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

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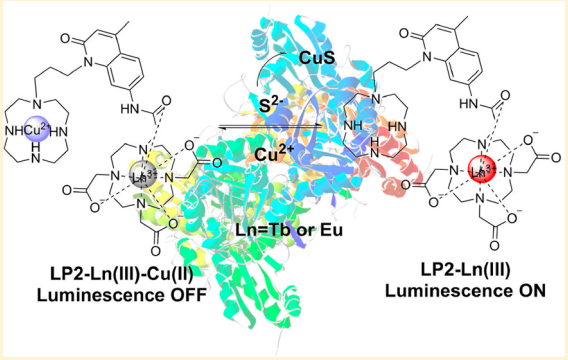
Supporting Information

ABSTRACT: Hydrogen sulfide (H_2S) 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 H_2S, LP2-Cu(II)-Ln(III), a heterobinuclear metal complex that uses Cu(II) decomplexation to control millisecond-scale-lifetime-Tb(III)- or Eu(III)-emission intensity. LP2-Cu(II)-Ln(III) responded rapidly, selectively, and with high sensitivity to aqueous H_2S. The probe's potential for biological applications was verified by measuring the H_2S generated by the slow-releasing chemical-sulfide-donor GYY4147, by cystathionine γ-lyase (CSE), and by Na_2S-stimulated HeLa cells.



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 an aqueous solution to HS^- with a pK_{a1} of 6.9 and to S^{2-} with a pK_{a2} 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 is seen as 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) the reduction of quenching nitro or azide groups; (ii) the nucleophilic addition of HS^-, which unmasks or creates a fluorophore moiety; or (iii) unquenching that follows the precipitation of chelated metals (Table S1).[11,12] Ideally, probes should react selectively and rapidly with aqueous sulfide to give large changes in fluorescence. Good cell permeability is needed for microscopic imaging. The best performers variously exhibit high dynamic ranges, nanomolar limits of detection, response times shorter than 1 min, 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, although 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, the addition of sodium sulfides rapidly boosts H_2S levels in a manner that may not reflect conditions in biological systems, where the analyte's concentration is modulated enzymatically.[14]

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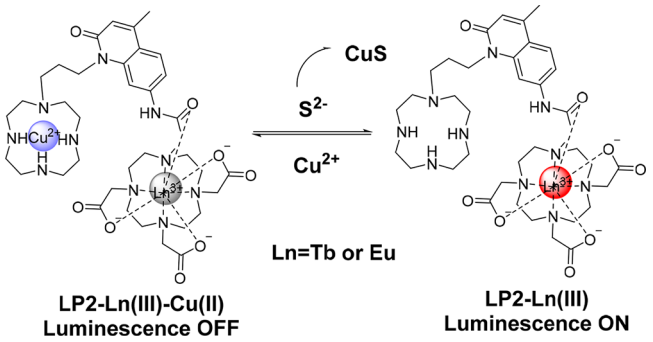
Herein, we describe the preparation and sulfide-detecting properties of a lanthanide-based luminescent sensor that exhibits an extremely fast, selective, and sensitive response to H_2S. As depicted in Scheme 1, LP2-Cu(II)-Ln(III) (Ln = Tb or Eu) is a heterobinuclear complex that consists of a lanthanide 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). Sensitized Ln(III) emission is quenched in the presence of Cu(II). In the presence of H_2S, 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 H_2S donors including fast-releasing Na_2S, slow-releasing GYY4147, and the enzyme cystathionine γ-lyase (CSE). LLPS-Tb(III)-Cu(II) responded to Na_2S 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 H_2S from GYY4147, whereas a conventional fluorescent probe, azidomethyl coumarin (AzMC), failed. We also detected time-dependent changes in H_2S levels from CSE in vitro and from HeLa cells stimulated with Na_2S. The results show that LLPS-Tb(III)-Cu(II) is a versatile luminescent probe for H_2S that may be used in a variety of biological assays.

