Published in final edited form as:

Inorg Chem. 2018 January 16; 57(2): 681–688. doi:10.1021/acs.inorgchem.7b02533.

Time-Gated Luminescence Detection of Enzymatically Produced Hydrogen Sulfide: Design, Synthesis and Application of a Lanthanide-Based Probe

Yao Yao^a, Loruhamá Delgado-Rivera^b, Hamid Samareh Afsari^a, Liang Yin^b, Gregory R. J. Thatcher^b, Terry W. Moore^b, and Lawrence W. Miller^{a,*}

^aDepartment of Chemistry, University of Illinois at Chicago, 845 W. Taylor Street, MC 111, Chicago, Illinois 60607 (USA)

^bDepartment of Medicinal Chemistry and Pharmacognosy, UICentre for Drug Discovery, and University of Illinois Cancer Center, University of Illinois at Chicago, Chicago, Illinois 60612 (USA)

Abstract

Hydrogen sulfide (H₂S) is now recognized as an important gaseous transmitter that is involved in a variety of biological processes. Here, we report the design and synthesis of a luminescent lanthanide biosensor for H₂S, LP2-Cu(II)-Ln(III), a heterobinuclear metal complex that uses Cu(II) decomplexation to control ms-scale lifetime Tb(III) or Eu(III) emission intensity. LP2-Cu(II)-Ln(III) responded rapidly, selectively and with high sensitivity to aqueous H₂S. The probe's potential for biological applications was verified by measuring the H₂S generated by the slow-releasing chemical sulfide donor GYY4147, by cystathionine γ -lyase (CSE) and by Na₂S-stimulated HeLa cells.

TOC SYNOPSIS

Fast and selective reaction of a heterobinuclear metal complex probe with aqueous sulphide precipitates Cu(II) and turns on Tb(III) or Eu(III) luminescence. Sensitive, time-gated detection of long-lived lanthanide emission permits analysis of slow H₂S generation by cystathionine γ -lyase, by a small molecule sulphide donor, GYY4147, and in living HeLa cells.

*To whom correspondence should be addressed: lwm2006@uic.edu.

SUPPORTING INFORMATION

Supporting table and figures, synthetic methods, NMR and MS data, supporting references.

INTRODUCTION

Hydrogen sulfide (H_2S) is an important gaseous transmitter that is involved in a broad spectrum of physiological and pathological processes.^{1–4} It exerts its effects in multiple organ systems^{4, 5} and also plays a role in the regulation of cellular metabolism and immunological responses.⁵ H_2S dissociates in aqueous solution to HS^- with a $\text{pK}_{\text{a}1}$ of 6.9 and to S^{2-} with a $\text{pK}_{\text{a}2}$ of 12. Thus, over 75% of dissolved H_2S exists as HS^- at physiological pH. H_2S is predominantly synthesized from three sulfur-containing precursors (cysteine, homocysteine, cystathionine) by two pyridoxal 5'-phosphate-dependent enzymes: cystathionine- β -synthase (CBS) and cystathionine γ -lyase (CSE).^{3, 5, 6} Although CBS is seen as abundant in the central nervous system and CSE more abundant outside of it, both CBS and CSE are widely distributed across different cell types.^{2, 7} Altered H_2S metabolism has been correlated with many diseases, including hypertension, sleep apnea, acute pancreatitis, and various types of cancers.^{1, 4, 6, 8} Consequently, there is significant interest in both the fundamental biology of H_2S and in drug discovery efforts to identify enzyme-specific modulators of H_2S production.^{9, 10}

The biomedical relevance of H_2S has led chemists to develop numerous small molecule sensors for use in vitro, in cell culture or in vivo. Most reported probes are reaction-based and exhibit turn-on fluorescence mediated by one of three processes: i) reduction of quenching nitro- or azide groups; ii) nucleophilic addition of HS^- that unmask or creates a fluorophore moiety; or iii), un-quenching that follows precipitation of chelated metals (Table S1).^{11, 12} Ideally, probes should react selectively and rapidly with aqueous sulfide to give a large change in fluorescence. Good cell permeability is needed for microscopic imaging. The best performers variously exhibit high dynamic range, nanomolar limits of detection, response times shorter than one minute or good cell permeability, although very few, if any demonstrate all of these features.

Sensor performance parameters are commonly assessed in aqueous solutions using Na_2S or NaHS as quick release H_2S donors. Upon dissolution, either salt will rapidly speciate into H_2S , HS^- and S^{2-} . It should be noted, however, that H_2S is rapidly lost from an open solution by volatilization, and one study measured a half-life of only about 5 min for aqueous sulfide.¹³ Therefore, while the amount of sulfide salt added to solution can be precisely controlled, the actual reactive sulfide concentration at the time a measurement is

made may be highly uncertain, calling into question reported values of detection limits and probe reaction kinetics. Moreover, addition of sodium sulfides rapidly boosts H₂S levels in a manner that may not reflect conditions in biological systems, where the analyte's concentration is modulated enzymatically.¹⁴

