193.88, 164.98, 110.08, 61.70, 30.91; HRMS (ESI-TOF) calcd. for C\textsubscript{4}H\textsubscript{6}NO\textsubscript{2}S\textsuperscript{+} [M+H]\textsuperscript{+} 132.0114, found 132.0114.
Figure S4. $^{13}$C NMR of NTA1.

Scheme S2. NTA ROMP monomer Synthesis
A roundbottom flask was equipped with a stir bar and charged with 1 (2.20 g, 12.2 mmol), iminodiacetic acid (1.62 g, 12.20 mmol), NaOH (1.46 g, 36.6 mmol), and water (55 mL) to give a turbid solution. The slurry was stirred at rt for 14 h, at which point the reaction mixture had become a clear yellow solution. Reaction progress was monitored by TLC, eluting with 10 % MeOH in CH$_2$Cl$_2$. Once the reaction was complete, the reaction mixture was then acidified by dropwise addition of concentrated HCl (10 mL). The clear yellow solution was extracted with EtOAc (3 x 30 mL), and the organic layers were combined and washed with 1 N HCl (2 x 15 mL) and brine (1 x 10 mL). The organic solution was then dried over Na$_2$SO$_4$ and concentrated under vacuum to give the crude product as a white solid. The product was purified by recrystallization from hexanes/EtOAc to afford the product as a white solid (1.40 g, 6.33 mmol, 52 % yield). $^1$H NMR (DMSO-$d_6$): δ 12.90 (s, 2H), 4.48 (s, 2H), 4.38 (q, 2H), 4.22 (s, 2H), 1.22 (t, 3H). $^{13}$C NMR (DMSO-$d_6$): δ 189.08, 169.69, 169.58, 67.52, 54.77, 50.78, 13.92. HRMS (ESI-TOF) calcd. for C$_7$H$_{10}$NO$_5$S [M-H]$^-$ 220.0273, found 220.0285.

Figure S5. $^1$H NMR of thiocarbmate diacetic acid 3.
Exo-norbornene alcohol was synthesized according to literature procedures. A roundbottom flask was equipped with a stirbar and charged with dicarboxylic acid 3 (2.00 g, 9.04 mmol), pyridine (750 μL, 9.04 mmol) and CH₂Cl₂ (75 mL) giving a light yellow, slightly turbid solution. The flask was placed in an ice bath and a solution of EDC (1.73 g, 9.04 mmol) and exo-norbornene alcohol (9.37 g, 45.2 mmol) in CH₂Cl₂ (25 mL) was added dropwise via addition funnel. The reaction mixture was allowed to stir at rt for 14 h. Reaction progress was monitored by TLC, eluting with 5 % MeOH in CH₂Cl₂. The resulting clear yellow solution was washed with 1 N HCl (2 x 10 mL) and brine (1 x 10 mL). The organic solution was then dried over Na₂SO₄ and concentrated in vacuo to yield the crude product as a yellow oil. The product was purified on a silica column, eluting with 1-10 % MeOH in CH₂Cl₂ to give the pure product as a light yellow oil (1.93 g, 4.70 mmol, 52 % yield). ¹H NMR (CDCl₃): δ 6.29 (t, 2H), 4.64 (d, 2H), 4.49 (quin, 2H), 4.24-4.37 (m, 4H), 3.78 (quin, 2H), 3.27 (s, 2H), 2.72 (s, 2H), 1.22-1.56 (m, 6H). ¹³C NMR (CDCl₃): δ 190.44, 190.33, 178.38, 170.01, 168.47, 137.93, 69.10, 68.86, 62.66,
62.34, 55.16, 51.42, 50.58, 48.03, 45.38, 42.90, 42.85, 37.66, 37.53, 14.18, 14.10. HRMS (ESI-TOF) calcd. for C\textsubscript{18}H\textsubscript{21}N\textsubscript{2}O\textsubscript{7}S\textsuperscript{-}[M-H]\textsuperscript{-} 409.1036, found 409.1075.

**Figure S7.** H NMR of norbornene thiocarbamate acetic acid 4.
Figure S8. $^{13}$C NMR of norbornene thiocarbamate acetic acid 4.

