A near-infrared “turn-on” fluorescent probe with a self-immolative linker for the in vivo quantitative detection and imaging of hydrogen sulfide

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A near-infrared (NIR) fluorescent probe with a self-immolative linker is introduced as an in vivo hydrogen sulfide (H2S) detection tool. Detection of H2S, an endogenously produced gaseous signaling compound, is of great importance for understanding its biological functions. The NIR-Az probe allows for both concentration- and time-dependent detection of H2S, demonstrating its utility in monitoring H2S levels. This approach provides a powerful tool for probing H2S chemistry in living systems.

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ABSTRACT
Hydrogen sulfide is a critical biological messenger, but few biocompatible methods are available for its detection in vivo. Here, we describe the design and synthesis of a novel azide-functionalized near-infrared (NIR) fluorescent probe, NIR-Az, for in vivo H2S detection. The NIR-Az probe is a powerful approach for probing hydrogen sulfide chemistry in biological systems.

1. Introduction
Hydrogen sulfide, an endogenously produced gaseous signaling compound and critical biological messenger, has recently been recognized as a gasotransmitter along with nitric oxide and carbon monoxide. For many pathologies, the production of endogenous H2S has been demonstrated to be closely correlated with particular diseases, such as relaxing vascular smooth muscle, inducing the vasodilation of isolated blood vessels, and reducing blood pressure. Although the hydrogen sulfide level in biological systems is known to be related to numerous physiological and pathological processes, many underlying molecular events remain unknown. Furthermore, the concentration of hydrogen sulfide has been demonstrated to be closely correlated with particular diseases; for example, hydrogen sulfide is excessively produced in patients with sepsis and is found at very low levels in patients with Down syndrome and Alzheimer's disease. The hydrogen sulfide concentration in the blood ranges from 10 to 100 μM, thereby providing a powerful approach for probing hydrogen sulfide chemistry in biological systems.

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sulfide precipitation (Doeller et al., 2005; Han et al., 2006; Jayarajan and Annachhatre, 2013; Tangerman, 2009; Vosoughi et al., 2015; Yang et al., 2016), which often require complex sample processing. Moreover, hydrogen sulfide is rapidly catabolized, thus resulting in continuous changes in its concentration and hence making accurate analysis difficult (Lin et al., 2015). The existing detection methods have limitations in terms of their response rate, accuracy, and lack of real-time determination; the most important impediment in sensing hydrogen sulfide is the lack of efficient sensing agents that allow for its rapid and accurate detection.

Among the recently developed technologies for the biological detection of hydrogen sulfide, fluorescence-based methods allow for greater selectivity and convenience, less invasiveness, high in situ sensitivity, and the ability to perform real-time imaging (Huang et al., 2016; Hammers et al., 2015; Li et al., 2015b; Sathyadevi et al., 2015). Several fluorescent probes have been designed on the basis of the reactions of hydrogen sulfide in solutions or in living cells resulting in the reduction of azide or nitro groups on a fluorogenic moiety such as rhodamine, fluorescein or naphthalimide (Li et al., 2015a; Liu et al., 2012; Montoya and Pluth, 2012; Thorson et al., 2013). The known unique reduction of an azide group with hydrogen sulfide can be useful in developing a sulfide-sensitive agent (de Silva et al., 1997). Moreover, the strong nuclophilic character of the fluorogenic moiety accelerates the reduction of the azide group (Yang et al., 2003). However, these organic hydrogen sulfide probes are not applicable to bioimaging beyond a certain depth because of the shallow tissue penetration depth of ultraviolet (UV)/visible light (as an excitation source). In most circumstances, these fluorescent probes are also not suitable for long-term assays because of photo-bleaching. Near-infrared (NIR) dye-based probes are attractive for bioimaging because of their spectral activity in the NIR region, which results in minimal photodamage and minimum interference from background autofluorescence in living systems. Unlike visible light, which penetrates only a few hundred micrometers into the tissue surface, NIR light can penetrate millimeters to centimeters into living tissue (Iverson et al., 2013; Kim et al., 2009). Therefore, the use of NIR fluorophores, that is, molecules that convert one NIR wavelength into another, can provide a high signal-to-background ratio (SBR) (Choi et al., 2013). Thus, the development of new types of fluorescent probes that can be used for hydrogen sulfide detection under various physiological conditions is required, preferably probes showing absorption and fluorescence emission spectra and high photostability within the NIR region.