Herein, we describe the preparation and sulfide detecting properties of a lanthanide-based luminescent sensor that exhibits an extremely fast, selective and sensitive response to H₂S. As depicted in Scheme 1, LP2-Cu(II)-Ln(III) (Ln = Tb or Eu) is a heterobinuclear complex that consists of a lanthanide ion chelated by 1,4,7,10-tetraazacyclododecane-1,4,7-tetraacetic acid (DO3A), an antenna chromophore (7-amino-4-methyl quinolinone, cs124) and a Cu(II) receptor 1,4,7,10-tetraazacyclododecane (cyclen). When in the presence of Cu(II), sensitized Ln(III) emission is quenched. In the presence of H₂S, Cu(II) is removed from the complex, resulting in a strong turn-on response. We assessed the performance of this sensor using a variety of H₂S donors including fast-releasing Na₂S and slow releasing GYY4147 and the enzyme cystathionine γ -lyase (CSE). LLPS-Tb(III)-Cu(II) responded to Na₂S within seconds to give a >100-fold change in emission intensity and a detection limit of ~10 nM. The sensitivity and fast reaction kinetics made it possible to detect the gradual, low-level release of H₂S from GYY4147, whereas a conventional fluorescent probe, azidomethyl coumarin (AzMC) failed. We also detected time-dependent changes in H₂S level from CSE *in vitro* and from HeLa cells stimulated with Na₂S. The results show that LLPS-Tb(III)-Cu(II) is a versatile luminescent probe for H₂S that may be used in a variety of biological assays.

RESULTS AND DISCUSSION

Sensor design and synthesis

We set about to develop a lanthanide-based H₂S sensor because an initial goal of this work was to design a probe that was well suited for high throughput screening (HTS) for inhibitors of CSE or CBS. Emissive Tb(III) or Eu(III) complexes are often incorporated into HTS assays because their ms-scale emission signals can be separated from ns-scale fluorescence background generated by sample containers, biological media, biomolecules or library compounds.¹⁵ These non-specific signals can reduce fluorescence-based HTS assay robustness by generating false positive or negative results.¹⁶ Multiwell plate readers that are capable of pulsed excitation and time-gated detection can eliminate background fluorescence, enabling assays with high signal-to-noise ratios and sub-picomolar limits of detection. A handful of lanthanide-based H₂S sensors have been reported to date that employed azide reduction, H₂S-trapping or copper precipitation as means to alter ligand-to-metal energy transfer efficiency and thereby produce a change in luminescence intensity.^{17–20} As with conventional fluorescent species, the sensitivities and response times of the reported lanthanide H₂S sensors varied widely with no single probe achieving maximal performance in all criteria (Table S1).¹¹

Our sensor design required three elements: 1) a chelated Tb(III) or Eu(III) ion; 2) an antenna chromophore that could absorb light and transfer excitation energy to the chelated lanthanide ion;²¹ and 3) a H₂S-trapping moiety that could modulate sensitized Tb(III) or Eu(III) emission. For sensing, we chose the demetallation strategy because of its notably fast

response times (<1 min).^{20, 22, 23} We incorporated a cyclen-Cu(II) moiety based on the report of Sasakura et al. which showed that fluorescein linked to cyclen-Cu(II) was highly selective for H₂S over other sulfur species.²² The well-established kinetic inertness of DOTA-based metal complexes led us to choose DO3A for Ln(III) chelation. We selected cs124 as the antenna chromophore because it effectively sensitizes Tb(III) or Eu(III) when coupled to DO3A at the 7-amino position and because it remains an effective sensitizer when alkylated at the 1-amido positions.^{24, 25} This feature allowed us to bridge the cyclen and DO3A moieties. Reaction of cs124 (**2**) with haloalkyl-substituted, Boc-protected cyclens **1** or **6** was followed by coupling to chloroacetyl chloride and alkylation of ^tBu-DO3A (Scheme 2). Removal of protecting groups yielded ligands LP1 and LP2 in 25% overall yield. Stable 1:1 Tb(III) or Eu(III) complexes were obtained by reacting ligands and metals in buffer at elevated temperature followed by reverse-phase HPLC purification.

Characterization of lanthanide probes

We first investigated the quenching effects of Cu(II) on overall Tb(III) luminescence intensity. Excitation of LP1-Tb(III) or LP2-Tb(III) at 330 nm in the absence of Cu(II) resulted in characteristic Tb(III) emission spectra (Fig. 1a, S1). After adding one equivalent of Cu(II), we observed essentially complete quenching of LP2-Tb(III) luminescence but only partial quenching of LP1-Tb(III) emission (Fig. S1). A Job's plot confirmed that LP2-Ln(III) responded to Cu(II) in 1:1 stoichiometry (Fig. S2).

Next, we examined the sensitivity of our compounds for H₂S. LP1-Ln(III)-Cu(II) showed low sensitivity due to the poor quenching effect of Cu(II), but LP2-Ln(III)-Cu(II) turned out to be extremely sensitive to H₂S. As shown in Fig. 1a, LP2-Tb(III)-Cu(II) showed immediate 40-fold and 150-fold turn-on responses in the presence of 10 μM and 50 μM of Na₂S, respectively. Similar results were achieved by LP2-Eu(III)-Cu(II) (Fig. S3). To further establish the utility of this probe for the detection of sulfide in aqueous solution, time-gated measurements were carried out in 96-well microtiter plates. An excellent linear correlation between the Na₂S concentrations and the luminescence response was observed (Fig. 1b). The linear relationship established a quantitative detection method for sulfide with a calculated detection limit of 10 nM.

