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A two-photon fluorescent probe with near-infrared emission for hydrogen sulfide imaging in biosystems†

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Hydrogen sulfide (H_2S) is emerging as an important gasotransmitter but remains difficult to study. Here we report a novel two-photon fluorescent probe with NIR emission for H_2S detection. It was successfully used to realize H_2S imaging in bovine serum, living cells, tissues as well as in living mice.

Hydrogen sulfide (H_2S), which is believed to have been critical to the origin of life on earth, remains important in physiology and cellular signalling.[1] H_2S is now considered the third member of the gasotransmitter family, along with nitric oxide (NO) and carbon monoxide (CO).[2] It is generated from L-cysteine in reactions catalyzed by cystathionine-synthase (CBS) or cystathionine-lyase (CSE), and converted within different organs and tissues.[3] As a signal molecule, H_2S appears to be involved in various physiological processes, including modulation of neuronal transmission, regulation of release of insulin, relaxation of the smooth muscle and reduction of the metabolic rate.[4] For example, in animal models of critical illness, H_2S donors protect from lethal hypoxia and reperfusion injury and exert anti-inflammatory effects.[5] The physiologically relevant H_2S concentration is estimated to range from nano- to millimolar levels.[6] Once the cells cannot maintain the level within the physiological range, H_2S is involved in diseases, such as Alzheimer's disease, Down syndrome, diabetes and other diseases of mental deficiency.[7] Therefore, accurate and reliable measurement of H_2S concentrations *in vivo* is needed to provide useful information to study the function of H_2S in depth.

In recent years, a few analytical technologies have been developed to detect various environmental, medical and cellular H_2S.[8] Among these detection technologies, fluorescence-based assays have found widespread application in the fluorescence imaging of various analytes in living cells, tissues, and organisms because of the rapid, nondestructive, selective, and sensitive advantages of emission signals.[9] Actually, although the related research work on H_2S fluorescent probes has just begun from the year 2011, there are a few examples based on three different H_2S specific reactions. They made use of its reducibility, nucleophilicity and strong complexation ability with Cu^{2+}, respectively.[10–12] Among these reactions, it is a good choice to introduce an azido group into probes to be reduced by H_2S due to the simple synthesis, relatively good selectivity, suitable reaction time, and non-cell toxicity.[10]

Compared with the traditional one-photon microscopy imaging technique (OPM), TPM (two-photon microscopy) utilizes two-photons of lower energy to obtain the excited state of a fluorophore, and thus has more advantageous features over OPM including less phototoxicity, better three dimensional spatial localization, deeper penetration depth and lower self-absorption.[13] However, almost all reported TP probes have short emission wavelengths ranging from 380 nm to 550 nm which limit their biological applications. Therefore, it is of great value to develop a new TP fluorescent probe with NIR (near-infrared) emission for H_2S determination which will definitely have more practical applications.

Keeping the above ideas in mind, by introducing a styrene group to increase its emission wavelength to the NIR region, we synthesized a conjugate extended benzopyran derivative **1-NH$_2$**, which exhibited the desired TP absorbing property. We tested the photophysical properties of the compound in different solvents. **1-NH$_2$** showed unique spectroscopic characteristics, such as emission wavelength in the NIR region, a large Stokes shift (>100 nm in different solvents), good $\Phi\delta_{max}$ values (50 GM at 820 nm in DMSO) and stable spectroscopic properties over a biologically relevant pH range (Table S1 and Fig. S1a, ESI†). Upon irradiation from a 500 W *I-W* lamp for more than 4 h, the maximal fluorescence intensity of **1-NH$_2$** remained nearly constant which is much better than a commercial Cy5 NIR dye in terms of photostability (Fig. S1b, ESI†). On the basis of compound **1-NH$_2$**, we next prepared a reactive H_2S probe **1** by replacing the amino group with an azido group. **1** can selectively react with H_2S to produce **1-NH$_2$** as the final product and thus result in significant fluorescence changes. Further fluorescence bioimaging investigations have indicated that **1** can be used as a fluorescent probe for monitoring H_2S in living cells, tissues and *in vivo* in mice. To the best of our knowledge, this is the first successful fluorescent probe for H_2S detection in living mammals.

