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Therapeutic Delivery of H_2S via COS: Small Molecule and Polymeric Donors with Benign Byproducts

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Supporting Information

ABSTRACT: Carbonyl sulfide (COS) is a gas that may play important roles in mammalian and bacterial biology, but its study is limited by a lack of suitable donor molecules. We report here the use of *N*-thiocarboxyanhydrides (NTAs) as COS donors that release the gas in a sustained manner under biologically relevant conditions with innocuous peptide byproducts. Carbonic anhydrase converts COS into H_2S, allowing NTAs to serve as either COS or H_2S donors, depending on the availability of the enzyme. Analysis of the pseudo-first-order H_2S release rate under biologically relevant conditions revealed a release half-life of 75 min for the small molecule NTA under investigation. A polynorbornene bearing pendant NTAs made by ring-opening metathesis polymerization was also synthesized to generate a polymeric COS/H_2S donor. A half-life of 280 min was measured for the polymeric donor. Endothelial cell proliferation studies revealed an enhanced rate of proliferation for cells treated with the NTA over untreated controls.

Since nitric oxide (NO) was first proposed to be endothelium-derived relaxing factor in 1986,[1] an increasing number of scientists have been drawn to the field of signaling gases. These short-lived gaseous mediators, classified as gasotransmitters, exist in most tissues throughout the body and play a critical role in cellular signaling and homeostasis.[2] To meet the definition of a gasotransmitter, a gas must be enzymatically generated and regulated, have specific molecular targets and physiological functions, and be freely permeable to cellular membranes.[3] Three species are currently classified as gasotransmitters: NO, carbon monoxide (CO), and hydrogen sulfide (H_2S). However, evidence suggests that a variety of other gases may be involved in cellular processes. Gases currently under investigation include ammonia, methane, sulfur dioxide, and carbonyl sulfide (COS).[4−6] Of these, COS is perhaps the least studied in biological systems, in large part due to a lack of suitable donor molecules.

COS is the most abundant sulfur compound in the atmosphere (500 ppt), generated naturally from emissions from hot springs, trees, and volcanoes.[7] Despite its low overall atmospheric concentration, high concentrations near volcanoes may have enabled COS to serve as a condensing agent in prebiotic times for coupling amino acids.[8,9] Investigation into the role of COS in biology continues today, with COS-generating enzymes (thiocyanate hydrolases) identified in bacteria and COS generation and utilization hypothesized in mammals.[10] The study of COS biology is complicated by its short half-life in vivo, primarily due to its fast hydrolysis by carbonic anhydrase (CA). CA is ubiquitous in plants and mammals and efficiently carries out its primary function of generating carbonic acid from water and carbon dioxide (CO_2). Because of its structural similarity to CO_2, COS is also a substrate for CA, which the enzyme rapidly converts into CO_2 and H_2S.[5] In fact, COS itself is relatively nontoxic: its toxicity is a result of this conversion into toxic levels of H_2S.[11]

Gasotransmitter donors have become vital tools for studying the biology of NO, CO, and H_2S. Donors are typically small molecules that release gasotransmitters under physiologically relevant conditions over a defined period of time.[3] Chemists strive to synthesize gasotransmitter donors that mimic the body's natural signaling through precise exogenous application. Today, many small molecule donors are available that release NO, CO, or H_2S, all triggered by various stimuli with wide-ranging half-lives of release. For example, a variety of H_2S donors have been reported in recent years, with release triggers including water,[12,13] thiols,[14−16] light,[17,18] and enzymes.[19] Gasotransmitter donors can be covalently attached to drugs,[20,21] which extends the capacities of existing therapeutics, or to polymers, providing localized release. Multiple types of NO, CO, and H_2S-releasing polymers have been reported recently.[22−24] In contrast, COS donors have received little attention, which has impeded studies on COS biology or delivery of H_2S via COS release.

To address this gap, we set out to design a molecule that could readily generate COS in a controlled fashion, easily attach to a polymer scaffold, and conveniently decompose to yield biologically innocuous byproducts. We envisioned that this COS-releasing small molecule and the resulting polymer could serve as either COS donors (in the absence of CA) or as H_2S donors (in the presence of CA) to facilitate biological studies. There exist a handful of methods to generate COS, including

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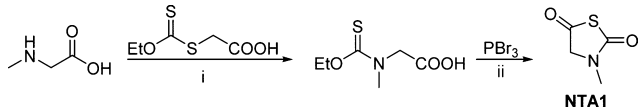
from potassium thiocyanate and sulfuric acid in the presence of water[7] and a recently published metal-catalyzed reaction of CO and elemental sulfur.[25] Although useful for generating solutions of the gas, these methods are not capable of generating COS in a biological environment. In the lone example of COS generation in a biological system, Pluth and co-workers recently reported on a class of turn-on fluorescent H_2S sensors that regenerate the detected H_2S through a decomposition reaction involving COS.[26] Considering our requirements for sustained release under biologically relevant conditions and nontoxic byproducts, we envisioned that *N*-thiocarboxyanhydrides (NTAs) might provide a suitable platform from which to build small molecule and polymeric COS donors.

