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Development of a Highly Selective Fluorescence Probe for Hydrogen Sulfide

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Supporting Information

ABSTRACT: Hydrogen sulfide (H_2S) has recently been identified as a biological response modifier. Here, we report the design and synthesis of a novel fluorescence probe for H_2S, HSip-1, utilizing azamacrocyclic copper(II) ion complex chemistry to control the fluorescence. HSip-1 showed high selectivity and high sensitivity for H_2S, and its potential for biological applications was confirmed by employing it for fluorescence imaging of H_2S in live cells.

Although hydrogen sulfide (H_2S) is a toxic gas with the characteristic smell of rotten eggs, recent studies have regarded H_2S as the third gaseous transmitter, in addition to nitric oxide (NO) and carbon monoxide (CO). H_2S appears to be involved in various physiological processes, including relaxation of vascular smooth muscles,[1] mediation of neurotransmission,[2] inhibition of insulin signaling,[3] and regulation of inflammation and O_2 sensing.[4,5] There are several methods for selective detection of H_2S, among which the most commonly used are the methylene blue method and the sulfide ion-selective electrode method.[6,7] However, these methods are destructive, requiring homogenization of samples. Therefore, for detailed studies of the physiological functions of H_2S, a new method to measure H_2S concentration in cells is required. We focused on fluorescence imaging, because it is suitable for nondestructive detection of targeted biomolecules in live cells or tissues with readily available instruments.[8] Thus, we set out to develop a sensitive and selective fluorescence probe for H_2S. This involves two substantial challenges. One is to attain sufficient selectivity over other biothiols; i.e., the probe has to be able to measure endogenous H_2S without interference from other biothiols, including reduced glutathione (GSH, present at levels of about 1−10 mM), L-cysteine (L-Cys, about 100 μM), and thiol-containing proteins.[9] The other challenge is to achieve sufficient sensitivity, because the concentration of H_2S required to elicit physiological responses has been reported to be 10 μM−1 mM, although there remains some controversy as to the intracellular H_2S levels generated in response to physiological stimuli.[1−3,10]

A few fluorescence probes for H_2S have been reported, utilizing sulfide anion-triggered removal of a 2,4-dinitrobenzenesulfonyl group or reduction of azide to amine.[11−13] However, the reactions are relatively slow and show poor selectivity for H_2S over reactive oxygen species (ROS), such as superoxide (O_2^-) or Na_2SO_3.[12,14] Recently, Chang et al. have reported that a dipicolylamine (DPA)−fluorescein complex with Cu^{2+} (DPA-AF+Cu^{2+}) showed a selective turn-on fluorescence response to sulfide anion.[15] We confirmed that DPA-AF+Cu^{2+} could detect 10 μM H_2S in an aqueous solution at pH 7.4 (Supporting Information, Figure S1), but this probe also showed fluorescence enhancement upon addition of 10 mM GSH. We hypothesized that improvement of the chelator−Cu^{2+} complex stability in the presence of biothiols would result in a good selectivity for H_2S at low concentrations in the presence of high concentrations of GSH. It is well known that azamacrocyclic rings form stable metal complexes with Cu^{2+}, and the paramagnetic Cu^{2+} center has a pronounced quenching effect on fluorophores.[16] On the basis of these facts, we expected that Cu^{2+} would be released from the azamacrocyclic ring when H_2S binds to the Cu^{2+} center, resulting in fluorescence enhancement, whereas the azamacrocyclic Cu^{2+} complex would retain its structure in the presence of high concentrations of GSH, showing no fluorescence enhancement.

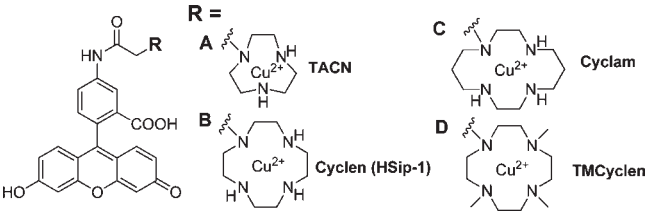

Figure 1. Structures of four macrocyclic fluorescein−Cu^{2+} conjugates: (A) TACN-AF+Cu^{2+}, (B) Cyclen-AF+Cu^{2+} (HSip-1), (C) Cyclam-AF+Cu^{2+}, and (D) TMCyclen-AF+Cu^{2+}.

On the basis of this hypothesis, we designed and synthesized four sensor probes based on a fluorescein scaffold conjugated with an azamacrocyclic Cu^{2+} complex, employing 1,4,7-triazacyclononane (TACN), 1,4,7,10-tetraazacyclododecane (Cyclen), 1,4,8,11-tetraazacyclotetradecane (Cyclam), and *N,N′,N″*-trimethylcyclen (TMCyclen) as chelators for Cu^{2+} instead of DPA (Figure 1 and Supporting Information, Schemes S1−S4).