Scheme 1. Chemical Structure of LP2-Ln(III) and Its Mechanism of Sensing Cu(II) and Sulfide

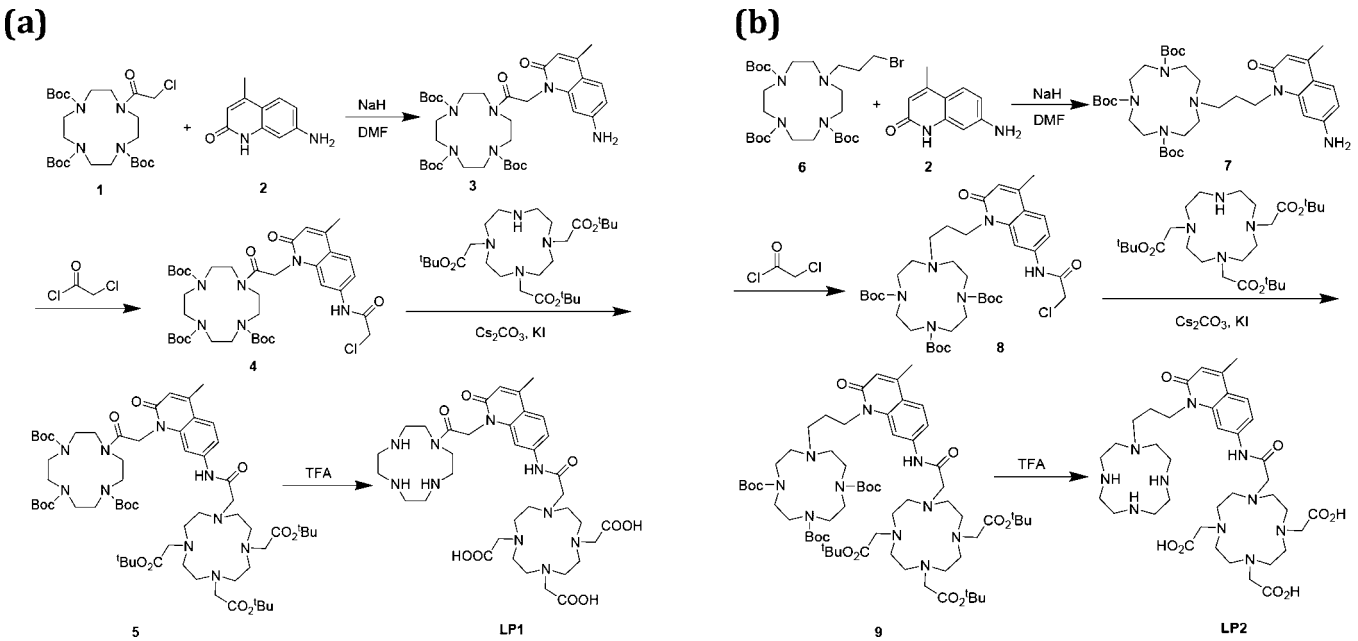


RESULTS AND DISCUSSION

Sensor Design and Synthesis. We set about to develop a lanthanide-based H_2S 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 millisecond-scale emission signals can be separated from the nanosecond-scale fluorescence background generated by sample containers, biological media, biomolecules, or library compounds.[15] These nonspecific 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 subpicomolar limits of detection. A handful of lanthanide-based H_2S sensors have been reported to date that employ azide reduction, H_2S 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 H_2S sensors varied widely with no single probe achieving maximal performance in all criteria (Table S1).[11]

Our sensor design required three elements: (i) a chelated Tb(III) or Eu(III) ion, (ii) an antenna chromophore that could absorb light and transfer its excitation energy to the chelated lanthanide ion,[21] and (iii) a H_2S-trapping moiety that could modulate sensitized Tb(III) or Eu(III) emission. For sensing, we chose the demetalation 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 H_2S over other sulfur species.[22] The well-