Herein, we report the selective, sensitive detection of hydrogen sulfide in living cells and mice with a new azide-functionalized NIR probe, NIR-Az, through analyte-dependent nuclophilic amination and successive self-immolation, thus leading to dose-dependent “turn-on” fluorescence. In comparison with conventional always-on fluorescent probes, turn-on fluorescent probes exhibit noticeable advantages, including a high SBR, which results in enhanced contrast and sensitivity of the probe in the process of analyte detection and biological imaging (Fong et al., 2016; Jia et al., 2016; Jiang et al., 2016a, 2016c). Unfortunately, however, this benefit has mostly been applied to visible light-emitting probes. In our study, the introduction of a self-immolative linker between the azide moiety and phenolic dihydroxanthene fluorophore from cyanine dyes can greatly reduce steric hindrance and enhance the accessibility of NIR-Az to hydrogen sulfide in the amination reaction. We developed the NIR-Az probe by treating chloro-substituted cyanine with 4-chlororesorcinol in the presence of a base (compound 1), followed by the addition of compound 3. Compounds 2 and 3 used in the synthesis of compound 1 were newly synthesized, as shown in Scheme S1. The formation of compound 1 includes a sequence of several reactions such as nucleophilic substitution, the retro-Knoevenagel reaction, a cyclization reaction and a dehydration reaction (Yuan et al., 2012). To enable self-immolation through the intramolecular 1,6-elimination of the p-aminobenzyl moiety, an azide group was introduced to make the chromophore responsive to hydrogen sulfide. If the self-immolative process can be triggered by hydrogen sulfide, a marked change in fluorescence signal will be obtained (Chan et al., 2012; Steiger et al., 2016). The current work describes the superior chemical stability and optical properties of a newly synthesized fluorescent probe, NIR-Az, and its use in time- and concentration-dependent ratiometric fluorescence monitoring of hydrogen sulfide in living cells and mice, thereby highlighting its value as an NIR fluorescent platform. Furthermore, because of its good biocompatibility and rapid cell internalization, this probe also exhibits the potential for real-time monitoring of endogenous hydrogen sulfide activity in living cells and animals.

2. Materials and methods

2.1. Synthesis of compound 1

4-Chlororesorcinol (270 mg, 1.87 mmol) and triethylamine (Et3N) (0.65 mL, 4.67 mmol) were placed in a flask containing dry dimethylformamide (DMF) (2.7 mL), and the mixture was stirred at room temperature under an argon atmosphere for 30 min. IR-780 iodide (500 mg, 0.75 mmol) in dry DMF (1.0 mL) was added to the mixture via a syringe, and the reaction mixture was heated and stirred at 50 °C for 4 h. The solution was then removed under reduced pressure. The crude product was purified by silica column chromatography using CH2Cl2/EtOH (30:1) as the eluent to produce compound 1 as a blue-green solid (283 mg, yield 66%). 1H NMR (CDCl3, 600 MHz): δ 8.39 (d, 1H, J = 14.4 Hz), 7.45 (s, 1H), 7.39–7.36 (m, 3H), 7.24 (t, 1H, J = 7.2 Hz), 7.12 (d, 1H, J = 7.2 Hz), 7.09 (s, 1H), 6.01 (d, 1H, J = 14.4 Hz), 4.07 (t, 2Hm, J = 7.2 Hz), 2.74 (t, 2H, J = 5.4 Hz), 2.66 (t, 2H, J = 5.4 Hz), 1.93 (m, 4H), 1.71 (s, 6H), 1.07 (s, 3H, J = 7.8 Hz) ppm. 13C{1H} NMR (CDCl3, 120 MHz): δ 171.86, 167.48, 162.05, 155.53, 142.41, 140.65, 140.12, 137.63, 128.64, 127.43, 125.99, 124.80, 122.28, 121.98, 115.13, 114.21, 110.40, 103.81, 98.78, 49.10, 45.99, 29.67, 28.60, 28.33, 24.42, 20.73, 20.68, 11.7 ppm. HRMS (m/z): Calcd. for [M]+ 446.18813, found 446.18965.