NTAs were first reported in 1971 by Hirschmann et al. as an alternative to *N*-carboxyanhydrides (NCAs) for use in the solution-phase synthesis of oligopeptides.[27] More recently, NTAs have been synthesized from *N*-alkyl amino acids and polymerized to afford polypeptoids.[28] Because the ring-opening polymerization of NTAs occurs with loss of COS, we envisioned that NTAs could be suitable COS donors upon ring-opening by a biological nucleophile such as an amine. After COS release, conversion into the known gasotransmitter H_2S is expected to occur by one of two routes: (1) the rapid, enzymatic conversion by CA; or (2) hydrolysis, a relatively slower process.

To test our hypothesis that NTAs could be used as COS/H_2S donors, we began by synthesizing the NTA derivative of sarcosine (**NTA1**) through the route shown in Scheme 1. With

Scheme 1. Synthesis of NTA1[a]



[a]Conditions: (i) NaOH, H_2O, 98% yield; (ii) CH_2Cl_2, 0 °C, 53% yield.

modifications of literature procedures,[28] we were able to obtain **NTA1** in two steps with minimal purification. Although COS release from NTAs during ring-opening polymerization has been hypothesized, direct detection of COS generation from ring-opening of NTAs has not been reported. To identify COS evolution, **NTA1** was added to an aqueous solution of glycine in an airtight vial. GC−MS analysis of samples taken from the vial headspace after 5 min revealed the presence of COS (m/z = 60). A small peak at m/z = 34 was also observed, which is consistent with slow hydrolysis of COS into H_2S (Figures S14 and 15).[29] Confirmation of the expected dipeptide byproduct upon nucleophilic attack by glycine was realized by LC-MS. (Figure S16).

After confirming COS release, we next measured the H_2S release kinetics of **NTA1** both in the presence and absence of CA. Currently, there are no simple and reliable methods for measuring the rate COS release in solution. However, the fast conversion of COS into H_2S in the presence of CA allows for indirect quantification of COS release kinetics using methods commonly employed to detect and quantify H_2S. To evaluate the rate of H_2S evolution, a colorimetric assay known commonly as the methylene blue assay was chosen.[30] In the presence of 300 nM CA and 1 mM glycine (biologically relevant values) in PBS buffer (pH = 7.4), the pseudo-first-order release half-life of H_2S for **NTA1** was measured to be 75 min (Figure S17). Further studies of H_2S release using an H_2S-selective electrochemical probe gave a peaking time of 45 min (Figure 1A). Additional release studies performed in complete

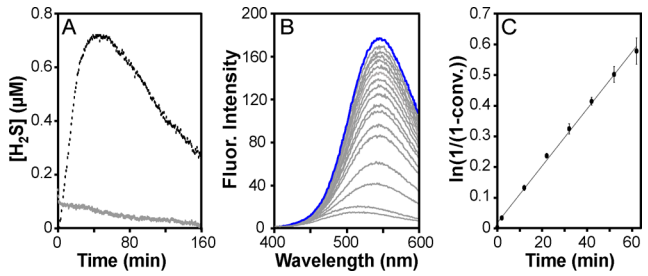


Figure 1. (A) H_2S-selective electrochemical probe release curves for **NTA1** (10 μM) in the presence of glycine (1 mM) with 300 nM CA (black curve) or without CA (gray curve) in PBS buffer (pH = 7.4). (B) Representative fluorescence spectra (λ_{ex} = 340 nm) exhibiting an increase in intensity over time as H_2S reduces the nonemissive Da-N_3 probe into fluorescent Da-NH_2. (C) First-order kinetic plot of Da-NH_2 generation over time, as measured by the change in fluorescence emission intensity at 535 nm. Line represents the pseudo-first-order kinetic fit using the equation: conv. = $1 - e^{[-kt]}$.

endothelial cell media exhibited a faster release profile and a quicker return to baseline (Figure S19). No measurable release was observed in the absence of CA. Although a half-life cannot be determined using this electrochemical method due to oxidation and volatilization of the gas, it is a reliable way to measure instantaneous H_2S concentration.