Norbornene-NTA ROMP monomer NB-NTA

A flame-dried, two-necked round-bottom flask was equipped with an N$_2$ inlet, a septum, and a stir bar. The flask was charged with 4 (0.302 g, 0.736 mmol) and dry CH$_2$Cl$_2$ (4 mL) under N$_2$ flow. The clear and colorless reaction mixture was then cooled to 0°C on an ice bath. Once cool, PBr$_3$ (0.09 mL, 0.96 mmol) was added dropwise to the reaction mixture via syringe. The reaction mixture was allowed to stir for 10 minutes at 0°C. The ice bath was then removed, and the reaction mixture was allowed to warm to rt as it stirred for an additional 30 minutes. The reaction mixture was then transferred to a separatory funnel where it was washed with saturated bicarbonate solution added slowly (2 x, 2 mL) and brine (1 x, 2 mL). The organic solution was then dried over Na$_2$SO$_4$ and concentrated in vacuo to yield the crude product as a yellow oil. The crude product was purified on a silica column, eluting with CH$_2$Cl$_2$ to give the pure product as a light yellow oil (0.135 g, 0.371 mmol, 50% yield). $^1$H NMR (CDCl$_3$): $\delta$ 6.29 (t, 2H), 4.36 (s, 2H), 4.32 (t, 2H), 4.22 (s, 2H), 3.77 (t, 2H), 3.27 (m, 2H), 2.70 (s, 2H), 1.53 (m, 1H), 1.24 (m,
$^1$H) $^{13}$C NMR (CDCl$_3$): $\delta$ 193.25, 178.09, 167.34, 166.29, 137.91, 62.83, 60.19, 48.01, 45.38, 44.48, 42.85. HRMS (ESI-TOF) calcd. for C$_{16}$H$_{16}$N$_2$O$_6$Na$^+$ [M+Na]$^+$ 387.0632, found 387.0621.

Figure S9. $^1$H NMR of NB-NTA.

Figure S10. $^{13}$C NMR of NB-NTA.
Exo-norbornene carboxylic acid was prepared by literature procedure. A roundbottom flask was equipped with a stirbar and charged with exo-norbornene carboxylic acid (2.021 g, 9.14 mmol), poly(ethylene glycol) monomethylether (Mₙ = 550 Da) (1.675 g, 3.05 mmol), and DMAP (0.112 g, 0.914 mmol). Dry CH₂Cl₂ (30 mL) was added, giving a clear yellow, slightly turbid solution. EDC (1.290 g, 9.14 mmol) was added in one portion, and the reaction mixture was allowed to stir at rt for 14 h. Reaction progress was monitored by TLC, eluting with 5 % MeOH in CH₂Cl₂. Once complete, the reaction mixture was transferred to a separatory funnel and washed with 1 N HCl (2 x, 15 mL) and brine (1 x, 15 mL). The organic layer was dried over Na₂SO₄ and concentrated under vacuum to yield the crude product as a light pink oil. The crude product was purified on a silica column, eluting with 5 % MeOH in CH₂Cl₂ to afford a clear, colorless oil (1.972 g, 2.56 mmol, 84 % yield).

Figure S11. ¹H NMR of NB-PEG monomer.
**Synthesis of polyNTA1 by ROMP**

A 20 mL scintillation vial equipped with a screw-cap lid was charged with **NB-NTA** (10.5 mg, 29.0 µmol), **NB-PEG** (200 mg, 260 mmol), and dry CH₂Cl₂ (3.5 mL). **G3** catalyst, prepared according to a literature procedure, was dissolved in dry CH₂Cl₂ in a second vial at a concentration of 8.40 mg/mL. The G3 solution (0.5 mL) was added rapidly by syringe to the monomer solution with vigorous stirring. The reaction mixture was stirred at rt for 1 h. A few drops of ethyl vinyl ether were then added to quench the polymerization, and then the reaction mixture was stirred for an additional 20 min. The polymer was isolated via precipitation from hexanes.

**Figure S12.** ¹H NMR of polyNTA1.
Figure S13. SEC analysis (RI trace) of polyNTA1.

Scheme S3. Proposed scheme for COS release from a generalized NTA structure

![Scheme S3](image)

GC-MS detection of COS:

A 1.5 mL vial was charged with 0.2 mL of a solution of NTA1 (5 mM in DMSO), 0.8 mL glycine solution (12.5 mM in PBS buffer) and sealed with a screw cap lid with a rubber septum. The reaction mixture was allowed to sit at rt for 5 min, at which point an air-tight syringe was used to sample 100 μL of the vial headspace. This volume was injected into the GC-MS immediately. A control vial containing 0.8 mL glycine (12.5 mM in PBS buffer) and 0.2 mL DMSO was sampled using the same procedure at the 5-minute mark.
Figure S14. GC-MS mass chromatogram of NTA1 reaction with glycine after 5 min.