We then evaluated the selectivity of our lanthanide probes towards H₂S over other thiols. In contrast to the robust and immediate response obtained by LP2-Tb(III)-Cu(II) in the presence of Na₂S, almost no response was observed using 1 mM of L-cysteine, Homocysteine and 10 mM glutathione (GSH) (Fig. 1c). LP1-Ln(III)-Cu(II), on the other hand, can be fully turned on not only by Na₂S but also by biothiols (Fig. S4), indicating the lower affinity between cyclen moiety in LP1 and Cu(II), which is likely caused by the amide bond in the cyclen moiety. This was also confirmed by the fact that only one equivalent of sulfide was required to turn on the LP1-Ln(III)-Cu(II) completely, but more than one equivalent was needed to reach the full decomplexation of LP2-Ln(III)-Cu(II) (Figs. S5, S6). In addition, LP2-Tb(III)-Cu(II) showed good selectivity over other thiols (2-mercaptoethanol, dithiothreitol), inorganic sulfur compounds (S₂O₃²⁻, S₂O₄²⁻, SO₃²⁻), inorganic nucleophiles (Br⁻, I⁻, HCO₃⁻, SO₄²⁻), reactive species (H₂O₂, NO) and metal ions (Zn²⁺, Ca²⁺, Fe²⁺, Fe³⁺) (Fig. 1d).

Sensing mechanism

We used mass spectrometry to show that sulfide sensing is due to CuS precipitation and not because of Cu(II) reduction or Ln(III) interaction. In solution containing only LP2-Tb(III), the most prominent peak occurred at m/z 929.3, corresponding to $[\text{LP2-Tb(III)}+\text{H}^+]^+$ (Fig. S7). A new intense peak at m/z 990.2 corresponding to $[\text{LP2-Tb(III)}+\text{Cu(II)}-\text{H}^+]^+$ was observed shortly after Cu(II) addition, and the peak of $[\text{LP2-Tb(III)}]$ disappeared (Fig. S8). Addition of sodium sulfide resulted in the disappearance of the $[\text{LP2-Tb(III)}+\text{Cu(II)}-\text{H}^+]^+$ peak and the reappearance of the peak assigned to $[\text{LP2-Tb(III)}+\text{H}^+]^+$ (Fig. S9). At no time did we observe peaks indicative of $[\text{LP2}+\text{Cu(II)}]$ or $[\text{LP2}+2\text{Cu(II)}]$.

Time-gated detection of exogenous H_2S in living cells

We also considered the suitability of our probe for time-gated detection of H_2S in living mammalian cells. HeLa cells were incubated in 5 mM LP2-Tb(III), washed in PBS, re-immersed in culture medium and imaged using a time-gated luminescence microscope. The absorption spectrum of LP2 peaks at 330–340 nm (Fig. S10), so it was necessary to use a relatively low power LED emitting at 340 nm as a pulsed excitation source. Nevertheless, we could observe weak Tb(III) luminescence distributed throughout the cytoplasm and nucleus of treated cells that appeared to be stable for at least 20 min (Fig. S11). Unfortunately, we were unable to reliably detect H_2S -dependent intensity changes in single cell images, most likely due to the weak signal (not shown). Therefore, we attempted to detect cellular H_2S using a multiwell plate reader. HeLa cells were seeded at 10,000 cells/well in 96-well plates and incubated at 37 °C and 5% CO_2 overnight. The seeding medium was replaced with growth media containing 250 μM LP2-Tb(III)-Cu(II), and the cells were incubated for 45 mins. The cells were then washed with PBS buffer 4 times and various concentrations of Na_2S was added. Within minutes, we observed a robust increase in time-gated Tb(III) luminescence that varied in proportion to the amount of Na_2S added (Fig. 2). The results clearly indicate that LP2-Tb(III)-Cu(II) can detect intracellular H_2S following stimulation with Na_2S .

Time-gated detection of enzymatic H_2S production

Drug discovery efforts require robust HTS assays, and there are few assay systems for measuring CSE or CBS inhibition (none for activation).^{5, 26, 27} Among the reported H_2S sensors, azidomethyl coumarin (AzMC) has been used in pilot screens for inhibitors of CBS and CSE by ourselves and others.^{26–28} However, it has a relatively slow response time due to the 2nd-order dependence of azide reduction on $[\text{HS}^-]$.²⁹ This, in turn, introduces a significant lag phase into the kinetics of H_2S detection and makes AzMC unsuitable for detecting weak inhibitors in assays run at low substrate concentration ($\sim 0.1 * K_M$).³⁰ Moreover, as with any fluorescent sensing strategy, non-specific fluorescence from library compounds or other assay components can obscure the AzMC signal which further increases the likelihood of missing weak inhibitors.^{16, 30, 31} Given the demonstrated limitations of existing sensors, we evaluated LP2-Ln(III)-Cu(II) for its ability to sense enzymatically generated H_2S in buffer.

