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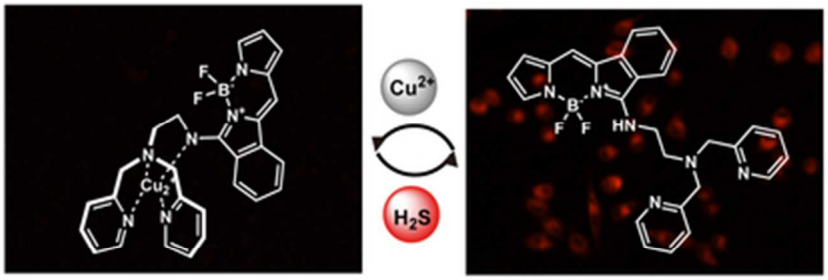

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ARTICLE TYPE

A Highly Sensitive and Water Soluble Fluorescent Probe for Rapid Detection of Hydrogen Sulfide in Living Cells

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A highly sensitive and specific fluorescent probe has been developed for the rapid detection of H_2S in living cells. This probe exhibits the unique qualities of quick response, low detection limit, excellent water solubility and good membrane permeability. Its potential for biological applications has been demonstrated by imaging H_2S in living cells.

Hydrogen sulfide (H_2S) is traditionally viewed as a toxic gas with the characteristic odor of rotten-eggs. However, more recent studies have established it as the third gaseous transmitter after nitric oxide (NO) and carbon monoxide (CO).[1] H_2S at physiological level exerts fine modulatory control over cellular functions by signaling an array of processes such as neuromodulation in the brain and smooth muscle relaxation in the vascular system.[2, 3] Endogenous H_2S is synthesized in mammalian tissues in a controlled fashion and dysregulation of H_2S production or metabolism is reported being linked to a variety of disease phenotypes including Alzheimer's disease,[4] diabetes,[5] hypertension,[6] *etc*. To date, many details on the physiological and pathological roles of H_2S are still under active investigation. Therefore, it is important to have robust assays for H_2S detection in its native environment to facilitate detailed biological function studies.

Current methods available for the detection of H_2S, including colorimetric, electrochemical,[6] and gas chromatography assays,[7] are limited by low resolution, complicated requirements for sample preparation and poor biocompatibility, which forbid the real time monitoring of H_2S production, trafficking, and consumption in living cells, tissues, and whole organisms. As such, fluorescent molecular probes offer an appealing approach for the detection of H_2S attributed to their nondestructive attribute and high sensitivity.[8, 9] A number of fluorescent probes have recently been developed to measure H_2S in living cells or in whole blood. These probes, judiciously designed by making use of the nucleophilic or reducing properties of H_2S, undergo chemoselective reactions with H_2S in the native cellular environment and their fluorescence properties are altered as a consequence.[10, 11] However, most of these probes are limited by two substantial challenges. One is their sluggish reaction kinetics with H_2S, as exemplified by our previous probe **ZS1**, which utilized an aldehyde–acrylate group to trap H_2S and required 50 minutes to reach its highest fluorescent response.[12] The other is their poor water-solubility. Being poor water-soluble, most probes can only be used in mixed solvents containing a large portion of non-biocompatible organic solvents for fluorescent spectrometry analysis, which greatly limits their application. Given the diffusive and highly reactive nature of H_2S, more sensitive probes with faster response and improved water-solubility are still highly desirable.

