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Time-gated detection of cystathionine γ -lyase activity and inhibition with a selective, luminogenic hydrogen sulfide sensor

Yao Yao,

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

Dr. Chen Kong,

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

Dr. Liang Yin,

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

Dr. Atul D. Jain,

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

Prof. Dr. Kiira Ratia,

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

Prof. Dr. Gregory R. J. Thatcher,

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

Prof. Dr. Terry W. Moore,

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

Prof. Dr. Tom Driver, and

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

Prof. Dr. Lawrence W. Miller

Department of Chemistry, University of Illinois at Chicago, 845 W. Taylor Street, MC 111, Chicago, Illinois 60607 (USA). Institute of Next Generation Matter Transformation, College of Chemical Engineering, Huaqiao University, 668 Jimei Blvd. Xiamen, Fujian, 361021, P. R. China

Abstract

Herein, we report the design, synthesis and characterization of a lanthanide^{III} complex-based probe for the time-gated luminescence detection of hydrogen sulfide (H₂S) in aqueous media. The probe's unique sensing mechanism relies on the selective reduction of azide to amine by sulfide, followed by intramolecular cyclization to form a quinolinone. The quinolinone is a sensitizer that absorbs near-UV light and transfers excitation energy to coordinated Tb^{III} or Eu^{III} ions to trigger a strong "turn-on" luminescence response with ms-scale lifetimes characteristic of lanthanide complexes. Using this probe, we developed a robust, high throughput screening (HTS) assay for detecting H₂S generated by cystathionine γ -lyase (CSE), one of the main producers of H₂S in mammalian cells. In a 240-compound screen to identify potential CSE inhibitors, the Eu^{III} analog of the sensor showed a low false positive rate and high Z'-factor (> 0.7).

Graphical Abstract

Time-gated sensing: An emissive lanthanide probe LLPS-Ln^{III} was developed that gives a strong turn-on response to hydrogen sulfide. H₂S reduces an aryl azide moiety to trigger an intramolecular cyclization to form a quinolinone that efficiently sensitizes long-lifetime Tb^{III} or Eu^{III} luminescence (see picture). Time-gated detection permits sensitive, high-throughput screening for inhibition of H₂S-

Keywords

High-throughput screening; Hydrogen sulfide; Lanthanides; Luminescence; Sensors

Hydrogen sulfide (H₂S) is a gasotransmitter that acts as a second messenger in various cell signaling pathways.^[1] Three main pathways for enzymatic H₂S generation have been identified, including synthesis by cystathionine β -synthase (CBS), cystathionine γ -lyase (CSE) and cysteine aminotransferase/3-mercaptopyruvate sulfurtransferase (3MST).^[2] There is recent evidence that inhibiting formation of H₂S by these enzymes, and CSE in particular, may be a promising strategy in a number of therapeutic indications, including sleep apnea,^[3a] sepsis,^[3b] chronic heart failure,^[3c] and neuropathic pain.^[3d] Efforts are underway to discover novel selective inhibitors of these enzymes.^[4] Consequently, probes that can sense H₂S generated by enzymes with good selectivity and sensitivity would be very useful for drug discovery efforts, as well as for fundamental biological studies.

Numerous fluorescent probes^[5] have been described that can detect and quantify H₂S in biochemical preparations or live cells (Figure S1). In 2013, the Barrios group used the fluorogenic probe 7-azido-4-methylcoumarin (AzMC) to identify inhibitors of the H₂S-releasing enzyme CBS.^[5c] One disadvantage of fluorogenic probes is that their sensitivity is diminished when other fluorescent species are present in the sample. For example, fluorescent library compounds can generate interfering background when performing high

throughput screening (HTS), and autofluorescence of cultured cells can obscure the signal of imaging probes. Non-specific fluorescence background, however, can be eliminated by using time-gated detection in combination with ligand-sensitized complexes of lanthanide cations such as Tb^{III} or Eu^{III} that have ms-scale excited state lifetimes.^[6] With time-gating, pulsed light is used to excite the sample, and long-lived emission is detected after a μ s-scale delay during which ns-scale fluorescence from organic fluorophores decays. The high signal-to-background ratios afforded by lanthanide probes have made them a mainstay for luminescence-based bioassays and HTS and have generated considerable interest in time-gated cellular microscopy.^[7] Some bio/chemi-luminescent probes for H₂S that circumvent some of the same problems associated with biological background signals have also been reported in recent years.^[8]