The primary function of CSE is the cleavage of cystathionine to give cysteine, α -ketobutyrate, and ammonia.³² In principle, a variety of CSE-catalyzed reactions can lead to

H₂S formation from the substrates cysteine or homocysteine. We initiated CSE-mediated H₂S generation by adding homocysteine (1 mM) to assay buffer that contained the probe (1 μM), cofactor pyridoxal 5'-phosphate (10 μM), and CSE at varied concentrations. According to results of an experimental study and kinetic analysis performed by Chiku et al, the predominant mechanism of product formation under these conditions would be α,γ-elimination of homocysteine with a K_M of 2.7 mM to yield H₂S and homoserine.³³ Several minutes after enzyme addition, we observed an apparently linear increase in probe luminescence intensity that continued for ~10 min and then levelled off toward a maximum (Fig. 3a and Fig. S12). No significant signal change was observed when the well-studied CSE inhibitor, L-propargyl glycine (L-PAG) was included in the reaction mixture.⁵ The slopes of the linear portions of the curves increased with enzyme concentration, indicating that the LP2-Tb(III)-Cu(II) signal mirrors the progress of the enzymatic reaction. However, the interval between reaction initiation and appearance of an observable signal, which decreased with increased enzyme level, was surprising given the reaction conditions and fast response of the LP2 probe toward Na₂S. A similar lag to detection was observed with AzMC (see below). The plateaus in the intensity vs. time curves seen at later time points are likely due to probe depletion, as there should be no appreciable substrate depletion under these conditions.³³ However, the maximum signal intensity reached increased with enzyme concentration up to at least 500 nM (Fig. 3). It is possible that the LP2 probe binds to secondary products like polysulfides [84] without Cu(II) precipitation such that the probe remains quenched yet cannot react with H₂S.

We next compared the performance of LP2 to that of AzMC²⁷ under identical conditions. Following addition of CSE to reaction buffer (final conc., 300 nM), about 10 min elapsed before a detectable signal was observed with either probe (Fig. 4a,b). The lag to detection is not entirely surprising with AzMC because of the relatively long response times reported for this compound (10 min – 1 h).²⁷ However, further investigation will be required to determine whether the detection lag is due to probe responses or an artifact of the enzyme reaction conditions. The maximum rate of linear increase in signal intensity for LP2, about 15%/min, was about 2.4-fold higher than the ~6%/min rate seen with AzMC. In addition, LP2-Tb(III)-Cu(II) showed a stronger turn-on than AzMC assay (12-fold vs 5-fold, respectively, Fig. 4c). Moreover, while AzMC fluorescence intensity increased linearly and never reached a maximum during the 1 h experiment, LP2 luminescence was again observed to plateau. This difference could simply reflect a lack of AzMC depletion during the time course measured due to its relatively higher concentration (20 μM) that of LP2 (1 μM), or because each AzMC reacts with two H₂S.²⁹

Time-gated detection of H₂S released from GYY4137

GYY4137 (Morpholin-4-ium 4 methoxyphenyl(morpholino) phosphinodithioate) has been used as a H₂S donor in biological studies due to its slow-releasing property, which better mimics the gradual H₂S release of enzymatic synthesis compared with Na₂S.^{14, 34} In solution under physiological conditions, H₂S release was shown to be slow and at a low level, with less than 10% equivalent release after 7 days.³⁵ We observed an immediate increase in signal after addition of GYY4137 (to 50 or 100 μM) to a solution of LP2 (1 μM). The abrupt signal increase was due to H₂S release in the donor stock solution during

preparation. After the initial spike, the signal increased steadily because of the slow release of H₂S (Fig. 5a). We didn't observe the steady increase when the concentration of GYY4137 was 10 μ M, indicating that it cannot release enough H₂S to be sensed by the probe. As a comparison, AzMC failed to response to 100 μ M GYY4137 at all within 1 h (Fig. 5b). Under conditions of slow release and low H₂S concentrations, the ~10 nM detection limit and sub-minute response time favors LP2 over the slower reacting and less sensitive AzMC. While AzMC was non-responsive, we note that there have been reported substantially brighter azide-reduction probes based on rhodamine or fluorescein that may be expected to yield detectable signals under these experimental conditions.¹¹

CONCLUSION

Here we have presented a heterobinuclear, lanthanide-based probe, LP2-Ln(III)-Cu(II), that exhibits strong turn-on Tb(III) or Eu(III) luminescence following selective reaction with aqueous H₂S and precipitation of a chelated, quenching Cu(II) ion. We characterized the probe using a variety of H₂S donors and compared its performance directly to that of AzMC, a representative azide reduction-based sensor. As is typical when characterizing H₂S sensors, we measured common performance benchmarks in aqueous Na₂S solution including limit of detection, fold-change in signal and response time. The cell-permeable probe, LP2-Ln(III)-Cu(II), met or exceeded the performance of previously reported sensors, showing more than 100-fold change in signal within seconds and a low-nanomolar detection limit. Both the LP2 probe and AzMC yielded easily detectable responses to enzymatically generated H₂S but only after several minutes had elapsed following reaction initiation. This result was unexpected in that we anticipated a much shorter lag for LP2 and a much longer lag for AzMC because of the much slower kinetics of azide reduction relative to Cu(II) precipitation and the substantially higher reported detection limit of AzMC. By contrast, LP2 responded rapidly and strongly to the slow-releasing H₂S donor, GYY4147, while AzMC did not respond at all. Our results show that commonly reported figures of merit like response time or detection limit only provide a rough measure of relative sensor performance, and they do not necessarily reflect how well a given sensor will respond to small, slow changes in H₂S levels that are more reflective of biological systems. Further studies will explore response kinetics of this and similar probes to enzymatic H₂S production both *in vitro* and *in vivo*.