Scheme 2. Synthesis of Chelators LP1 and LP2



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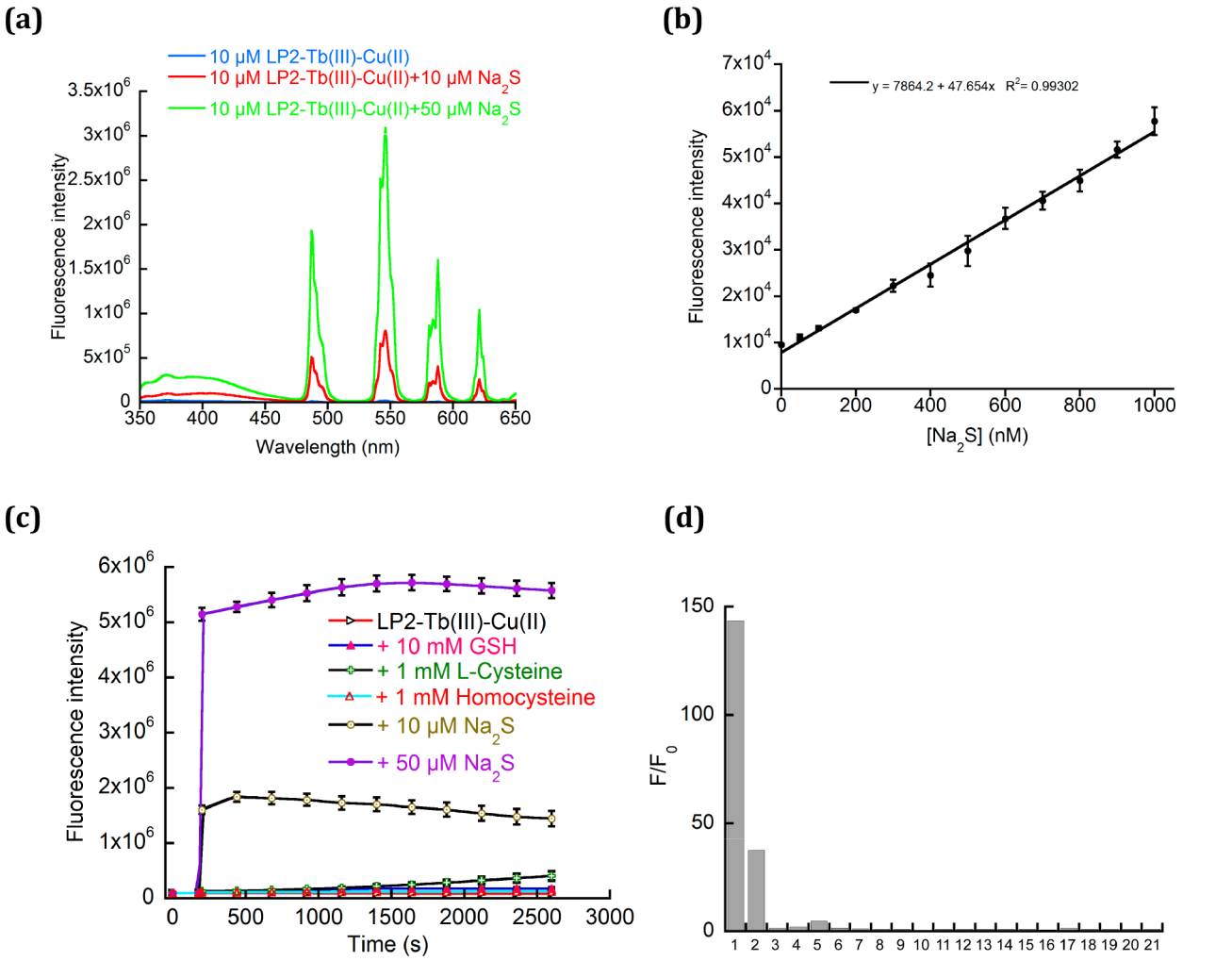

Figure 1. Characterization of LP2-Tb(III)-Cu(II). (a) Steady-state photoluminescence (SS PL) spectra of 10 μM LP2-Tb(III)-Cu(II) upon the addition of Na_2S. The data were acquired in 20 mM HEPES buffer (pH 7.4) in cuvettes with an excitation wavelength of 330 nm. (b) Linear increase in the time-gated luminescence response of 500 nM LP2-Tb(III)-Cu(II) with increasing sulfide concentrations in a 96-well plate in three replicates. The excitation and emission wavelengths were 340 and 540 nm, respectively, and the delay time was 400 μs. The error bars are SD. (c) Time-gated photoluminescence vs time following the addition of the indicated analytes to LP2-Tb(III)-Cu(II) (1 μM in 20 mM HEPES buffer, pH 7.4, 37 °C) in three replicates. The analytes were added at t = 180 s. The excitation and emission wavelengths were 340 and 540 nm, respectively, and the delay time was 400 μs. The error bars are SD. (d) Fold-change in the 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 $Na_2S_2O_3$, (9) 1 mM $Na_2S_2O_4$, (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+}, and (21) 100 μM Fe^{3+}. The intensities were measured 20 min after the addition of the analytes to the cuvettes at 37 °C.