2.2. Synthesis of NIR-Az

Compound 1 (75 mg, 0.13 mmol) and K2CO3 (55 mg, 0.39 mmol) were placed in a flask containing dry CH2Cl2/CH3CN (1:1, 1.0 mL), the mixture was stirred at room temperature under an argon atmosphere for 30 min, and then compound 3 (89 mg, 0.39 mmol) was added. After an overnight reaction, the solvent was removed under reduced pressure. The crude product was purified by silica column chromatography using CH2Cl2/EtOH (60:1) as the eluent to produce the NIR-Az compound as a blue solid (47 mg, yield 51%). 1H NMR (CDCl3, 600 MHz): δ 8.66 (d, 1H, J = 15 Hz), 7.62 (d, 2H, J = 8.4 Hz), 7.60 (d, 1H, J = 7.8 Hz), 7.49 (t, 1H, J = 7.8 Hz), 7.43–7.40(m, 2H), 7.38 (d, 1H, J = 14.4 Hz), 7.14 (s, 1H), 7.12 (s, 1H), 7.06 (d, 2H, J = 8.4 Hz), 6.55 (d, 1H, J = 15 Hz), 5.44 (s, 2H), 4.49 (t, 2H, J = 7.2 Hz), 2.78 (t, 2H, J = 6 Hz), 2.73 (t, 2H, J = 6 Hz), 2.01–1.97 (m, 2H), 1.94–1.91 (m, 2H), 1.89 (s, 6H), 1.11 (t, 3H, J = 7.8 Hz). 13C{1H} NMR (CDCl3, 120 MHz): δ 177.99, 160.84, 156.74, 152.74, 145.84, 142.12, 141.36, 139.81, 132.87, 129.08, 129.04, 128.47, 127.71, 127.58, 122.96, 120.76, 119.15, 115.95, 115.03, 112.70, 104.72, 101.94, 71.43, 51.13, 47.68, 29.25, 28.67, 24.75, 21.37, 20.14, 11.66, 1.01 ppm. HRMS (m/z): Calcd. for [M]+ 577.23648, found 577.23663.

2.3. Density functional theory (DFT) calculation

The software package ORCA 3.0.3 was used for the calculation. The geometries of the complexes were optimized by the DFT method using the BP86 functional. The basis set from Ahlrichs (def2-SVP) was used for all atoms. The resolution-of-identity approximation for both Coulomb integrals and HF exchange integrals were used with the matching auxiliary basis set.
2.4. Quantum yield

The fluorescence quantum yields for NIR-Az were determined by using Rhodamine 6 G (Φ_F=0.95 in ethanol) as a fluorescence standard. The quantum yield was calculated using the following equation:

\[ \Phi_{F,X} = \Phi_{F,S} (A_X/A_S) (n_S/n_X)^2 \]

where \( \Phi_F \) is the fluorescence quantum yield, \( A \) is the absorbance at the excitation wavelength, \( F \) is the area under the corrected emission curve, and \( n \) is the refractive index of the solvents used. The subscripts \( S \) and \( X \) indicate the standard and unknown, respectively.