METHODS

General considerations

All reagents were commercially obtained and unless otherwise stated, were used without further purification. Deionized and distilled water was used throughout (18 M Ω cm⁻¹). ¹H NMR and ¹³C NMR spectra were recorded at ambient temperature using 500 MHz spectrometers. The data are reported as follows: chemical shift in ppm from internal tetramethylsilane on the scale, multiplicity (br = broad, s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet), coupling constants (Hz) and integration. High-resolution mass spectra were obtained by peak matching. Analytical thin layer chromatography was performed on 0.25 mm extra hard silica gel plates with UV254 fluorescent indicator.

Medium pressure liquid chromatography (MPLC) was performed to force flow the indicated solvent system down columns that had been packed with 60Å (40 – 60 µm) mesh silica gel (SiO₂). Reverse-phase high-performance liquid chromatography (HPLC) was performed using a Beckman System Gold instrument equipped with an analytical-scale pump (model 128), a UV-vis detector (model 168), and a C18 analytical column (Grace Vydac, cat.no. 218TP54, 5 µm, 4.6 mm i.d. × 250 mm). Emission spectra were measured by using the FluoroMax-3 spectrofluorometer (Jobin Yvon). Absorption spectra were measured on Cary 300 bio UV-visible spectrophotometer. Time-gated luminescence measurements using 96-well microtiter plates as cuvettes were carried out on a PerkinElmer Victor 1420 multilabel counter with the settings of delay time, 0.4 ms; window time (counting time), 0.7 ms; cycling time, 1.2 ms; excitation wavelength, 340 nm; and emission wavelengths, 545 and 615 nm for Tb(III) and Eu(III) complexes, respectively.

Determination of Detection Limit

500 nM LP2-Tb(III)-Cu(II) in 20 mM HEPES buffer (pH 7.4) was added into 10 wells in 96 well-plate. The luminescence was recorded in PerkinElmer Victor 1420 multilabel counter after incubation at 37°C for 5 mins (Ex/Em=340/540 nm; delay time, 400 µs). Then LP2-Tb(III)-Cu(II) was treated with Na₂S at various concentrations, and the luminescence was measured after incubation for 5 mins at 37°C. Each data point represents at least three trials. The detection limit was determined to be concentration at which the luminescent intensity equals that of [blank + 3σ]³⁶, Where σ is the standard deviation of the blank measurement.

Cell culture

HeLa cells were maintained in DMEM (+) (DMEM supplemented with 10% FBS, 1X MEM non-essential amino acids and 15 mM HEPES) at 37 °C and 5% CO₂. The cells were passaged with 0.25% trypsin/2.21 mM EDTA.

Cellular delivery of the probe

For the time-gated Image—Cells were trypsinized and seeded at 23,000 cells/well in Poly-Lysine treated 8-well chambered coverglass (Nunc, 12-565-470) and incubated at 37 °C and 5% CO₂ overnight. The following day, growing medium was removed and LP2-Tb(III) (final conc. 5 mM) in fresh growing medium was added and then the cells were incubated for 45 min at 37 °C and 5% CO₂. The cells were washed with PBS 3 times and immersed in growing media for imaging.

For 96-wells plates—Cells were trypsinized and seeded at 10,000 cells/well in 96 well (Costar, #3916, black, flat bottom, tissue culture treated) incubated at 37 °C and 5% CO₂ overnight. The following day, growing medium was removed and LP2-Tb(III)-Cu(II) (final conc. 250 µM) in fresh growing medium was added to each well and then the cells were incubated for 60 min at 37 °C and 5% CO₂. The cells were washed with PBS 4 times and different concentration of Na₂S was added. The signal of each well at 545 nm was monitored in plate reader every 3 mins for 30 mins. At least 10 wells used to measure the signal. The plate reader was set up to automatically shake the plate for 10 sec prior to the first data point.

Time-gated Image

Images were acquired using a time-gated luminescence microscope that uses a UV LED for pulsed excitation and an intensified CCD camera (ICCD) for gated detection. The operation and characterization of the microscope system have been described in detail.³⁷ Images were summations of 32 frames and large variations in signal resulting from ion-feedback noise of the intensifier removed by a feature of the camera control software. The UV LED source and the ICCD timing parameters were the same for both time-resolved images. Following parameters have been used for imaging: excitation pulse width, 1500 μ s, pulse period, 3000 μ s, delay time, 10 μ s, intensifier on-time, 1480 μ s. Both time-gated images were acquired at an intensifier gain of 889 V. A 340 nm UV LED and 540 $\pm$ 10 nm filter have been used for excitation and emission respectively.

CSE expression and purification

Human CSE cDNA was cloned into pET-28b to generate N-terminal His-tagged fusion protein, as previously described⁶. The expression vector was transformed and plated onto LB agar plates, supplemented with kanamycin (50 μ g/mL). Recombinant CSE was expressed in the E. coli strain BL21 (DE3) and purified based on previously described procedures with slight modifications. The protein was purified with nickel agarose beads and subsequently eluted with 300 mM imidazole in buffer containing 20 mM Tris-HCl (pH 8.0) and 300 mM NaCl. The protein was estimated to be at least 90% pure from SDS-PAGE analysis. Protein concentration was then determined by BCA protein assay. CSE was used in the enzyme assays as the His-tagged fusion protein.