Next, we examined the sensitivity and selectivity of the synthesized compounds for H_2S over GSH by measuring the fluorescence increment upon addition of 10 μM H_2S or 10 mM GSH. TACN-AF+Cu^{2+} showed high sensitivity and low selectivity (Figure S2), while Cyclam-AF+Cu^{2+} (Figure S3) and TMCyclen-AF+Cu^{2+} (Figure S4) showed low sensitivity and high

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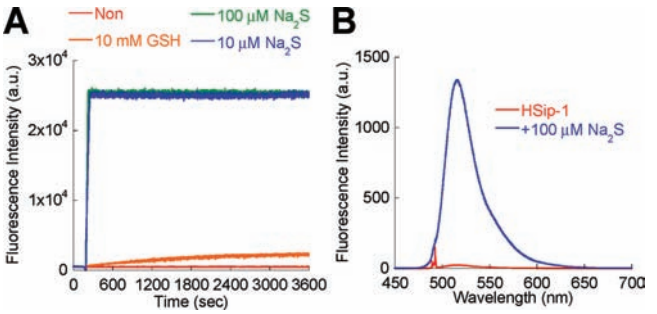

Figure 2. (A) Time course of reactions of HSip-1 with no addition (red) or addition of 10 mM GSH (orange), 100 μM Na_2S (green), or 10 μM Na_2S (blue) in 30 mM HEPES buffer (pH 7.4) at 37 °C. Na_2S and GSH were added at 180 s. Ex/Em = 491/516 nm. (B) Fluorescence spectra of 1 μM HSip-1 before (red) and after (blue) reaction with 100 μM Na_2S in 30 mM HEPES buffer (pH 7.4).

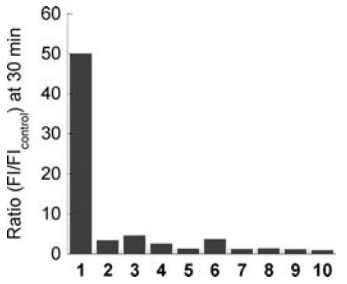

Figure 3. Fluorescence enhancement of 1 μM HSip-1 in the presence of thiols, inorganic sulfur compounds, and sodium ascorbate. Bars represent relative fluorescence intensity 30 min after addition of thiols, inorganic sulfur compounds, or sodium ascorbate. The reactions of 1 μM HSip-1 with (1) 10 μM Na_2S, (2) 10 mM GSH, (3) 1 mM L-Cys, (4) 1 mM DL-Hcy, (5) 1 mM 2-ME, (6) 100 μM DTT, (7) 1 mM NaSCN, (8) 1 mM Na_2SO_3, (9) 1 mM $Na_2S_2O_3$, and (10) 10 mM sodium ascorbate were performed in 30 mM HEPES buffer (pH 7.4) at 37 °C.

selectivity. Fortunately, Cyclen-AF+Cu^{2+} (HSip-1 = hydrogen sulfide imaging probe-1) showed excellent properties as a fluorescence probe for H_2S (Abs_{max}/Em_{max} = 491/516 nm, Φ_{fl} = 0.019 in 30 mM HEPES buffer at pH 7.4); i.e., it showed a large and immediate increment of fluorescence intensity by 50-fold upon addition of 10 μM H_2S, whereas almost no fluorescence increment was observed upon addition of 10 mM GSH (Figure 2). In addition, the fluorescence enhancement induced by H_2S was retained even in the presence of 10 mM GSH (Figure S5). HSip-1 also showed high selectivity over other thiols (1 mM L-Cys, DL-Hcy, 2-mercaptoethanol (2-ME), and 100 μM dithiothreitol (DTT)), inorganic sulfur compounds (1 mM NaSCN, Na_2SO_3, and $Na_2S_2O_3$), and a reducing condition (10 mM sodium ascorbate) (Figures 3 and S6). Moreover, HSip-1 did not show any fluorescence enhancement in response to ROS or reactive nitrogen species (RNS), such as hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$), peroxynitrite ($ONOO^-$), hypochlorite (^-OCl), superoxide ($O_2^{\bullet-}$), singlet oxygen ($^1O_2$), and nitric oxide ($^{\bullet}NO$). Upon addition of Angeli's salt (NO^- donor), a small fluorescence enhancement was observed (Figures S7 and S8). Thus, HSip-1 offers high selectivity for H_2S compared with previously reported fluorescence probes utilizing a 2,4-dinitrosulfonyl group or an azide group.