2.5. Fluorometric analyses

Fluorescence intensity was measured at 37 °C using a Scinco FS-2 spectrometer. A 10-μM acetonitrile (ACN) solution of NIR-Az was diluted with phosphate-buffered saline (PBS) buffer (10 mM, pH 7.4, 30% acetonitrile, v/v), and then NaHS was added as a hydrogen sulfide source. The resulting solution was incubated for 30 min. The fluorescence intensity was measured (\( \lambda_{\text{ex}}=680 \text{ nm} \)) with the excitation and emission slit widths set at 5 nm and 10 nm, respectively. The emission spectra were recorded from 700 to 820 nm at a velocity of 1200 nm min^{-1}. Data are shown as the mean ± SD (n=3).

2.6. Macrophage culture and imaging

Raw 264.7 murine macrophages were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in an atmosphere of 5% CO₂ and 95% air at 37 °C. After 24 h, the cover slips were rinsed lightly 3 times with Dulbecco’s phosphate-buffered saline (DPBS) to remove the medium, and then the cells were cultured in DPBS for later use. For the verification procedure, 5 μM NIR-Az was added to the above cellular samples, which were then incubated for 30 min. The samples were then lightly rinsed 3 times with DPBS, and the cells were incubated with NaHS (0, 20, 40 and 80 μM) in the medium for 60 min. Prior to imaging, the cells were washed 3 times with DPBS. Fluorescence images were acquired on a confocal microscope (Olympus Fluoview 1000) using a 40× oil-immersion objective.

2.7. Cytotoxicity test (CCK-8 assays)

HeLa cells were plated in flat-bottomed 96-well plates at a density of 5000 cells/well in 200 μL of DMEM (GIBCO, 11885) supplemented with 10% (v/v) FBS and 1% penicillin/streptomycin in a humidified incubator with 5% CO₂ in air at 37 °C. After the cells were incubated for 24 h, NIR-Az (with 30% acetonitrile as a cosolvent) was added to the cellular sample plate. After incubation for 1 h, 10 μL of CCK-8 solution (Dojindo, Japan) was added to each plate well, and the cells were further incubated for 30 min. The absorbance at 450 nm was measured with a microplate reader (SpectraMax M2/Molecular Devices).

2.8. Fluorescence imaging in living mice

Balb/c nude mice (15–22 g) were anesthetized by i.p. injection of xylazine (10 mg kg^{-1}) and ketamine (80 mg kg^{-1}). For the concentration-dependence experiment, the mice were i.p. injected with NIR-Az (50 μM, in 20 μL of DMSO) and then i.p. injected with different concentrations of NaHS (0, 10, 20, 40, and 80 μM).
amounts of NaHS (0, 50, 100 or 200 μM, in 100 μL of PBS). After 30 min, the mice were imaged using an IVIS Lumina II in vivo imaging system with an excitation filter of 675 nm and an emission filter of 695–770 nm. For the time-dependence experiment, the mice were i.p. injected with NIR-Az (50 μM, in 20 μL of DMSO) and then i.p. injected with NaHS (100 μM, in 100 μL of PBS). The mice were then imaged using an IVIS Lumina II in vivo imaging system at different times (0, 0.5, 1, 2 or 3 h) with a 675-nm excitation filter and a 695- to 770-nm emission filter. All animal care was carried out in accordance with the guidelines from the Korea Research Institute of Bioscience and Biotechnology (KIRIBB), and all experimental protocols were approved by KIRBB-IACUC (approval number: KIRBB-AEC-15036).