In the present work, we are interested in devising new probes by taking advantage of the extremely strong affinity and facile kinetic profile between S^{2-} and Cu^{2+}. Indeed, several fluorescent probes have been developed employing this strategy, including **HSip-1** from Nagano group, **L1Cu** from Zeng group and **Cu-1** from Shen group (Fig. 1).[13] However, they are limited by their poor membrane permeability or poor water solubility.[13] Herein we disclose a highly sensitive and selective H_2S probe, namely **ZS2-Cu**, with good water solubility and excellent membrane permeability. The probe was assembled by complexing Cu^{2+} with the BODIPY-based ligand **ZS2** (Fig. 1). Our design of **ZS2-Cu** was based on the following facts: 1) BODIPY fluorophore represents a desirable fluorescent scaffold for the design of live-organism imaging probes owning to its prominent photophysical properties and excellent membrane permeability;[14] 2) *N*, *N*-bis(2-pyridylmethyl)ethylenediamine may serve as a coordinating group for Cu^{2+} to quench the fluorescence of **ZS2** after binding Cu^{2+} because of the paramagnetic character of the cation;[15] 3) in the presence of S^{2-}, Cu^{2+} would be rapidly de-coordinated from the probe to release ligand **ZS2** and therefor restore the strong fluorescence of the system, yielding a quick and sensitive response towards S^{2-}; 4) in addition to its function to chelate Cu^{2+}, *N*, *N*-bis(2-pyridylmethyl)ethylenediamine also helps to improve the water solubility of the probe. Besides, fluorescent ligand **ZS2** is readily accessible due to the well documented nucleophilic reaction between α-chloroBODIPY and *N*-centered nucleophiles.[16]

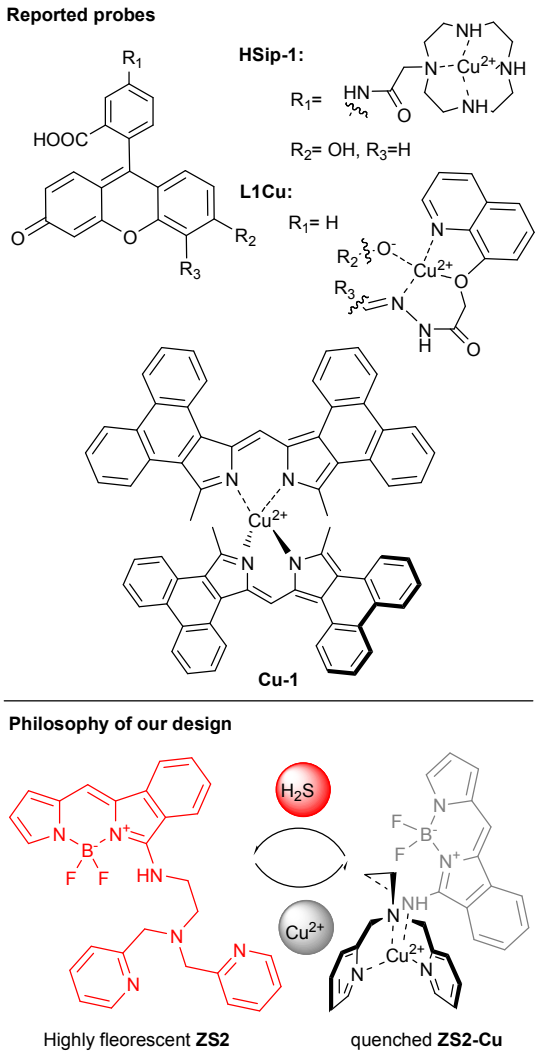
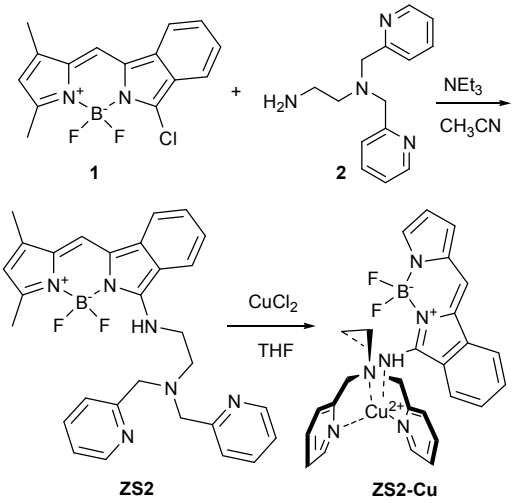