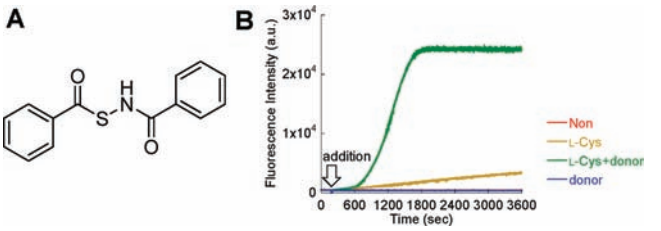

Figure 4. (A) Structure of cysteine-activated H_2S donor. (B) Time course of reactions of 1 μM HSip-1 with no addition (red) or addition of 50 μM H_2S donor (blue), 1 mM L-Cys (yellow), or 50 μM H_2S donor + 1 mM L-Cys (green) in 30 mM HEPES buffer (pH 7.4) with 0.1% tetrahydrofuran as a cosolvent at 37 °C. H_2S donor and L-Cys were added at 180 s, as indicated by an arrow. Ex/Em = 491/516 nm.

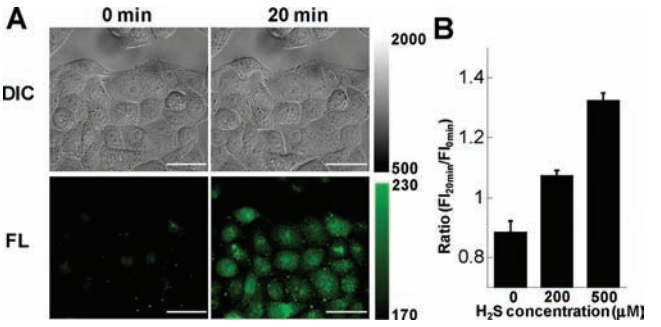

Figure 5. Visualization of H_2S in live cells using HSip-1 DA. HeLa cells were incubated with 30 μM HSip-1 DA in DMEM containing 0.3% DMSO for 30 min. (A) Differential interference contrast (DIC) and fluorescence (FL) images were captured after addition of 500 μM Na_2S in HBSS solution. (B) Average $FI_{20\ min}/FI_{0\ min}$ intensity ratios in fluorescence images after addition of 0, 200, or 500 μM Na_2S in HBSS buffer. The excitation and emission wavelengths were 470−490 and 515−550 nm, respectively. Scale bar, 10 μm. Representative fluorescence images from replicate experiments (n = 3) are shown. Error bars are ±SD.

Xian et al. have reported cysteine-activated H_2S donors which mimic the slow and continuous endogenous biosynthesis of H_2S from L-Cys mediated by enzymes such as cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE).[17] We synthesized this H_2S donor (Figure 4A) to examine the feasibility of using HSip-1 to monitor pseudo-enzymatic H_2S production. We could detect time-dependent H_2S release associated with decomposition of the H_2S donor by using HSip-1 in the presence of 1 mM L-Cys (Figure 4B).

The spectroscopic properties of HSip-1, as well as its selectivity for H_2S, seemed appropriate for cellular application, so we next considered the suitability of HSip-1 for fluorescence imaging of cellular H_2S. However, this probe is membrane-impermeable because of its hydrophilicity derived from the carboxyl group and he cyclen−Cu^{2+} complex moiety. Therefore, we employed a standard diacetylation approach to improve the membrane permeability and synthesized diacetylated HSip-1 (HSip-1 DA); this was confirmed to be a membrane-permeable precursor that is hydrolyzed to HSip-1 by intracellular esterases (Scheme S2). Next, HeLa cells were incubated with 30 μM HSip-1 DA containing 0.3% DMSO as a cosolvent in Dulbecco's modified Eagle's medium (DMEM) for 30 min and then washed with Hanks' Balanced Salt Solutions (HBSS), and various concentrations of Na_2S (0, 200, or 500 μM) were added to the medium. Upon addition of 500 μM Na_2S, a large intracellular fluorescence enhancement was observed, while no significant fluorescence

increment was seen in the absence of Na_2S (Figure 5). The results of CCK-8 assay showed that HSip-1 DA exhibits no cytotoxicity at concentrations up to 100 μM (Figure S9).

In summary, we have developed a novel fluorescence probe for H_2S, HSip-1, based on azamacrocyclic Cu^{2+}complex chemistry. HSip-1 can sensitively detect H_2S in aqueous solution with high selectivity over biothiols, inorganic sulfur compounds, ROS, and RNS and has excellent photophysical properties for biological applications, arising from its fluorescein scaffold. We confirmed that HSip-1 could be used both for detection of pseudo-enzymatic H_2S release in a cuvette and for real-time fluorescence imaging of intracellular H_2S in live cells. In addition, we also confirmed that HSip-1 could detect H_2S produced by 3-mercaptopyruvate sulfurtransferase (3MST), or lysate of 3MST-expressing cells, and 3-mercaptopyruvate, i.e., H_2S-producing enzyme and its substrate (Figure S10).[18] We anticipate that HSip-1 will be useful for high-throughput screening of CBS, CSE, and 3MST agonists and antagonists, as well as for detailed investigation of a wide range of biological functions of H_2S.

■ ASSOCIATED CONTENT

Supporting Information. Synthesis; experimental details; characterization of developed compounds; and experiments using living cells. This material is available free of charge via the Internet at http://pubs.acs.org.

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