3. Results and discussion

3.1. Synthesis of the fluorescent probe

The treatment of the chloro-substituted cyanine “IR 780 iodide” with 4-chlororesorcinol in the presence of the base triethylamine at 50 °C for 4 h in DMF did not produce a straightforward nucleophilic substitution product of the chlorine atom. Instead, a phenolic dihydroxanthene NIR-emitting fluorophore, compound 1, with a chlorine atom, which originated from 4-chlororesorcinol, was obtained and characterized by 1H and 13C NMR (Fig. S1). The mechanistic pathway for the formation of compound 1 is plausibly represented in Scheme 1b. The cyanine structure of IR 780 iodide and 4-chlororesorcinol could lose its Fischer base via a retro-Knoevenagel reaction, thereby yielding an assumed half-cyanine intermediate a; this possibility is based on the chemistry of the formation of a cyanine dye that would condense with the Fischer base and yield a cyanine dye (Wolinska et al., 2009). The Knoevenagel-type condensation reaction is reversible. The half-cyanine intermediate a subsequently became the much more stable compound 1 through cyclization and dehydration reactions. Hydrogen sulfide participates in nucleophilic substitution as a reactive nucleophile in biological systems. Most hydrogen sulfide probes based on the reduction of aromatic azides have shown a delayed response time (>20 min) to hydrogen sulfide (Lin et al., 2015). To improve the reaction rate, an electron-withdrawing group, fluorine, can be introduced at the ortho-position of the aromatic azide (Wei et al., 2014). Along with physiological properties of the aromatic azide group, the introduced functional group on the enol site of our probe affected properties such as the fluorescence intensity, response time, selectivity and cell permeability. The NIR-Az probe can be easily obtained through the reaction between compound 1 and compound 3 (Scheme 1a). Although azide derivatives typically display low fluorescence intensities, the fluorescence-on response is obtained after the reduction to the fluorescent amine counterpart, which is strongly based on the thiolate-triggered reaction in the presence of hydrogen sulfide (Srikun et al., 2008; Zhang et al., 2011). A possible hydrogen sulfide-selective signaling mechanism is shown in Scheme 1a. We verified that the fluorescence of NIR-Az by reaction with hydrogen sulfide appeared in two steps, with the reaction mechanism strongly supported by the electrospray-ionization mass spectrometry spectra (Fig. S5). After the addition of hydrogen sulfide, the phenolic dihydroxanthene fluorophore released via the amination and self-immolative reaction from NIR-Az, as previously ascribed to its two types of molecular ion peak ([1]+ and [1- Na]+) at m/z 446.1 (m/z calculated for C28H28NO2Cl: 468.1) and m/z 468.1 (m/z calculated for C28H28NO2ClNa: 486.2), thus leading to the generation of strong fluorescence emission at 720 nm upon photoirradiation. The relative intensity of the [1- Na]+ peak is approximately 5 times that of the peak at m/z 446.1 corresponding to a hydroxylated fragmented molecule, [1]+. This intensity difference may indicate that the [1- Na]+ corresponding to the peak at m/z 468.1 is more stable than the [1]+ peak.

DFT calculation was carried out to estimate the electronic structure of the key compounds, including NIR-Az (Fig. 1). In the molecular orbital distribution, clear differences were observed before and after the self-immolation. Remarkably, in the case of NIR-Az, a dense electron cloud was localized on the azide unit at the HOMO, whereas the electron cloud was delocalized over the phenolic dihydroxanthene unit at the LUMO. In other words, the azide unit significantly contributes to the highest energy occupied molecular orbital in NIR-Az, which may lead to the drastic change in fluorescence after the self-immolation triggered by hydrogen sulfide. The bandgap energies were calculated to be 1.16, 1.67, and 1.61 eV for NIR-Az, [1]+, and [1- Na]+, respectively, which reasonably matched the experimental findings.