Detection of H₂S generated from CSE

100 μ L of enzyme solution (50 mM Tris, pH 7.4, 10 μ M pyridoxal 5'-phosphate, 0.1 mg/mL BSA, 1 μ M LP2-Ln(III)-Cu(II), 100, 300 or 500 nM CSE) was added into 96-well plate and incubated at 37 °C for 15 mins. Immediately following the addition of substrates (homocysteine, final concentration 1 mM), the fluorescence of the mixture was monitored for about 50 mins. The plate reader was set up to automatically shake the plate for 10 sec prior to the each measurement (every 3 mins).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful to the National Institutes of Health (NHLBI UH2 L123610 and NIGMS R01 GM081030) and the UICentre for Drug Discovery; University of Illinois at Chicago, Office of the Vice Chancellor for Research.

The authors declare no competing financial interests.

References

1. Prabhakar NR. Sensing hypoxia: physiology, genetics and epigenetics. *J Physiol.* 2013; 591(9): 2245–2257. [PubMed: 23459758]

2. Donovan LM, Moore MW, Gillombardo CB, Chai S, Strohl KP. Effects of hydrogen sulfide synthesis inhibitors on posthypoxic ventilatory behavior in the C57BL/6J mouse. *Respiration; international review of thoracic diseases*. 2011; 82(6):522–529. [PubMed: 21952225]
3. Brancalione V, Esposito I, Gargiulo A, Vellecco V, Asimakopoulou A, Citi V, Calderone V, Gobetti T, Perretti M, Papapetropoulos A, Bucci M, Cirino G. D-Penicillamine modulates hydrogen sulfide (H₂S) pathway through selective inhibition of cystathionine-gamma-lyase. *Br J Pharmacol*. 2016; 173(9):1556–1565. [PubMed: 26890936]
4. Li Q, Sun B, Wang X, Jin Z, Zhou Y, Dong L, Jiang LH, Rong W. A crucial role for hydrogen sulfide in oxygen sensing via modulating large conductance calcium-activated potassium channels. *Antioxid Redox Signal*. 2010; 12(10):1179–1189. [PubMed: 19803741]
5. Asimakopoulou A, Panopoulos P, Chasapis C, Coletta C, Zhou Z, Cirino G, Giannis A, Szabo C, Spyroulias G, Papapetropoulos A. Selectivity of commonly used pharmacological inhibitors for cystathionine β synthase (CBS) and cystathionine γ lyase (CSE). *Br J Pharmacol*. 2013; 169(4): 922–932. [PubMed: 23488457]
6. Sun Q, Collins R, Huang S, Holmberg-Schiavone L, Anand GS, Tan CH, van-den-Berg S, Deng LW, Moore PK, Karlberg T, Sivaraman J. Structural basis for the inhibition mechanism of human cystathionine gamma-lyase, an enzyme responsible for the production of H(2)S. *J Biol Chem*. 2009; 284(5):3076–3085. [PubMed: 19019829]
7. Prabhakar NR, Semenza GL. Gaseous messengers in oxygen sensing. *J Mol Med (Berl)*. 2012; 90(3):265–272. [PubMed: 22349394]
8. Sonke E, Verrydt M, Postenka CO, Pardhan S, Willie CJ, Mazzola CR, Hammers MD, Pluth MD, Lobb I, Power NE, Chambers AF, Leong HS, Sener A. Inhibition of endogenous hydrogen sulfide production in clear-cell renal cell carcinoma cell lines and xenografts restricts their growth, survival and angiogenic potential. *Nitric Oxide*. 2015; 49:26–39. [PubMed: 26068241]
9. Wallace JL, Wang R. Hydrogen sulfide-based therapeutics: exploiting a unique but ubiquitous gasotransmitter. *Nat Rev Drug Discov*. 2015; 14(5):329–345. [PubMed: 25849904]
10. Zhang Y, Tang ZH, Ren Z, Qu SL, Liu MH, Liu LS, Jiang ZS. Hydrogen sulfide, the next potent preventive and therapeutic agent in aging and age-associated diseases. *Mol Cell Biol*. 2013; 33(6): 1104–1113. [PubMed: 23297346]
11. Lin VS, Chen W, Xian M, Chang CJ. Chemical probes for molecular imaging and detection of hydrogen sulfide and reactive sulfur species in biological systems. *Chem Soc Rev*. 2015; 44(14): 4596–4618. [PubMed: 25474627]
12. Hartle MD, Pluth MD. A practical guide to working with H₂S at the interface of chemistry and biology. *Chem Soc Rev*. 2016; 45(22):6108–6117. [PubMed: 27167579]
13. DeLeon ER, Stoy GF, Olson KR. Passive loss of hydrogen sulfide in biological experiments. *Anal Biochem*. 2012; 421(1):203–207. [PubMed: 22056407]
14. Zhao Y, Biggs TD, Xian M. Hydrogen sulfide (H₂S) releasing agents: chemistry and biological applications. *Chemical communications*. 2014; 50(80):11788–11805. [PubMed: 25019301]
15. Mathis G. HTRF (R) technology. *J Biomol Screen*. 1999; 4(6):309–313. [PubMed: 10838427]
16. Inglese J, Johnson RL, Simeonov A, Xia M, Zheng W, Austin CP, Auld DS. High-throughput screening assays for the identification of chemical probes. *Nat Chem Biol*. 2007; 3(8):466–479. [PubMed: 17637779]
17. Tropiano M, Faulkner S. A lanthanide based sensor for the time-gated detection of hydrogen sulfide. *Chemical communications*. 2014; 50(36):4696–4698. [PubMed: 24671372]
18. Dai Z, Tian L, Song B, Ye Z, Liu X, Yuan J. Ratiometric time-gated luminescence probe for hydrogen sulfide based on lanthanide complexes. *Anal Chem*. 2014; 86(23):11883–11889. [PubMed: 25388079]
19. Xiao Y, Yang Y, Zhao G, Fang X, Zhao Y, Guo P, Yang W, Xu J. A terbium-based time-resolved luminescent probe for sulfide ions mediated by copper in aqueous solution. *Inorg Chem Commun*. 2014; 50:31–34.
20. Liang Z, Tsoi T, Chan C, Dai L, Wu Y, Du G, Zhu L, Lee C, Wong W, Law G. A smart “off-on” gate for the in situ detection of hydrogen sulphide with cu (ii)-assisted europium emission. *Chem Sci*. 2016; 7:2151–2156.