Neither the colorimetric nor the electrochemical assay were suitable to quantify the H_2S release rate in the absence of CA because COS hydrolysis is sufficiently slow to limit the use of these methods. To make a comparison between the H_2S release rates with and without CA, additional analysis of the H_2S release profile of **NTA1** was performed using the azide derivative of dansyl sulfonamide developed by Wang et al.[31] The dansyl azide (Da-N_3) probe exhibits pronounced selectivity for the hydrosulfide anion (HS^-) over other anions and reactive species commonly found in biological assays with a response time on the order of seconds. The probe acts through a turn-on fluorescence mechanism whereby HS^- reduces the nonfluorescent azide in solution to give the corresponding fluorescent amine (Da-NH_2). Although Da-NH_2 is a known CA inhibitor,[32] we used the initial rates of Da-N_3 reduction by **NTA1** to compare the rates of H_2S production in the presence and absence of CA.

Fluorescence assays were performed under similar conditions to those described above for the methylene blue and electrochemical probe experiments, monitoring the increase in emission intensity at 535 nm over 10 h (Figure 1B). Pseudo-first-order analysis of Da-NH_2 production in the first 60 min of the experiment in the presence of CA gave an H_2S release half-life of 120 min (Figure 1C). The discrepancy between the half-lives based on methylene blue and fluorescence is likely due to some inhibition of CA by Da-NH_2. Performing the assay in the absence of CA gave a release half-life of 990 min, in excellent agreement with previously measured COS hydrolysis rates in aqueous media.[29]

Next, we set about preparing an NTA-containing polymer for potential use in COS or H_2S-releasing films, hydrogels, nanoparticles, or other materials that could serve to localize gas delivery. A polymerization technique was needed that would be compatible with the reactive NTA moiety. Ring-

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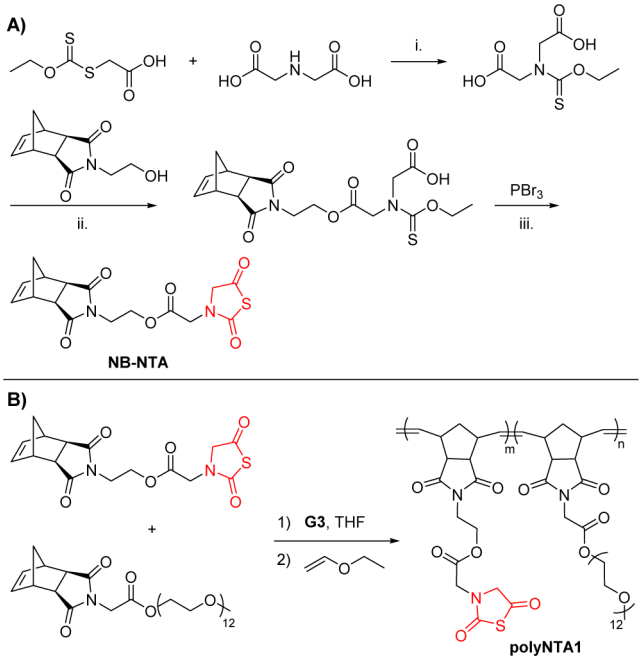
opening metathesis polymerization (ROMP) is typically performed under mild conditions (room temperature, no potential nucleophiles or radical initiators), proceeds rapidly to high conversions, and is highly functional group tolerant.[33] Therefore, we designed a synthesis to couple a ROMP-active norbornene to a COS donor. ROMP monomer **NB-NTA** was prepared similarly to **NTA1** in three steps starting from iminodiacetic acid (Scheme 2A).

Scheme 2. Synthesis of Norbornene-NTA Monomer and Copolymer polyNTA1[a]



[a]Conditions: (i) NaOH, H_2O, 52% yield; (ii) EDC, pyridine, CH_2Cl_2, 52% yield; (iii) CH_2Cl_2, 0 °C, 50% yield.

Polymerizations of **NB-NTA** were carried out in CH_2Cl_2 initiated by Grubbs' third generation catalyst $(H_2IMes)(pyr)_2(Cl)_2Ru{=}CHPh$ (**G3**). The resulting homopolymer was insoluble in water, so a poly(ethylene glycol) (PEG) norbornene comonomer was introduced into the feed at a 9:1 ratio with respect to **NB-NTA** (Scheme 2B). The polymerization yielded a water-soluble random copolymer (**polyNTA1**) with a number-average molecular weight (M_n) of 37 kDa and a dispersity of 1.06 (Figure S13).

H_2S release from copolymer **polyNTA1** was analyzed using the H_2S-selective electrochemical probe (Figure 2A). A rapid initial release was observed, followed by a plateau after approximately 2 h and sustained release out to at least 20 h. Additionally, the methylene blue assay (Figure 2B) revealed an H_2S release half-life of 280 min (Figure 2C), a 3-fold increase over the small molecule **NTA1**. This result is consistent with similar gasotransmitter-releasing copolymers in which the release of the polymeric donor is sustained over a longer period than the corresponding small molecules.[23] We speculate that this phenomenon may be due to the steric crowding imparted by the polymer backbone as well as the pendant PEG chains in **polyNTA1**.