3.2. Spectral response to hydrogen sulfide

The spectroscopic properties of NIR-Az were obtained under physiological conditions, and the hydrolysis of NIR-Az by hydrogen sulfide at different time scales was monitored by fluorimetry. We investigated the fluorescence spectra of NIR-Az and its reaction with hydrogen sulfide by using NaHS (a common hydrogen sulfide source) in PBS buffer (10 μM, pH 7.4) at 37 °C. NIR-Az exhibited UV absorption bands at approximately 550–680 nm, and this was followed by an increase in a new absorbance peak at 700 nm after treatment with hydrogen sulfide (Fig. S6). However, the fluorescence emission band of NIR-Az itself was very weak (data not shown), a result consistent with its non-emissive character because of the quenching effect of the 7-hydroxy substitution at the phenolic dihydroxanthene fluorophore via photoinduced electron transfer (Chen et al., 2015). Fig. 2a shows the changes in the fluorescence emission spectra when hydrogen sulfide was added to the PBS buffer containing the NIR-Az probe (10 μM). The treatment of NIR-Az with NaHS for 30 min resulted in a marked increase in fluorescence intensity. These spectral changes clearly demonstrate that the fluorescence turn-on by the self-immolative process was triggered by hydrogen sulfide (up to 200-fold enhancement at 720 nm). We determined the time-dependent fluorescence responses of NIR-Az after the addition of 20 equiv. of hydrogen sulfide by establishing a correlation between the fluorescence signal at 720 nm and the corresponding time; the results showed that the reaction was completed within approximately 30 min of incubation. The background fluorescence of NIR-Az was extremely weak, and within minutes, a marked increase in fluorescence was observed because of the reaction of NIR-Az with hydrogen sulfide. We determined the pseudo-first-order rate constant, kobs, to be 1.45×10−3 s−1 for NIR-Az by fitting the data using a single exponential function. This result revealed that the turn-on response intensity of NIR-Az reached a steady state after approximately 30 min of incubation. The time-dependent fluorescence response demonstrates that NIR-Az can detect hydrogen sulfide both qualitatively and quantitatively. Furthermore, the time scale enables NIR-Az to detect hydrogen sulfide in real-time fluorescence imaging in biological systems, such as living cells and animals. Inspired by our initial results, we further examined the fluorescence signal change of NIR-Az in the presence of various concentrations of hydrogen sulfide. After the addition of increasing concentrations of hydrogen sulfide (0–50 μM), we observed a fluorescence-signal increase produced by a large fluorescence turn-on response, where the ratio of emission intensities (I720 nm/I680 nm) varied from 0.042 to 1.95, over 30 min of reaction time without any background correction (Fig. 2a). This enhancement factor is high for an NIR fluorescent probe and demonstrates the ability of NIR-Az to quantify different hydrogen sulfide concentrations. As predicted, NIR-Az after hydrogen sulfide treatment exhibited a high quantum yield (Φfl=0.72) in aqueous medium when excited at the λmax (680 nm) of NIR-Az; this value is relatively large for an NIR dye. The linear relationship of the fluorescence responses suggests that NIR-Az can be used to determine reaction-time- and concentration-dependent fluorescence responses of hydrogen sulfide through measurement of the fluorescence at 720 nm. The linear function also allows for easy and exact analysis, and good linearity was observed between the triggered
fluorescence and the hydrogen sulfide concentration in the range of 0–50 μM, with a detection limit of less than 0.26 μM (Fig. 2b); this detection limit is below the previously reported range of hydrogen sulfide concentrations (20–100 μM) found in mammalian blood (Hyšpler et al., 2002).