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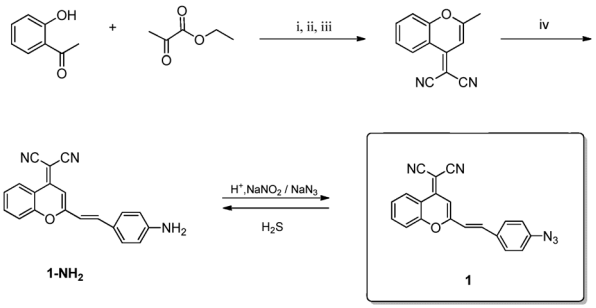

Scheme 1 Synthesis of probe **1** and the proposed mechanism of response of **1** to hydrogen sulfide. (i) Ethyl pyruvate with Na in $CH_3COOC_2H_5$, 4 h, 53%; (ii) AcOH, H_2SO_4, 30 min, 76.9%; (iii) malononitrile, AcOH, H_2SO_4, 14 h, 32.5%; (iv) toluene, piperidine, AcOH, 3 h, 42.9%.

The benzopyran derivative **1** (Scheme 1) was prepared from 1-(2-hydroxyphenyl)ethanone by a five-step procedure under mild conditions with a good yield. The structures of **1** and other major intermediates were well characterized using ^{1}H NMR, ^{13}C NMR, and HPLC-MS (see ESI†).

We then tested the optical properties of probe **1** in PBS buffer (0.1 M, pH 7.4, 50% DMSO). UV-vis spectra of **1** (5 μM) exhibited two absorptions at around 400 nm and 425 nm. After treatment with 50 equiv. of NaHS (a commonly used hydrogen sulfide source) at 37 °C, the absorption at 400 nm and 425 nm apparently decreased, whereas a new absorption peak appeared at about 505 nm. Such a large red shift of 90 nm in the absorption behavior changed the color of the solution from yellow to orange red, allowing colorimetric detection of H_2S by the naked eye (Fig. S2, ESI†). Accordingly, the emission at 670 nm strongly appeared upon excitation at 520 nm (Fig. 1a), which means that the azido group of **1** can be converted efficiently into fluorescent **1-NH$_2$**. Within 60 min of reaction under these conditions, a 65-fold turn-on response of the fluorescent signal was observed (Fig. S4, ESI†). Different concentrations of NaHS were then added to the test solution, and the fluorescence intensity increased linearly with the concentration of H_2S from 25 μM up to 250 μM (Fig. 1b and S5, ESI†). Thus, the detection limit (3σ/slope) was as low as 3.05 μM.

The turn-on response of probe **1** for H_2S was tested over reactive oxygen species (ROS), biologically relevant sulfide species (RSS) and reducing reagents including hypochlorite (ClO^-), sulfite (SO_3^{2-}), bisulfite (HSO_3^-), thiosulfate ($S_2O_3^{2-}$), dithionite ($S_2O_4^{2-}$), cysteine (Cys), homocysteine (Hcy), glutathione (GSH), I^-, Fe^{2+} and Cu^+. In the test system, 50 equiv. of H_2S and 100 equiv. of other analytes were added, respectively, to a 5 μM solution of **1** and the fluorescence response was monitored over 60 min. As show in Fig. 2, only H_2S could cause robust fluorescence intensity enhancement while others exhibited almost no changes in fluorescence behaviour. The excellent selectivity for H_2S over other relative analytes shows that probe **1** has potential applications for detection of H_2S in a complex biological environment. The pH titration curve (Fig. S1a, ESI†) also reveals that probe **1** reaches almost a constant minimal value at pH 2.5–10, demonstrating that **1** can work in a wide pH range without influence.