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 position.[24,25] This feature allowed us to bridge the cyclen and DO3A moieties. Reaction of cs124 (**2**) with the haloalkyl-substituted, Boc-protected cyclens, **1** or **6**, was followed by coupling it to chloroacetyl chloride and by the alkylation of ^{t}Bu-DO3A (Scheme 2). The removal of the protecting groups yielded ligands **LP1** and **LP2** in a 25% overall yield. Stable 1:1 Tb(III) or Eu(III) complexes were obtained by reacting the ligands and metals in buffer at an elevated temperature, followed by reverse-phase HPLC purification.

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

Next, we examined the sensitivity of our compounds for H_2S. 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 H_2S. As shown in Figure 1a, LP2-Tb(III)-Cu(II) showed immediate 40- and 150-fold turn-on responses in the presence of 10 μM and 50

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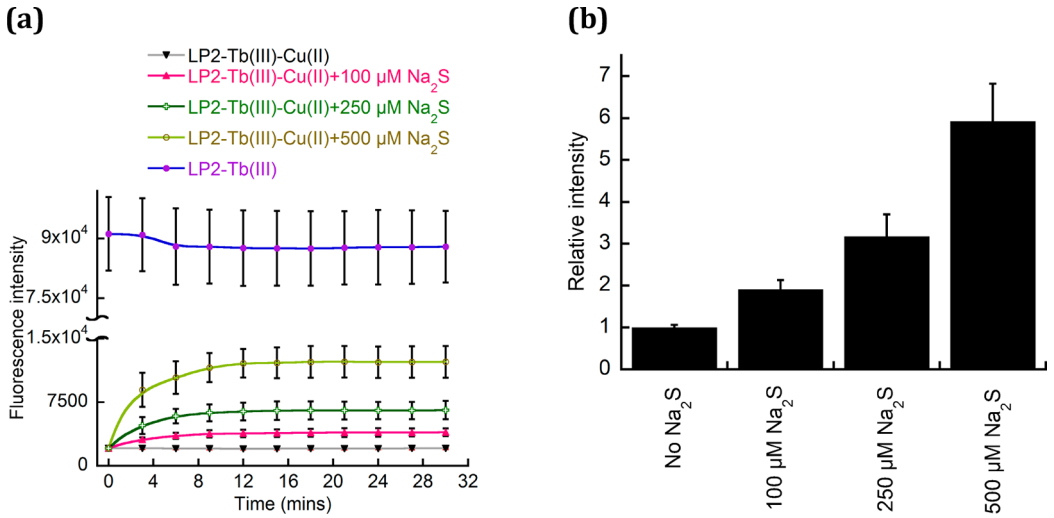

Figure 2. (a) Time-gated detection of Na_2S in HeLa cells in a plate reader using the probe LP2-Tb(III)-Cu(II). Cells were incubated with the probe (250 μM) for 45 min in a growth medium and then washed with PBS buffer four times. Then different concentrations of Na_2S were added. The excitation and emission wavelengths were 340 and 540 nm, respectively, and the delay time was 400 μs. (b) Relative intensities (without any background subtraction) of the cells under different concentrations of Na_2S. The error bars are SD. Twelve replicates were performed.