21. Bunzli JCG. On the design of highly luminescent lanthanide complexes. *Coord Chem Rev.* 2015; 293:19–47.
22. Sasakura K, Hanaoka K, Shibuya N, Mikami Y, Kimura Y, Komatsu T, Ueno T, Terai T, Kimura H, Nagano T. Development of a highly selective fluorescence probe for hydrogen sulfide. *J Am Chem Soc.* 2011; 133(45):18003–18005. [PubMed: 21999237]
23. Sun M, Yu H, Li H, Xu H, Huang D, Wang S. Fluorescence signaling of hydrogen sulfide in broad pH range using a copper complex based on BINOL-benzimidazole ligands. *Inorg Chem.* 2015; 54(8):3766–3772. [PubMed: 25839192]
24. Pillai S, Kozlov M, Marras SA, Krasnoperov LN, Mustaev A. New cross-linking quinoline and quinolone derivatives for sensitive fluorescent labeling. *J Fluoresc.* 2012; 22(4):1021–1032. [PubMed: 22450725]
25. Li M, Selvin PR. Luminescent Polyaminocarboxylate Chelates of Terbium and Europium: The Effect of Chelate Structure. *J Am Chem Soc.* 1995; 117:8132–8138.
26. Druzhyna N, Szczesny B, Olah G, Módos K, Asimakopoulou A, Pavlidou A, Szoleczky P, Gerö D, Yanagi K, Törö G. Screening of a composite library of clinically used drugs and well-characterized pharmacological compounds for cystathionine β -synthase inhibition identifies benzerazide as a drug potentially suitable for repurposing for the experimental therapy of colon cancer. *Pharmacol Res.* 2016; 113:18–37. [PubMed: 27521834]
27. Thorson MK, Majtan T, Kraus JP, Barrios AM. Identification of Cystathionine β -Synthase Inhibitors Using a Hydrogen Sulfide Selective Probe. *Angew Chem, Int Ed Engl.* 2013; 52(17):4641–4644. [PubMed: 23512751]
28. Yao Y, Kong C, Yin L, Jain AD, Ratia K, Thatcher GR, Moore TW, Driver TG, Miller LW. Time - Gated Detection of Cystathionine γ -Lyase Activity and Inhibition with a Selective, Luminogenic Hydrogen Sulfide Sensor. *Chem - Eur J.* 2017; 23(4):752–756. [PubMed: 27734530]
29. Henthorn HA, Pluth MD. Mechanistic insights into the H₂S-mediated reduction of aryl azides commonly used in H₂S detection. *J Am Chem Soc.* 2015; 137(48):15330–15336. [PubMed: 26540330]
30. Acker MG, Auld DS. Considerations for the design and reporting of enzyme assays in high-throughput screening applications. *Perspect Sci.* 2014; 1(1):56–73.
31. Simeonov A, Jadhav A, Thomas CJ, Wang Y, Huang R, Southall NT, Shinn P, Smith J, Austin CP, Auld DS. Fluorescence spectroscopic profiling of compound libraries. *J Med Chem.* 2008; 51(8):2363–2371. [PubMed: 18363325]
32. Aitken SM, Kirsch JF. The enzymology of cystathionine biosynthesis: strategies for the control of substrate and reaction specificity. *Arch Biochem Biophys.* 2005; 433(1):166–175. [PubMed: 15581575]
33. Chiku T, Padovani D, Zhu W, Singh S, Vitvitsky V, Banerjee R. H₂S biogenesis by human cystathionine gamma-lyase leads to the novel sulfur metabolites lanthionine and homolanthionine and is responsive to the grade of hyperhomocysteinemia. *J Biol Chem.* 2009; 284(17):11601–11612. [PubMed: 19261609]
34. Li L, Whiteman M, Guan YY, Neo KL, Cheng Y, Lee SW, Zhao Y, Baskar R, Tan CH, Moore PK. Characterization of a novel, water-soluble hydrogen sulfide-releasing molecule (GYY4137): new insights into the biology of hydrogen sulfide. *Circulation.* 2008; 117(18):2351–2360. [PubMed: 18443240]
35. Lee ZW, Zhou J, Chen CS, Zhao Y, Tan CH, Li L, Moore PK, Deng LW. The slow-releasing hydrogen sulfide donor, GYY4137, exhibits novel anti-cancer effects in vitro and in vivo. *PLoS One.* 2011; 6(6):e21077. [PubMed: 21701688]
36. MacDougall D, Crummett WB. Guidelines for data acquisition and data quality evaluation in environmental chemistry. *Anal Chem.* 1980; 52(14):2242–2249.
37. Afsari HS, Dos Santos MC, Lindén S, Chen T, Qiu X, en Henegouwen PMvB, Jennings TL, Susumu K, Medintz IL, Hildebrandt N. Time-gated FRET nanoassemblies for rapid and sensitive intra-and extracellular fluorescence imaging. *Sci Adv.* 2016; 2(6):e1600265. [PubMed: 27386579]