3.3. Selectivity

After establishing the time- and concentration-dependent reactivity of NIR-Az with hydrogen sulfide, we evaluated the selectivity profile of the probes for hydrogen sulfide and various biologically relevant species, such as sulfur, oxygen, and nitrogen species (RSONs). We investigated the fluorescence response by hydrogen sulfide for NIR-Az only and for the mixed solution of NIR-Az and analytes. Sulfur-containing inorganic ions (S$_2$O$_3^{2-}$, SO$_4^{2-}$, SO$_3^{2-}$, and SCN$^-$), an inorganic salt (NaH$_2$PO$_4$), an organosulfur compound (α-lipoic acid), a reactive oxygen species (H$_2$O$_2$), reactive nitrogen species (NO, NO$_3$, and NO$_2$), thiols (L-cys, homo-cys, and glutathione) and L-ascorbic acid were used as analytes and were demonstrated to be chemically inert toward the probes. Overall, 16 analytes were screened, and no obvious responses were observed for most of the analytes at a concentration of 200 μM, comparable to that of sulfide. On the basis of previous reports using hydrogen sulfide as a reductant for azides (Lin and Chang, 2012), we expected that NIR-Az would exhibit high selectivity for hydrogen sulfide over RSONs, including biologically relevant thiols. On the basis of the strong hydrogen sulfide-sensing properties of NIR-Az, a selectivity test of the fluorescence response was conducted by using fluorescence titration of various analytes. As shown in Fig. 3, a pronounced fluorescence enhancement was observed for the solution with hydrogen sulfide, and no significant changes occurred for solutions with other analytes, thereby indicating the excellent selectivity of the hydrogen sulfide-mediated azide-reduction mechanism. The fluorescence intensity for NaHS over other analytes was approximately 20-fold for NIR-Az, whereas the fluorescence color of the probe solution changed from colorless to bright red under 675 nm laser irradiation in the presence of NaHS (Fig. 3b). Therefore, the results demonstrate that NIR-Az has a high selectivity for hydrogen sulfide, thus indicating its potential utility for studies of various biological samples. To verify the influence of pH on the fluorescence of NIR-Az,
the fluorescence intensities of the sensing reaction in different solutions (pH 1.0–12.0) were measured (Fig. S7). The fluorescence spectra of NIR-Az were recorded in various pH solutions (ACN/PBS 1:4 v/v). The pH-dependence response was estimated on the basis of the fluorescence intensity ratio at 720 nm for excitation at 680 nm. Under pH 6 or alkaline conditions, NIR-Az responded to hydrogen sulfide with a marked fluorescence change, whereas there was no obvious response for acidic solutions (pH < 5). This result shows that NIR-Az not only can detect hydrogen sulfide but also is considerably more stable under physiological conditions (pH 5–12).

3.4. Live cell and mouse imaging

To establish the potential efficacy of NIR-Az in biological applications, given its excellent hydrogen sulfide-sensing properties, we attempted fluorescence imaging to detect hydrogen sulfide in living cells through confocal microscopy. CCK-8 assays were conducted, and the results showed that more than 95% of HeLa cells survived after 1 h (1–30 μM incubation); after 24 h, the cell viability remained at approximately 90%, thus demonstrating that NIR-Az is minimally cytotoxic to cultured cell lines (Fig. S8). Because the high selectivity and sensitivity of NIR-Az were demonstrated for hydrogen sulfide in vitro, the ability of NIR-Az to detect changes in the hydrogen sulfide levels in living cells was examined in a RAW264.7 cell model (Fig. 4).

The incubation of RAW264.7 cells with NIR-Az (5 μM) for 30 min at 37 °C was followed by the addition of different concentrations of NaHS (0, 20, 40 and 80 μM) and then incubation for another 30 min. After the excess NaHS was removed, the cells were subsequently imaged with a confocal fluorescence microscope. As shown in Fig. 4a, RAW264.7 cells treated with only NIR-Az as a control showed no fluorescence at 655–755 nm for excitation at 635 nm. However, in the presence of NIR-Az and NaHS, the cells showed strong fluorescence, with an intensity that increased with increasing NaHS concentration. The relationship between the average fluorescence intensity and added NaHS is shown in Fig. 4e. The cell body regions in the visual field were selected as regions of interest (ROIs), and the average fluorescence intensity was determined via confocal laser-scanning microscopy with various hydrogen sulfide concentrations. These results demonstrated that NIR-Az has excellent membrane permeability and potential in visualizing hydrogen sulfide in living cells, and thus applications of this probe can probably be extended to assays involving biological fluids such as serum, blood, or tissue homogenates.