The favorable fluorescence properties of **1** for H_2S prompted us to further establish its utility for the determination of sulfide in biosystems. **1** was first evaluated to detect H_2S in commercial fetal bovine serum. Upon addition of H_2S (40 equiv.) to the serum solution of **1** (5 μM), the fluorescence intensity increased quickly and reached saturation after 40 min of reaction (Fig. S6a, ESI†). Bovine serum solutions containing NaHS at different concentrations (5, 10, 20, and 40 equiv.) were also prepared. These serum solutions were then incubated with 5 μM probe **1**. After 40 min, a good linear relationship between fluorescence intensity and NaHS concentrations was also obtained in this solution, indicating that **1** can respond to H_2S levels in serum without addition of any co-solvents or detergents (Fig. S6b, ESI†). Importantly, the 5 μM concentration of **1** used in this experiment was much lower than that of other reported H_2S probes (100–200 μM).[10a,11a,b] The low concentration of the probe will be able to greatly reduce the damage to biological systems.

We next acquired fluorescence imaging of H_2S in living cells utilizing an Olympus FV1000-IX81 confocal microscope. Using 800 nm TP excitation in a scanning lambda mode, MCF-7 cells incubated with only probe **1** (5 μM) for 30 min at 37 °C in PBS exhibited weak fluorescence at an emission window of 575–630 nm (Fig. 3a), suggesting that **1** is immune to metabolism of various intracellular species under the assay conditions. Whereas addition of H_2S (50 equiv.) to the above cells resulted in a distinct change in the observed fluorescence after incubation for another 60 min (Fig. 3c). Photo imaging of H_2S in HeLa cells was also successfully realized with excitation at 515 nm (Fig. S8, ESI†). The effect of a hypoxic atmosphere on the probe was also examined according to the reported method.[14] Although hypoxia could cause low-level fluorescence intensity in living cells (Fig. S10, ESI†), the effect can be effectively avoided when performing an *in vivo* experiment. These data indicate that probe **1** is cell membrane permeable and capable

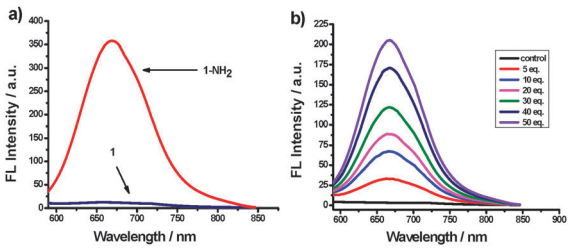

Fig. 1 (a) Fluorescence spectra of **1** (5 μM) and **1-NH$_2$** (5 μM) in PBS buffer (0.1 M, pH 7.4, 50% DMSO). (b) Fluorescence change in **1** (5 μM) after incubation with different concentrations of H_2S (5, 10, 20, 30, 40 and 50 equiv.) for 60 min. Conditions: PBS buffer (0.1 M, pH 7.4, 50% DMSO) at 37 °C, λ_{ex} = 520 nm, each spectrum was obtained after 60 min of reaction.

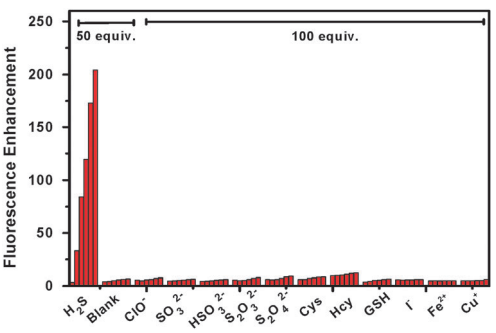

Fig. 2 Fluorescence responses of probe **1** (5 μM) to biologically relevant ROS, RSS and reducing reagents. Bars represent relative responses at 670 nm at 0, 5, 10, 20, 40 and 60 min after addition of analytes. Data are shown for 50 equiv. of H_2S and 100 equiv. of other analytes. Conditions: PBS buffer (0.1 M, pH 7.4, 50% DMSO) at 37 °C, λ_{ex} = 520 nm.