Figure 1.

Characterization of LP2-Tb(III)-Cu(II). (a) Steady-state photoluminescence (SS PL) spectra of 10 μM LP2-Tb(III)-Cu(II) upon addition of Na_2S . The data were acquired in 20 mM HEPES buffer (pH 7.4) in cuvettes. Ex = 330 nm. (b) Time-gated luminescence response of 500 nM LP2-Tb(III)-Cu(II) increases linearly with sulfide concentration in a 96-well plate. Ex/Em 340/540 nm; delay time, 400 μs . 3 replicates, error bars, SD. (c) Time-gated photoluminescence vs. time following addition of indicated analytes to LP2-Tb(III)-Cu(II) (1 μM in 20 mM HEPES buffer, pH 7.4; 37°C). Analytes were added at $t = 180$ s. Ex/Em 340/540 nm; delay time, 400 μs . 3 replicates, error bars, SD. (d) Fold-change in SS PL intensity of 20 mM HEPES buffer (pH=7.4) containing 10 μM LP2-Tb(III)-Cu(II) and different analytes. The analytes are: (1) 50 μM Na_2S , (2) 10 μM Na_2S , (3) 10 mM GSH, (4) 1 mM Homocysteine, (5) 1 mM L-cysteine, (6) 1 mM DTT, (7) 1 mM 2-mercaptoethanol, (8) 1 mM $\text{Na}_2\text{S}_2\text{O}_3$, (9) 1 mM $\text{Na}_2\text{S}_2\text{O}_4$, (10) 1 mM Na_2SO_3 , (11) 1 mM NaBr, (12) 1 mM NaI, (13) 1 mM NaHCO_3 , (14) 1 mM Na_2CO_3 , (15) 1 mM Na_2SO_4 , (16) 1 mM H_2O_2 , (17) 100 μM NO, (18) 100 μM Zn^{2+} , (19) 100 μM Ca^{2+} , (20) 100 μM Fe^{2+} , (21) 100 μM Fe^{3+} . The intensity was measured 20 min following analyte addition in cuvettes at 37 °C.

Figure 2.

(a) Time-gated detection of Na_2S in Hela cells in plate reader using probe LP2-Tb(III)-Cu(II). Cells were incubated with the probe (250 μM) for 45 mins in growing medium and then washed with PBS buffer 4 times. Then different concentration of Na_2S was added. Exc/Emi=340/540 nm. Delay time: 400 μs . (b) Relative intensities (without any background subtraction) of the cells under different concentration of Na_2S . Error bar: SD, 12 repeats.

Figure 3.

(a) Time-gated photoluminescence of LP2-Tb(III)-Cu(II) ($1 \mu\text{M}$) vs. time of CSE activity assay buffer ($10 \mu\text{M}$ pyridoxal 5'-phosphate, 0.1 mg/mL BSA, 1 mM homocysteine, 50 mM Tris, pH 7.4) containing indicated co-additives at 37°C . L-Propargylglycine ($50 \mu\text{M}$) was used as the inhibitor in the assay. Error bars represent standard deviation. Ex/Em 340/540 nm; delay time, $400 \mu\text{s}$. (b) Relative intensities of buffer solutions containing indicated additives at 50 mins.

Figure 4.

Comparisons of AzMC (a) and LP2-Tb(III)-Cu(II) (b) assay for CSE activity. (a) Steady-state detection of CSE activity using AzMC assay. (b) Time-gated detection of CSE activity using LP2-Tb(III)-Cu(II). (c) Fold-change in signal intensity at 50 mins of the assay. The assay was carried in the presence of AzMC (20 μ M) or LP2-Tb(III)-Cu(II) (1 μ M), Homocysteine (1 mM), PLP (10 μ M), CSE (300 nM), BSA (0.1 mg/mL) and HEPES buffer (50 mM, pH 7.4) in 96-well plate at 37°C.

Figure 5.

Comparisons of LP2-Tb(III)-Cu(II) (a) and AzMC (b) for detecting H₂S from GYY4137. (a) Time-gated detection of H₂S released from different concentrations of GYY4137 using 1 μM LP2-Tb(III)-Cu(II) in 20 mM HEPES buffer, pH 7.4. (b) Steady-state detection of H₂S released from different concentrations of GYY4137 using 20 μM AzMC in 20 mM HEPES buffer, pH 7.4.

Scheme 1.

Chemical structure of LP2-Ln(III) and its mechanism of sensing Cu(II) and sulfide.

Scheme 2.
Synthesis of chelators LP1 and LP2.