μM Na_2S, respectively. Similar results were achieved by LP2-Eu(III)-Cu(II) (Figure S3). To further establish the utility of this probe for the detection of sulfides in aqueous solutions, time-gated measurements were carried out in 96-well microtiter plates. An excellent linear correlation between the Na_2S concentrations and the luminescence response was observed (Figure 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 toward H_2S over other thiols. In contrast to the robust and immediate response obtained by LP2-Tb(III)-Cu(II) in the presence of Na_2S, almost no response was observed using 1 mM L-cysteine or homocysteine or 10 mM glutathione (GSH, Figure 1c). LP1-Ln(III)-Cu(II), on the other hand, can be fully turned on not only by Na_2S but also by biothiols (Figure S4), indicating the lower affinity between the 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 LP1-Ln(III)-Cu(II) completely, but more than one equivalent was needed to reach the full decomplexation of LP2-Ln(III)-Cu(II) (Figures S5 and S6). In addition, LP2-Tb(III)-Cu(II) showed good selectivity over other thiols (2-mercaptoethanol and dithiothreitol), inorganic sulfur compounds ($S_2O_3^{2-}$, $S_2O_4^{2-}$, and SO_3^{2-}), inorganic nucleophiles (Br^-, I^-, HCO_3^-, and SO_4^{2-}), reactive species (H_2O_2 and NO) and metal ions (Zn^{2+}, Ca^{2+}, Fe^{2+}, and Fe^{3+}), as shown in Figure 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 a solution containing only LP2-Tb(III), the most prominent peak occurred at m/z 929.3, corresponding to $[LP2+Tb(III)+H^+]^+$ (Figure S7). A new intense peak at m/z 990.2 corresponding to $[LP2+Tb(III)+Cu(II)-H^+]^+$ was observed shortly after Cu(II) addition, and the peak of [LP2+Tb(III)] disappeared (Figure S8). Addition of sodium sulfide resulted in the disappearance of the $[LP2+Tb(III)+Cu(II)-H^+]^+$ peak and the reappearance of the peak assigned to $[LP2+Tb(III)+H^+]^+$ (Figure S9). At no time did we observe peaks indicative of [LP2+Cu(II)] or [LP2+2Cu(II)].

Time-Gated Detection of Exogenous H_2S in Living Cells. We also considered the suitability of our probe for the time-gated detection of H_2S in living mammalian cells. HeLa cells were incubated in 5 mM LP2-Tb(III), washed in PBS, reimmersed in culture medium, and imaged using a time-gated luminescence microscope. The absorption spectrum of the LP2 peaks was at 330−340 nm (Figure 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 nuclei of the treated cells, which appeared to be stable for at least 20 min (Figure S11). Unfortunately, we were unable to reliably detect H_2S-dependent intensity changes in single-cell images, most likely because of 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 a growth medium containing 250 μM LP2-Tb(III)-Cu(II), and the cells were incubated for 45 min. The cells were then washed with PBS buffer four times, and various concentrations of Na_2S were added. Within minutes, we observed a robust increase in time-gated Tb(III) luminescence, which varied in proportion to the amount of Na_2S added (Figure 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 and 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 second-order dependence of azide reduction on $[HS^-]$.[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 a low substrate concentration ($\sim 0.1 * K_M$).[30] Moreover, as with any fluores-

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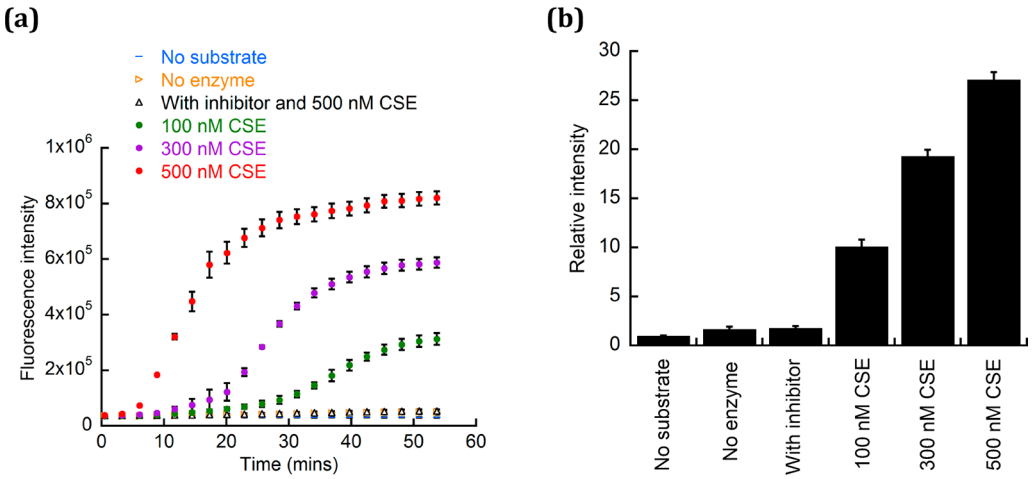

Figure 3. (a) Time-gated photoluminescence of LP2-Tb(III)-Cu(II) (1 μM) vs time for the CSE-activity assay in a buffer (10 μM pyridoxal 5′-phosphate, 0.1 mg/mL BSA, 1 mM homocysteine, 50 mM tris, pH 7.4) containing the indicated coadditives at 37 °C. L-Propargylglycine (50 μM) was used as the inhibitor in the assay. The error bars represent standard deviations. The excitation and emission wavelengths were 340 and 540 nm, respectively, and the delay time was 400 μs. (b) Relative intensities of buffer solutions containing the indicated additives at 50 min.