To test the ability of **NTA1** and **polyNTA1** to deliver H_2S in a biological system, we assessed their efficacy in promoting proliferation of brain-derived endothelial cells. Endothelial cell

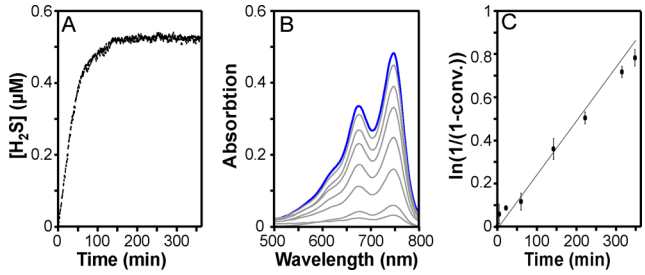


Figure 2. (A) H_2S-selective electrochemical probe release curves for **polyNTA1** (10 μM in NTA) in the presence of glycine (100 μM) with 300 nM CA in PBS buffer (pH = 7.4). Release was sustained out to 20 h. (B) Absorbance spectra from the methylene blue assay. The increase in intensity over time corresponds to in situ generation of methylene blue from H_2S. (C) First-order kinetic plot derived from the methylene blue data, measured by an increase in absorbance at 676 nm. Line represents the pseudo-first order kinetic fit using the equation: conv. = $1 - e^{[-kt]}$.

proliferation is an important first step in angiogenesis, the formation of new blood vessels from existing vessels, which is vital for several biological processes, most notably wound healing. H_2S promotes angiogenesis;[34] therefore, H_2S donors may be useful as wound healing agents.[35] Experiments were performed by treating endothelial cells with **NTA1**, Na_2S (an H_2S source used as a positive control), or the Sar-Gly dipeptide byproduct for 24 h, followed by measuring the percentage of BrdU positive cells in each treatment group. No CA enzyme was added to any of the treatment groups because the concentration of endogenous CA, which is widely distributed in mammalian cells, is sufficient to quickly convert COS into H_2S.[11] A significant increase in endothelial cell proliferation was observed for both **NTA1** and Na_2S at 100 μM (Figure 3)

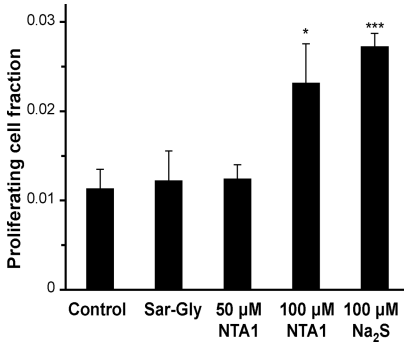


Figure 3. Endothelial cell proliferation data showing the ratio of proliferating cells in each treatment group. Cells were treated for 24 h in serum-free media, and quantification was performed by counting the number of $BrdU^+/Dapi^+$ cells (n = 7–8 for each treatment group). * indicates $p < 0.05$, *** indicates $p < 0.001$ relative to untreated control. Error bars represent standard error of the mean.

compared with the untreated control group. No increase was observed for the Sar-Gly byproduct. Caspase activity was also measured for **NTA1** to assess apoptosis, revealing no significant activity up to 100 μM (Figure S20). We also evaluated the effect of **polyNTA1** on endothelial cell proliferation (Figure S21). No increase in proliferation was observed, which may be a result of the slower rate of H_2S generation from the polymeric donor.

In summary, **NTA1** and **polyNTA1** were synthesized to afford donor compounds that released COS in a sustained

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manner; COS is then converted into H_2S in the presence of CA. In contrast to many H_2S donors that release potentially toxic byproducts, the products of release from **NTA1** are COS and an amino acid residue. Analysis of the H_2S release kinetics in the presence of CA gave release half-lives on the order of hours for both **NTA1** and **polyNTA1**. Treatment of endothelial cells with **NTA1** increased proliferation over relevant control groups. Taken together, these results demonstrate that NTAs offer a unique method to deliver H_2S in a therapeutic manner. Additionally, the polymeric delivery vehicles developed herein may enable localized, sustained H_2S delivery likely through intravenous or intraperitoneal injections for treatment of cardiovascular disease, cancer, and neurodegenerative disorders. We envision that NTAs will provide a chemical tool for studying COS in biological systems, facilitating a greater understanding of the roles that this gas plays in signaling biology.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b07204.

Synthetic and experimental details, characterization data, cell data, and supplementary H_2S release data (PDF)

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Author Contributions

The paper was written through contributions of all authors. All authors have given approval to the final version of the paper.

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Notes

The authors declare no competing financial interest.

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