Reported examples of lanthanide-based H₂S probes are rare,^[9] (Figure S2) and none of them have been utilized to study the activity of H₂S related enzymes. Herein, we describe the development of a lanthanide-based H₂S sensor that operates via a unique mechanism. This sensor exhibits a selective, >25-fold increase in long-lived emission and a sub-micromolar limit of detection for sulfide. In order to assess the probe's potential for HTS, we screened 240 compounds from the Prestwick Library of approved drugs to identify potential CSE inhibitors. The Eu^{III} analog of the sensor exhibited a high Z'-factor of 0.74 and a low false positive rate, comparable in performance to the commercially available fluorogenic H₂S probe, AzMC. By contrast, the Tb^{III} analog of the sensor had a markedly higher false positive rate that we attribute to charge transfer-mediated quenching of the relatively higher energy Tb^{III} excited state by library compounds.

Polyaminocarboxylate ligands that incorporate carbostyryl 124 (7-amino-4-methyl quinolinone) and its derivatives for lanthanide complexes with high quantum yields of emission, long luminescence lifetimes and good solubility in water.^[10] Therefore, we set out to design a turn-on sensor that contained a decadentate triethylenetetramine hexaacetic acid (TTHA) chelator and an appropriately positioned precursor moiety that would spontaneously form a quinolinone following reaction with sulfide. A previous study showed that the requisite quinolinone chromophore could be synthesized from o-aminocinnamate derivatives via photoirradiation-triggered double bond isomerization.^[11] These o-aminocinnamate derivatives, however, are thermally unstable and light-sensitive. Abe, Itoh and Saneyoshi overcame this weakness by preparing a lanthanide complex with a 2-methylcarboxylate, 2'-azidobiphenyl moiety that formed a phenanthridone following azide reduction.^[12] However, its signal could only be observed with time-gated detection, indicating a low overall quantum yield for lanthanide luminescence, possibly because the sensitizer was not coordinated to the metal center or because of a mismatch between sensitizer triplet state and lanthanide emissive state energy levels. We hypothesized that the methyl ester of 2-(2-azidophenyl)-1-cyclohexene-1-carboxylic acid would serve as an ideal lanthanide antenna precursor. In the presence of sulfide, the azide would be reduced to amine, which would cyclize with the proximal ester to produce a quinolinone with photophysical properties nearly identical to carbostyryl 124.

To test our hypothesis, we prepared the chelator, dubbed Luminogenic Lanthanide Probe for Sulfide (LLPS, Figure 1). The conformationally locked antenna was readily synthesized by

cross-coupling 2-azidoarylboronic pinacolate 1 with vinyl triflate 2—derived in one-step from the β -ketoester of cyclohexanone—using conditions developed in the Driver laboratory (Figure 2a).^[13]

To investigate its photophysical properties, solutions of LLPS-Ln^{III}, where Ln = Tb or Eu, were prepared. As shown in Figure S3, no absorption above 320 nm was detected in the ring-open form and strong absorbance peaking at 328 nm and 340 nm was detected in the ring-closed form. This showed that quinolinone was formed in the presence of Na₂S (a commonly employed H₂S precursor) because carbostyryl 124 has absorption peaks at 328 and 340 nm. The emission spectra also showed that the probe did not emit fluorescence upon 340 nm excitation without Na₂S. When Na₂S was added, however, typical lanthanide luminescence emission was observed (Figure S3). These results validated the design of the probe whereby sulfide-mediated reduction of the aryl azide triggers quinolinone formation to yield lanthanide luminescence.