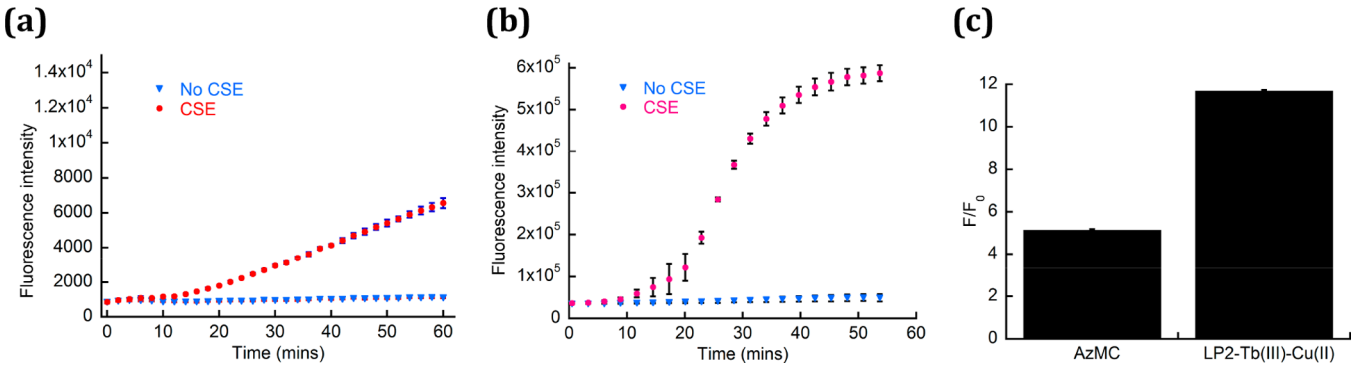

Figure 4. Comparisons of the AzMC (a) and LP2-Tb(III)-Cu(II) (b) assays for CSE activity. (a) Steady-state detection of CSE activity using the AzMC assay. (b) Time-gated detection of CSE activity using LP2-Tb(III)-Cu(II). (c) Fold-changes in signal intensities at 50 min of the assays. The assays were carried out in the presence of AzMC (20 μM) or LP2-Tb(III)-Cu(II) (1 μM), homocysteine (1 mM), PLP (10 μM), CSE (300 nM), BSA (0.1 mg/mL), and HEPES buffer (50 mM, pH 7.4) in 96-well plates at 37 °C.

cent-sensing strategy, nonspecific 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 a 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 H_2S formation from the substrates cysteine or homocysteine. We initiated CSE-mediated H_2S generation by adding homocysteine (1 mM) to the assay buffer that contained the probe (1 μM), the cofactor pyridoxal 5′-phosphate (10 μM), and CSE at varied concentrations. According to the 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 H_2S and homoserine.[33] Several minutes after enzyme addition, we observed an apparently linear increase in the probe luminescence intensity, which continued for ~10 min and then leveled off toward a maximum (Figures 3a and 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 the reaction initiation and the appearance of an observable signal, which decreased with increased enzyme levels, was surprising given the reaction conditions and fast response of the LP2 probe toward Na_2S. A similar lag to detection was observed with AzMC (see below). The plateaus in the intensity-versus-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 concentrations up to at least 500 nM (Figure 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 H_2S.

We next compared the performance of LP2 with that of AzMC[27] under identical conditions. Following the addition of CSE to the reaction buffer (final concn of 300 nM), about 10 min elapsed before a detectable signal was observed with either probe (Figure 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 to 1 h).[27] However, further investigation will be required to determine whether the