Fig. 1 Structures of reported probes and the philosophy of our design

With all these considerations, **ZS2** and **ZS2-Cu** were synthesized as outlined in Scheme 1. Nucleophilic substitution of α-Chloro-BODIPY (**1**) with *N*, *N*-bis(pyridin-2-ylmethyl)ethane-1,2-diamine (**2**), both of which were prepared according to literature procudeures,[17, 18] readily yielded **ZS2**. Treatment of **ZS2** with copper (II) chloride in THF in the dark gave **ZS2-Cu** quantitatively. The 1:1 stoichiometry of the complex was confirmed by both mass spectrometry and a Job's plot (Fig. S1). The nitrogen atom directly attached to the BODIPY core in **ZS2** was speculated being involved in the chelation by UV-vis spectra analysis. **ZS2** alone in PBS showed an absorption band with the maximum peak centered at 494 nm (ε= 17,150), which can be assigned to the lowest-lying π-π* transition of the BODIPY core, while the addition of Cu^{2+} caused a blue-shift of 18 nm (λ_{max}= 476 nm, ε= 17,940), presumably due to the decreased donation effect of the lone pair electrons on the *N* atom to the π system of BODIPY after chelation (Fig. S2).



Scheme 1 Synthetic procedures for **ZS2** and **ZS2-Cu**

With these probes in hand, we first measured their water solubility employing the UV spectrometry method. As expected, **ZS2-Cu** exhibited an improved water solubility of 1.28 ± 0.02 g/L. This advantage greatly facilitated following cuvette-based experiments which were carried out solely in aqueous solution without the need for organic co-solvent. The fluorescent properties of **ZS2** without or with the presence of Cu^{2+} were then investigated. As shown in Fig. 2, **ZS2** alone in PBS exhibited an intense emission centered at 546 nm (λ_{ex} 480 nm, **Φ** 0.1040), which was gradually attenuated by the addition of increasing amount of Cu^{2+}, indicating the coordination of **ZS2** with Cu^{2+}. The system reached 90% quenching when 1.0 eq of Cu^{2+} in total was added, in agreement with the 1:1 complexation stoichiometry. Significantly, the fluorescent turn-off response of **ZS2** was specific to Cu^{2+} with other cations including Ca^{2+}, Cd^{2+}, Cr^{3+}, Fe^{3+}, K^{+}, Na^{+}, Ni^{2+}, Pb^{2+}, Sn^{4+}, Zn^{2+} and Fe^{2+} triggering no obvious responses (Fig. S3). Besides, **ZS2** was able to respond to Cu^{2+} even in the presence of the above mentioned metal ions, implying it could be utilized as a Cu^{2+}-selective fluorescent sensor.

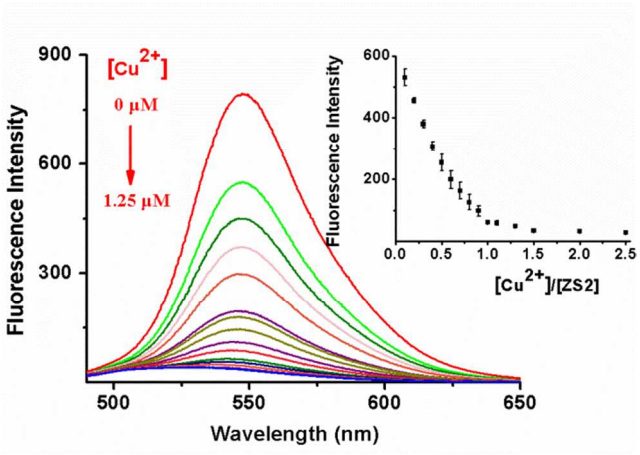


Fig.2 The turn-off responses of **ZS2** towards Cu^{2+}. The addition of increasing concentrations of Cu^{2+} gradually quenched the fluorescence of **ZS2** (0.5 μM). Spectra were taken in PBS (10 mM, pH 7.4, 25°C) immediately after the addition of Cu^{2+} (λex 480 nm). The slit width was 5 nm for both excitation and emission. Inner panel: correlation between the

emission intensity at 546 nm of the system (y) and the molar ratio of Cu^{2+} to **ZS2** (x)