Next, we examined the sensitivity of LLPS-Ln^{III} for H₂S. Upon treatment of 20 μ M of the LLPS-Ln^{III} with 100 μ M Na₂S, a robust increase in lanthanide emission intensity was observed. As shown in Figure S4–S5, it produced 3-fold turn-on response after one hour of exposure to Na₂S, which markedly increased to >25-fold after 8 hours. Also, the emission intensity of the probe gradually grew as the concentrations of Na₂S increased (Figure 2b). To further establish the utility of the 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 (Figure 2c). The linear relationship established a quantitative detection method for sulfide with detection limit of 200 nM (Figure S6).

We next evaluated the selectivity of the probe towards H₂S in comparison to other biologically relevant anions and reducing species. In contrast to the 26-fold luminescence response obtained using 100 μ M of Na₂S, almost no response was observed using 1 mM of cysteine, glutathione (GSH), Na₂SO₄ and other reducing agents (Figure 2d).

To investigate the origin of the sluggish response time of the probe, the reduction of the antenna with Na₂S was examined using ¹H NMR spectroscopy (Figure 3). We anticipated that the slow step would be cyclization to form the amide because Pluth reported that the aryl azide reduction by sulfide is fast (within one hour).^[14] If cyclization was slow, then accumulation of the aniline might be visible using spectroscopy. In line with this hypothesis, following the reaction progress using ¹H NMR spectroscopy revealed rapid (within 5 min) formation of an intermediate, whose spectrum was consistent with the aniline. This species slowly disappeared over time until after 4 h when quinolinone was the major species (52%) in solution. We've also found that increasing the temperature dramatically increased the reaction rate (Figure S7).

Lanthanide-based sensors are especially useful for drug screening applications because time-gated elimination of short-lived fluorescence from compound libraries reduces the rate of false positive results. To demonstrate the utility of LLPS-Ln^{III} for use in HTS, we first monitored the ability of this probe to detect formation of H₂S generated by full-length CSE.

Despite the relatively long time required for full-scale sensor response, both the Tb^{III} (Figure 4) and Eu^{III} (Figure S8) probes showed significant and easily observable increases in luminescence within 3 h in the presence of enzyme and substrate. Moreover, luminescence was substantially lower in the presence of the well-studied CSE inhibitor, L-propargyl glycine (L-PAG).^[15] This is noteworthy because there are relatively few robust assay systems for measuring CSE inhibition, and most of them rely on colorimetric^[5c] or fluorimetric^[15] readouts.

Encouraged by the success of detecting H₂S produced by CSE, we then carried out a small pilot screen for inhibitors of CSE using 240 compounds from the Prestwick Chemical Library. Three screens were performed (library conc., 40 μM) using LLPS-Tb^{III}, LLPS-Eu^{III} and, for comparison, AzMC. In all cases, the positive control compounds L-PAG showed full inhibition in the assay (Figure 5), and cycloserine (a known CSE inhibitor) was also identified as a top hit. One compound, (–)-Eseroline fumarate salt, was falsely identified as a hit for all three probes, most likely because the electrophilic fumarate can react with H₂S directly (Figure 6). Similar Z'-factors of 0.74 and 0.71 were calculated for LLPS-Eu^{III} and AzMC, respectively, while a lower value (0.58) was calculated for LLPS-Tb^{III} (Figure S9).