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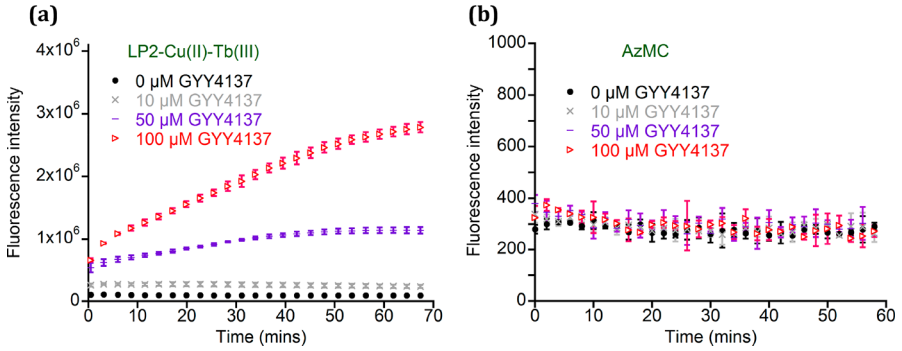

Figure 5. Comparisons of LP2-Tb(III)-Cu(II) (a) and AzMC (b) for detecting H_2S from GYY4137. (a) Time-gated detection of H_2S released from different concentrations of GYY4137 using 1 μM LP2-Tb(III)-Cu(II) in 20 mM HEPES buffer (pH 7.4). (b) Steady-state detection of H_2S released from different concentrations of GYY4137 using 20 μM AzMC in 20 mM HEPES buffer (pH 7.4).

detection lag of LP2 is due to probe responses or is an artifact of the enzyme-reaction conditions. The maximum rate of the 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 the AzMC assay (12-fold vs 5-fold, respectively, Figure 4c). Moreover, whereas 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) than that of LP2 (1 μM) or that fact that each AzMC reacts with two H_2S.[29]

Time-Gated Detection of H_2S Released from GYY4137. GYY4137 (morpholin-4-ium 4 methoxyphenyl-(morpholino) phosphinodithioate) has been used as a H_2S donor in biological studies because of its slow-release property, which better mimics the gradual H_2S release of enzymatic synthesis compared with that of Na_2S.[14,34] In solution under physiological conditions, H_2S release was shown to be slow and at a low level, with less than a 10% equivalent release after 7 days.[35] We observed an immediate increase in signal after the addition of GYY4137 (50 or 100 μM) to a solution of LP2 (1 μM). The abrupt signal increase was due to H_2S release in the donor stock solution during preparation. After the initial spike, the signal increased steadily because of the slow release of H_2S (Figure 5a). We did not observe the steady increase when the concentration of GYY4137 was 10 μM, indicating that it cannot release enough H_2S to be sensed by the probe. As a comparison, AzMC failed to respond to 100 μM GYY4137 at all within 1 h (Figure 5b). Under the conditions of a slow release and low H_2S concentrations, the ~10 nM detection limit and subminute response time favors LP2 over the more slowly reacting and less sensitive AzMC. Whereas AzMC was nonresponsive, we note that there have been reported substantially brighter azide-reduction probes based on rhodamine or fluorescein, which 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 a selective reaction with aqueous H_2S and the precipitation of a chelated, quenching Cu(II) ion. We characterized the probe using a variety of H_2S donors and compared its performance directly to that of AzMC, a representative azide-reduction-based sensor. As is typical when characterizing H_2S sensors, we measured common performance benchmarks in an aqueous Na_2S solution, including the limits of detection and the fold-changes in signal and response times. The cell-permeable probe, LP2-Ln(III)-Cu(II), met or exceeded the performances of previously reported sensors, showing a more than 100-fold change in the signal within seconds and a low-nanomolar detection limit. Both the LP2 probe and AzMC yielded easily detectable responses to enzymatically generated H_2S 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 H_2S donor, GYY4147, whereas AzMC did not respond at all. Our results show that commonly reported figures of merit, like response times or detection limits, only provide rough measures of relative sensor performance, and they do not necessarily reflect how well a given sensor will respond to small, slow changes in H_2S levels that are more reflective of biological systems. Further studies will explore the response kinetics of this and similar probes to enzymatic H_2S 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 MΩ cm^{-1}). ^1H NMR and ^{13}C NMR spectra were recorded at ambient temperature using 500 MHz spectrometers. The chemical shifts in parts per million from internal tetramethylsilane on a scale, the multiplicity (br = broad, s = singlet, d = doublet, t = triplet, q = quartet, or m = multiplet), the coupling constants (Hz), and integration are reported. 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 a UV254 fluorescent indicator. Medium-pressure liquid chromatography (MPLC) was performed to force-flow the indicated solvent system down columns that had been packed with 60 Å (40−60 μm) mesh silica gels (SiO_2). 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, catalogue 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

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luminescence measurements using 96-well microtiter plates as cuvettes were carried out on a PerkinElmer Victor 1420 multilabel counter with a delay time of 0.4 ms; a window time (counting time) of 0.7 ms; a cycling time of 1.2 ms; an excitation wavelength of 340 nm; and emission wavelengths of 545 and 615 nm for the Tb(III) and Eu(III) complexes, respectively.