Next, probe **ZS2-Cu** was examined for its ability to sense S^{2-} with NaHS as an aqueous sulfide source. As expected, **ZS2-Cu** alone in PBS was almost nonfluorescent (**Φ** 0.004), while the addition of NaHS triggered an immediate increment in fluorescence intensity. A maximum fluorescence enhancement factor of 19 was achieved at 546 nm when 2.0 eq of NaHS was added. Interestingly, the fluorescent spectrum of the system taken right after the addition of NaHS could overlap with that taken after an incubation time of 30 minutes, verifying rapid response of the probe. Moreover, both the shape and intensity of the emission band of the system resembled those of **ZS2**, indicating the de-coordination of Cu^{2+} from the probe and therefore the release of **ZS2** from the complex because of the higher affinity between Cu^{2+} and S^{2-}. Noteworthy, the fluorescence enhancement of the system was linear to the concentration of NaHS up to 20 μM, with the detection limit to be as low as 250 nM, indicating the great potential of **ZS2-Cu** to quantify H_2S (Fig. 3).

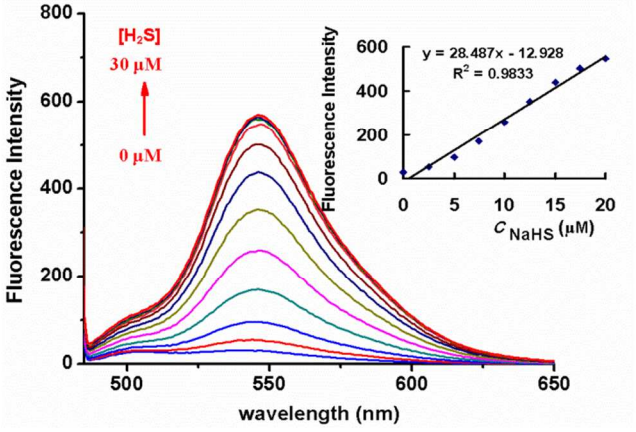


Fig. 3 The turn-on fluorescence responses of **ZS2-Cu** towards NaHS. The gradual addition of NaHS restored the fluorescence of the system. Spectra were taken in PBS buffer (10 mM, pH 7.4, 25°C) instantly after the addition of NaHS with excitation to be 480 nm. The slit width was 3 nm for both excitation and emission. Concentration of **ZS2-Cu** was 10 μM and those of NaHS ranged from 0 to 30 μM. Inner panel: regression equation between the fluorescence intensity at 546 nm (y) and the corresponding concentration of NaHS **(x)**

Furthermore, the specificity of **ZS2-Cu** was evaluated by measuring its response after exposure to various common anions and biothiols in PBS buffer. As shown in Fig. 4, among all the anions tested, including bio-relevant F^-, Cl^-, Br^-, I^-, $H_2PO_4^-$, NO_3^-, CO_3^{2-}, ClO^-, and sulfur oxyanions such as SO_3^{2-}, SO_4^{2-}, $S_2O_3^{2-}$, *etc*, no species other than S^{2-} was able to induce a fluorescence enhancement. Moreover, common biothiols such as reduced glutathione (GSH) and L-cysteine (L-Cys) which are the major interferents jeopardizing the specificity of reported H_2S probes, proved to be innocent in this case. Actually, **ZS2-Cu** exhibited more than 30-fold selectivity towards sulfide over other anions and biothios. Based on these preliminary results, we next investigated whether the fluorescent response of **ZS2-Cu** towards NaHS could be affected by the presence of other anions or biothiols. Much to our delight, **ZS2-Cu** was still able to respond to NaHS with 15-18 fold fluorescence increasement in the coexistence of other anions or biothiols and the resultant fluorescent profile was pretty much the same as that without the presence of other competitive analytes (Fig. S4). These results taken together demonstrated the high selectivity and sensitivity of **ZS2-Cu** for H_2S and its feasibility to detect H_2S in the complex biological samples.