Figure S15. SIM mode monitoring of NTA1 reaction with glycine after 5 min. A) H$_2$S abundance (m/z = 34.00) and B) COS abundance (m/z = 60.00).
Analysis of byproducts by LCMS

A one-dram vial was charged with **NTA1** (50.2 mg, 0.383 mmol) followed by addition of 1 mL glycine solution (0.46 M in 1X PBS) to give a colorless solution. After 8 h, 20 μL of the reaction mixture was injected onto the HPLC eluting 0 % ACN to 15 % ACN in water (0.1 % formic acid).

![Figure S16. LCMS UV absorbance (λ = 220 nm) chromatogram of NTA1 (0.38 M in phosphate buffered water) in the presence of glycine (0.42 M) after 8 h incubation.](image)

Methylene blue H$_2$S release assay:

Each assay described was run in triplicate in a one-dram vial containing 1.856 mL PBS buffer (pH = 7.4), 100 μL Zn(OAc)$_2$ solution (40 mM in H$_2$O), 20 μL **NTA1** or **poly(NTA1)** solution (10 mM in NTA functional group in DMSO), 20 μL CA solution (30 μM in PBS buffer), 4 μL glycine solution (500 mM in PBS buffer). Final concentrations were 100 μM **NTA1**, 2 mM Zn(OAc)$_2$, 1 mM glycine, and 300 nM CA. A control solution was run for each experiment containing all of the above components with the exception of **NTA1**. At predetermined timepoints, 100 μL was removed from each reaction vial and diluted with 100 μL FeCl$_3$ solution (30 mM in 1.2 M HCl) followed by 100 μL *N,N*-dimethyl-*p*-phenylenediamine solution (20 mM in 7.2 M HCl). Aliquots were stored until 90 minutes after the final aliquot had been taken to allow completion of methylene blue formation. The aliquots were transferred to a 96 well plate (250 μL/well) and their absorbance spectra were collected from 500 to 800 nm on a plate reader. Kinetic analysis was done by subtracting the absorbance of the control experiment (no methylene blue formation) from the absorbance of each aliquot at that specific timepoint at 676 nm.
**Figure S17.** Representative absorbance spectra for the methylene blue spectrophotometric H$_2$S release assay with absorbance increasing over time. Inset shows the pseudo-first order kinetics plot derived from absorbances at 676 nm.

**H$_2$S selective electrochemical probe calibration:**

A scintillation vial was charged with 1X PBS buffer (pH 7.4) (10 mL) and a small magnetic stir bar. The buffer solution was set to stir and the electrochemical probe was positioned in the vial. Once the probe current had equilibrated, a stock solution of NaSH (2 mM in H$_2$O) was added in 5 μL aliquots. Upon each addition of the NaSH stock solution to the vial, there was a spike in current which quickly stabilized. Upon current stabilization another addition was made until probe response no longer appeared linear. The calibration curve was constructed by plotting the apparent concentration of H$_2$S in solution against the stabilized probe current.
**Figure S18.** Calibration curve for H$_2$S release in 1X PBS buffer.

**H$_2$S selective electrochemical probe release studies:**

A scintillation vial was charged with 9.73 mL of 1X PBS buffer (pH 7.4), 4 μL of a glycine solution (0.5 M in H$_2$O), 20 μL of a solution of NTA1 (5 mM in DMSO), 250 μL of a solution of CA (6 μM in 1X PBS), and a small magnetic stir bar. Final concentrations were 10 μM NTA1, 100 μM glycine, and 300 nM CA. Once all of the reagents had been added, the probe was immediately inserted into the solution, and the probe current was recorded. The current initially spiked due to immersion in the solution, but stabilized quickly. This initial current spike is omitted from the reported data. Peaking time of H$_2$S in solution is measured at the point where the current readout is at a global maximum for the dataset.

**Figure S19.** H$_2$S selective electrochemical probe release data for NTA1 (10 μM) in complete endothelial cell media (10 mL). The faster peaking time is a result of a higher concentration of
free amines available in complete cell media. The rapid return to baseline is likely a result of sulfhydration of the various proteins in the cell media by the H₂S released leading to a lower instantaneous concentration of sulfide.