Determination of Detection Limit. LP2-Tb(III)-Cu(II) (500 nM) in 20 mM HEPES buffer (pH 7.4) was added into 10 wells in a 96 well-plate. The luminescence was recorded in PerkinElmer Victor 1420 multilabel counter after incubation at 37 °C for 5 min (Ex/Em = 340/540 nm; delay time, 400 μs). Then, LP2-Tb(III)-Cu(II) was treated with Na_2S at various concentrations, and the luminescence was measured after incubation for 5 min at 37 °C. Each data point represents at least three trials. The detection limit was determined to be the concentration at which the luminescent intensity equals that of the blank plus 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, 1× MEM nonessential amino acids, and 15 mM HEPES) at 37 °C and 5% CO_2. The cells were passaged with 0.25% trypsin/2.21 mM EDTA.

Cellular Delivery of the Probe. *Cellular Delivery for the Time-Gated Images.* Cells were trypsinized and seeded at 23 000 cells/well in a poly-lysine-treated 8-well-chambered coverglass (Nunc, 12-565-470) and incubated at 37 °C and 5% CO_2 overnight. The following day, the growth medium was removed, LP2-Tb(III) (final concn of 5 mM) in fresh growth medium was added, and then the cells were incubated for 45 min at 37 °C and 5% CO_2. The cells were washed with PBS three times and immersed in growth media for imaging.

Cellular Delivery for 96-Well Plates. Cells were trypsinized, seeded at 10 000 cells/well in a 96-well plate (Costar, #3916, black, flat bottom, tissue-culture treated), and incubated at 37 °C and 5% CO_2 overnight. The following day, the growth medium was removed, LP2-Tb(III)-Cu(II) (final concn of 250 μM) in fresh growth medium was added to each well, and then the cells were incubated for 60 min at 37 °C and 5% CO_2. The cells were washed with PBS four times, and different concentrations of Na_2S were added. The signal of each well at 545 nm was monitored in a plate reader every 3 min for 30 min. At least 10 wells used to measure the signal. The plate reader was set up to automatically shake the plate for 10 s prior to the first data point.

Time-Gated Images. Images were acquired using a time-gated luminescence microscope that used 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] The images were summations of 32 frames, and large variations in the signal, resulting from the ion-feedback noise of the intensifier, were 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. The following parameters were used for imaging: excitation pulse width, 1500 μs; pulse period, 3000 μs; delay time, 10 μs; and intensifier-on time, 1480 μs. Both time-gated images were acquired at an intensifier gain of 889 V. A 340 nm UV LED and 540 ± 10 nm filter were used for excitation and emission, respectively.

CSE Expression and Purification. Human CSE cDNA was cloned into pET-28b to generate an N-terminal-His-tagged fusion protein, as previously described.[6] The expression vector was transformed and plated onto LB agar plates, supplemented with kanamycin (50 μg/mL). Recombinant CSE was expressed in *Escherichia 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 a 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. The protein concentration was then determined by a BCA protein assay. CSE was used in the enzyme assays as the His-tagged fusion protein.

Detection of H_2S Generated from CSE. 100 μL of an enzyme solution (50 mM Tris; pH 7.4; 10 μM pyridoxal 5′-phosphate; 0.1 mg/mL BSA; 1 μM LP2-Ln(III)-Cu(II); and 100, 300, or 500 nM CSE) was added into 96-well plate and incubated at 37 °C for 15 min. Immediately following the addition of the substrate (homocysteine, final concn of 1 mM), the fluorescence of the mixture was monitored for about 50 min. The plate reader was set up to automatically shake the plate for 10 s prior to the each measurement (every 3 min).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.inorgchem.7b02533.

Supporting table and figures, synthetic methods, NMR and MS data, supporting references (PDF)

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Notes

The authors declare no competing financial interest.

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