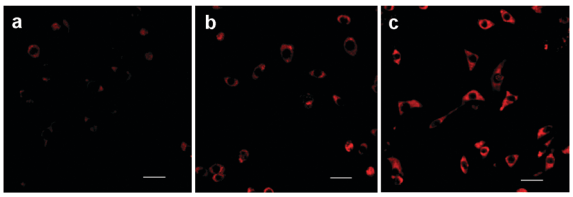

Fig. 3 Fluorescence imaging of H_2S in MCF-7 cells incubated with 5 μM probe **1**. Cells were incubated with **1** for 30 min (a), after which 50 equiv. of H_2S was added. After further incubation for 30 min (b) and 60 min (c) the cells were imaged. λ_{ex} = 800 nm, emission window (575 nm–630 nm). Scale bars: 20 μm.

of imaging H_2S in different living cells by using both OPM and TPM. Cytotoxicity test using MCF-7 cells showed that probe **1** and the corresponding product **1-NH$_2$** have almost no toxicity to living cells (Fig. S11, ESI†).

We further investigated the utility of probe **1** in tissue imaging. Since it takes a longer time to stain the tissues during which they may be deformed, an excess amount (20 μM) of **1** was used to facilitate staining. A fresh rat liver cancer slice incubated with 20 μM probe **1** for 60 min showed weak fluorescence in the whole region. After incubation with H_2S (50 equiv.) for another 60 min, there was an even and significant increase in fluorescence intensity excited at 800 nm (Fig. S12, ESI†). Furthermore, depth scanning demonstrated that the corresponding product **1-NH$_2$** was capable of tissue imaging at depths of 60–220 μm by TPM (Fig. S13, ESI†).

Finally, we examined the suitability of the sensor for visualizing H_2S in living animals. ICR mice were selected and divided into two groups. One group was given an s.p. (skin-pop) injection of probe **1** (40 μM, in 25 μL DMSO) on the back of ICR mice as the control experiment. The other group was then given an s.p. injection of 25 equiv. of NaHS (25 μL, 0.1 mM PBS) after the disposal of the control mice. The two groups were imaged using a NightOWL II LB983 small animal *in vivo* imaging system with a 530 nm excitation laser and a 655 ± 20 nm emission filter. Fig. 4 shows a representative fluorescent image of mice treated with probe **1** and NaHS at different times after injection and demonstrates that the fluorescence intensity became strong gradually within 4 hours. Whereas the control experiment shows almost no fluorescence, proving that probe **1** can detect H_2S *in vivo* without the interference of background signals (Fig. S14a, ESI†). Taken together these experiments established that **1** is a desired imaging agent used for visualizing H_2S *in vivo* for the first time.

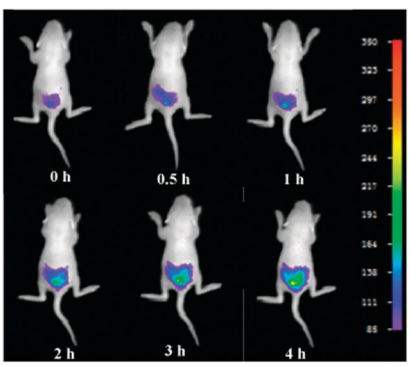

Fig. 4 Representative fluorescence images of mice (pseudo-color) given an s.p. injection of probe **1** (40 μM, in 25 μL DMSO) and then injected with 25 equiv. of NaHS (25 μL, 0.1 mM PBS). Images were taken after incubation of NaHS for different times (0, 0.5, 1, 2, 3 and 4 h).

In summary, we presented a new fluorescent probe **1** for specific H_2S detection with two-photon absorption and NIR emission. It exhibited a significant fluorescence response for H_2S over other reactive oxygen species and biologically relevant reactive sulfide species. Thus it is suitable for H_2S detection in bovine serum with a quite low probe concentration. Living cells and tissue imaging established the utility of this probe for tracking H_2S in biological systems using TPM microscopy. More importantly, the probe was first successfully utilized to realize H_2S imaging in living mice. Taken together, **1** is a robust imaging agent for H_2S detection both *in vitro* and *in vivo*. We expect this new probe to be useful in more chemical and biological applications.

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