While only two false positive hits were observed with LLPS-Eu^{III} and AzMC, eight were detected with LLPS-Tb^{III} (Figure 5). This result suggests that the luminescent product of LLPS-Tb^{III} with H₂S is more likely to be quenched by library components than that of LLPS-Eu^{III} with H₂S. Differential quenching of otherwise identical Tb^{III} and Eu^{III} species is consistent with the work of Kielar, *et al.* which showed that Tb^{III} complexes, compared with Eu^{III} complexes, are more susceptible to dynamic quenching by anti-oxidants like ascorbate, urate or catechols.^[16] The Tb^{III} ⁵D₄ and Eu^{III} ⁵D₀ excited states lie at 244 kJ/mol and 206 kJ/mol above their respective ground states and consequently, quenching of Tb^{III} is thermodynamically more favorable. We confirmed that the false positive hits identified in the screen efficiently quenched the product of Tb^{III} probe but not the product of Eu^{III} probe (Figure S10). We also observed similar results with TTHA-Cs124-Ln^{III}, a luminescent complex that has a nearly identical structure to the product of LLPS-Ln^{III} (Figure S10). A likely mechanism involves a charge transfer process mediated by formation of a transient excited state complex (exciplex) between the quencher and the heterocyclic sensitizing moiety. We observed non-linearity in complex lifetime as a function of quencher concentration as well as reduced quenching at higher temperature (Figure S11), and both of these results support the previously described, exciplex quenching model^[16].

In this work, we successfully designed and synthesized a novel lanthanide-based probe for the quantitative time-gated luminescence detection of sulfide in aqueous media, including CSE-generated H₂S. LLPS-Ln^{III} emits strongly following reaction with Na₂S with a limit of detection of about 200 nM. LLPS-Eu^{III} is the first lanthanide-based H₂S sensor that has been validated for HTS with a low false positive rate (2 in 240) and high Z' factor (> 0.7). Future studies will be aimed at improving the sensor response time by structurally altering the antenna to increase the rate of amide formation and by conjugating the probe to cell penetrating peptides to facilitate intracellular delivery and time-gated cellular imaging and analyses of H₂S metabolism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
Chemical structure of the probe LLPS-Ln^{III} and its luminescence response mechanism with H₂S.

Figure 2.

(a) Synthesis of the probe LLPS. (b) Steady-state luminescence response of 20 μM LLPS- Tb^{III} increase linearly with sulfide concentration in 20 mM HEPES buffer (pH 7.4) following overnight incubation. The inset shows the integrated intensity (540–550 nm) versus concentration of Na_2S . (c) Time-gated luminescence response of 50 nM LLPS- Eu^{III} increase linearly with sulfide concentration following overnight incubation in 96-well plate. (d) Fold-change in luminescence intensity of 20 mM HEPES buffer (pH=7.4) containing LLPS- Tb^{III} (20 μM) and Na_2S (100 μM) or other indicated species (1 mM) after overnight incubation.

Figure 3.

Partial ^1H NMR response of compound 3 to sodium sulfide. 30 mM compound 3 was treated with 50 mM Na_2S in DMSO-d_6 , affording 75:25 mixture of compound 5 and 6 in 5 mins (Figure 3b), 63:37 mixture in 1 hour (Figure 3c) and 48:52 mixture in 4 hours (Figure 3d).

Figure 4.

Utility of LLPS-Tb^{III} as H₂S probe and assay for CSE activity. (a) LLPS-Tb^{III} provides a sensitive assay for CSE activity in the presence of CSE (500 nM), LLPS-Tb^{III} (100 nM), Cys (2.0 mM), PLP (5 μM), HEPES pH 7.4 (50 mM), glutathione (10 mM). L-Propargylglycine (50 μM) was used as the inhibitor in the assay. Error bars represent standard deviation.

Figure 5.

Results from HTS for the inhibitors of CSE using probes (a) LLPS-Tb^{III}, (b) LLPS-Eu^{III} and (c) AzMC. Assays carried out in the presence of TRIS-HCl pH 8.5 (50 mM), BSA (0.1 mg/mL), PLP (100 μ M), Triton-X 100 (0.01%), homocysteine (2 mM), CSE (500 nM), library compound (40 μ M), LLPS-Ln^{III} (100 nM) or AzMC (20 μ M). Red are high signal control (without L-PAG), black are library compounds (40 μ M) and green are low signal control (with 100 μ M L-PAG).

Figure 6.

False positives found in HTS assay of LLPS-Tb^{III}. False positive for LLPS-Eu^{III} were Eseroline fumarate and Rifabutin and for AzMC Eseroline fumarate.