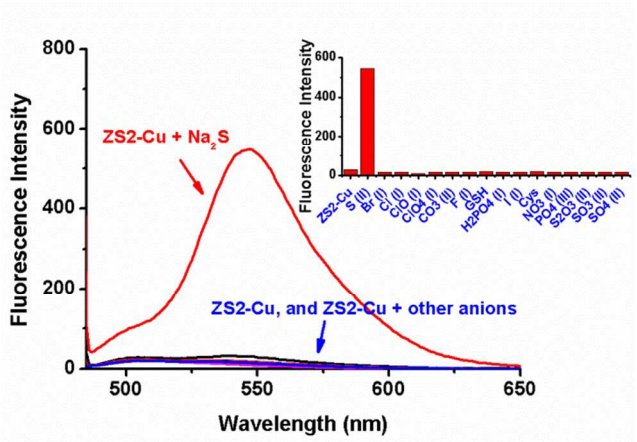


Fig.4 Fluorescence emission spectra of **ZS2-Cu** (10 μM) in PBS buffer (10 mM, pH 7.4, 25°C) right after the addition of NaHS or other biologically relevant anions and biothiols (all were kept at a final concentration of 20 μM) (λex = 480 nm). The slit width was 3 nm for both excitation and emission. Inner panel: emission intensity at 546 nm (y) when different species (x) were added

Finally, the ability of **ZS-2Cu** to visualize H_2S in living cells was assessed. HeLa cells were incubated with **ZS-2Cu** (5 μM) for 15 min and weak fluorescence was observed (Fig. 5a). The medium was then removed and cells were washed with PBS to remove any extracellualar probe. The cells were then treated with NaHS (100 μM) for 15 min which resulted in a dramatic increase in intracellular fluorescence (Fig. 5c). These results illustrated the excellent membrane permeability of **ZS2-Cu** and its ability to detect H_2S sensitively in living cells. It is noteworthy that live-cell imaging experiments also demonsrated that **ZS2** could act as a dual sensor for both Cu^{2+} and H_2S with the former gave a turn-off response and the latter restoring the strong fluorescent signal (Fig. S5).

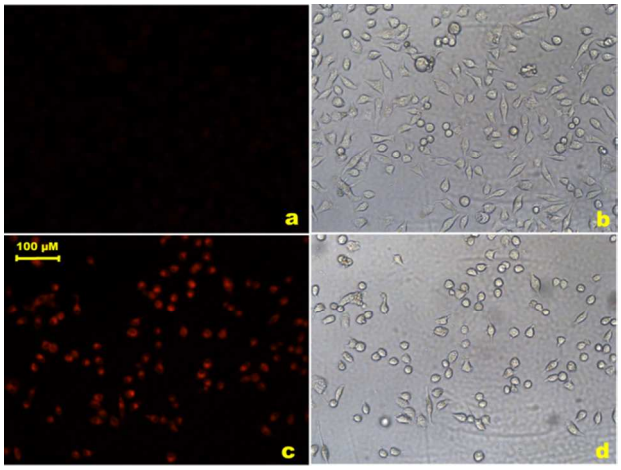


Fig.5 Imaging H_2S in live cells with **ZS-2Cu**. Intact HeLa cells were treated with **ZS-2Cu** (5 μM) for 15 min before imaging (a, b). Cells were then washed with PBS and incubated with NaHS (100 μM) for a further

15 min (c, d). Images were collected on a fluorescence microscope (LEICA DMI 4000B) upon excitation at 488 nm (a, c) or under bright field (b, d)

Conclusions

In summary, we have devised a highly specific and sensitive fluorescent probe for the rapid detection of H_2S in living cells employing the inorganic chemistry-based principle of precipitating copper with sulfide from the copper-complexing quenched probe and therefore releasing the fluorescent signal of the probe. Representing an appealing tool for H_2S analysis in biological samples, this probe exhibited rapid response towards H_2S, good water solubility and excellent membrane permeability. Besides, it responded to NaHS with a linear fluorescent enhancement and thus has the potential to be utilized for H_2S quantification. Its feasibility to monitor H_2S in living cells was also confirmed.

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Notes and references

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† Electronic Supplementary Information (ESI) available: experimental details for the synthese of **ZS2**, **ZS2-Cu**, NMR traces, biological methods and additional figures. See DOI: 10.1039/b000000x/

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