**Fluorescence H₂S release assay kinetics:**

Fluorescence assays were prepared in a 3 mL quartz cuvette with a threaded lid containing 1.80 mL 1X PBS buffer (pH 7.4), 0.1 mL glycine solution (30 mM in PBS buffer), 0.1 mL diethylenetriaminepentaacetic acid (DTPA) solution (2.5 mM in H₂O), 0.2 mL dansyl azide probe solution (3.76 mM in DMSO), and 0.020 mL CA solution (0.0375 mM in PBS). The fluorescence spectrum of this mixture was collected from 400 to 600 nm (λex = 340 nm) as the t = 0 timepoint. To this solution was added 0.3 mL of NTA1 solution (0.823 mM in DMSO). The cuvette was capped, and Parafilm was wrapped around the cap to limit any potential volatilization of COS/H₂S. The cuvette was then placed in the fluorometer, and fluorescence spectra were collected every 10 min from 400 to 600 nm. Kinetic analysis was done by subtracting the zero-time point fluorescence intensity from the fluorescence intensity at each time point at 540 nm. The pseudo-first-order half-life of H₂S release was determined by plotting time vs. ln(1/(1-% released), with t₁/₂ = ln(2)/slope.

**Brain-derived endothelial cell assays:**

Primary brain-derived endothelial cells (ECs) were plated in a 96-well plate containing serum-free media, pre-coated with 0.2% gelatin, at 20,000 cells/well. After 24 h incubation with the indicated treatments, cells were assessed for proliferation by adding 10 µM bromodeoxyuridine (BrdU; Sigma Aldrich, St. Louis, MO). Following 1 h incubation with BrdU, cells were fixed and stained with anti-BrdU (1:1000; ThermoFisher Scientific, Inc., Waltham, MA). ECs were counterstained with DAPI (1 mg/ml) and analyzed under TRITC/DAPI filters on an inverted IX-71 Olympus epi-fluorescence microscope equipped with a digital XM-10 camera and Cell Sense software package (Olympus, Valley, PA). For quantification of BrdU-positive ECs, four images per well were acquired and quantified as a ratio of BrdU/Dapi or proliferating cell fraction. For Caspase 3/7 assessment after 24 h incubation, cells were subjected to the Caspase-Glo 3/7 assay per manufactures instructions (Promega, Madison, WI). Data were graphed using GraphPad Prism, version 4 (GraphPad Software, Inc., San Diego, CA). Student’s two-tailed t-test was used for comparison of two experimental groups. For three or more groups, multiple comparisons were done using one-way ANOVA followed by Tukey test for multiple pairwise examinations. Changes were identified as significant if p was less than 0.05. Mean values were reported together with the standard error of mean (SEM).
Mouse brain endothelial cell caspase assay:

![Caspase assay graph](image)

**Figure S20.** Caspase assay of brain-derived endothelial cells treated with 5-100 µM NTA1.

Mouse brain endothelial cell BrdU assay of PolyNTA1:

![BrdU assay graph](image)

**Figure S21.** Endothelial cell proliferation data showing the ratio of proliferating cells in each treatment group. Cells were treated for 24 h in serum-free media, and quantification was performed by counting the number of BrdU+/Dapi+ cells ($n = 6–8$ for each treatment group). Error bars represent standard error of the mean. There was no statistically significant difference between these groups.

References:

1. Tao, X.; Deng, Y.; Shen, Z.; Ling, J. *Macromolecules* **2014**, *47*, 6173.
2. Auty, S. E. R.; Andren, O.; Malkoch, M.; Rannard, S. P. *Chem. Commun.* **2014**, *50*, 6574.
3. Qiao, Y.; Ping, J.; Tian, H.; Zhang, Q.; Zhou, S.; Shen, Z.; Zheng, S.; Fan, X. *J. Polym. Sci., Part A: Polym. Chem.* **2015**, *53*, 2116.
4. Conrad, R. M.; Grubbs, R. H. *Angew. Chem. Int. Ed.* **2009**, *48*, 8328.
5. Love, J. A.; Morgan, J. P.; Trnka, T. M.; Grubbs, R. H. *Angew. Chem. Int. Ed.* **2002**, *41*, 4035.