To further exploit these desirable attributes, we investigated the

Fig. 3. a) Fluorescence enhancement of NIR-Az in the presence of thiol, reactive sulfur, nitrogen, and oxygen species, and ascorbic acid. Reactions of (1) 10 μM NIR-Az with (2) 200 μM NaHS, (3) 1 mM L-cysteine, (4) 1 mM α-homocysteine, (5) 10 mM glutathione, (6) 200 μM Na2S2O3, (7) 200 μM Na2S2O4, (8) 200 μM Na2SO3, (9) 200 μM Na2SO4, (10) 200 μM NaHSO3, (11) 200 μM KSCN, (12) 200 μM H2O2, (13) 200 μM Angel’s salt (NO−), (14) 200 μM NaNO3, (15) 200 μM NaNO2, (16) 200 μM ascorbic acid, and (17) 200 μM α-lipoic acid in PBS buffer (10 mM, pH 7.4, 30% acetonitrile, v/v) at 37 °C for 30 min λ<sub>ex</sub>=680 nm, λ<sub>em</sub>=720 nm. b) Fluorescence images of 10 μM NIR-Az in the presence of thiol, reactive sulfur, nitrogen, and oxygen species, and ascorbic acid. λ<sub>ex</sub>=675 nm, λ<sub>em</sub>=700 nm.

Fig. 4. Confocal fluorescence images of living RAW264.7 cells preincubated with NIR-Az (5 μM, 30 min), then incubated with NaHS for 30 min. Incubation was performed at 37 °C under a humidified atmosphere containing 5% CO2. The fluorescence was determined at 655–755 nm with excitation at 635 nm. (a) NIR-Az (only), (b) NIR-Az+20 μM NaHS (c) NIR-Az+40 μM NaHS, and (d) NIR-Az+80 μM NaHS. The scale bar is 50 μm. (e) The cell body regions in the visual field were selected (n=10 cells) as the regions of interest (ROIs).
suitability of NIR-Az to detect hydrogen sulfide in living animals. After probe injection, Balb/c nude mice were imaged using an IVIS Lumina II in vivo imaging system. As shown in Fig. 5a, the mice treated with both NIR-Az and NaHS exhibited a much higher fluorescence readout than those treated with only the probe, showing an increase in fluorescence intensity with increasing NaHS concentration, in accordance with the results of the concentration dependence in living RAW264.7 cells. The fluorescence intensity from the abdominal area of the mice was quantified, and the data indicated that the mice loaded with the probe and 20 equivalents of NaHS exhibited a maximum fluorescence intensity 8-fold higher than that of the mice loaded with only the probe (Fig. 5b).

The good photostability and robust chemical stability of NIR-Az also facilitated the observation of the time-dependent reaction of hydrogen sulfide in living animals. The mice were injected with NIR-Az and NaHS, and images were taken at different times after the injection. As shown in Fig. 5c and d, the fluorescence intensity in the mice is time dependent, and the mice were still alive after 3 h, thus indicating that the probe has minimal cytotoxicity. Interestingly, these results are consistent with those of the time- and concentration-dependence titrations of the probe after incubation with hydrogen sulfide in the buffer. Therefore, NIR-Az can be readily used to detect and visualize hydrogen sulfide in vivo for real-time analysis in a non-invasive manner, which is important given that hydrogen sulfide has emerged as a key gaseous in vivo signaling molecule involved in many physiological processes, such as inflammation, endoplasmic reticulum stress, and apoptosis by sulfhydration of proteins.

4. Conclusions

In this study, we successfully developed a new approach for the construction of an NIR fluorescent sensor based on a novel class of NIR dyes represented by NIR-Az, exhibiting a selective fluorescence enhancement response to hydrogen sulfide over other physiological species. The probe NIR-Az was highly selective for hydrogen sulfide among 16 analytes tested and other common reducing species, with a detection limit of less than 0.26 μM in PBS buffer solution. The fluorescence enhancement of NIR-Az after hydrogen sulfide treatment was more than 200-fold, and its quantum yield after hydrogen sulfide treatment was 0.72. The time-dependent fluorescence response demonstrated that NIR-Az can detect hydrogen sulfide both qualitatively and quantitatively. The obtained linear relationship for the concentration covered the reported endogenous concentration range of hydrogen sulfide. NIR-Az provided a high turn-on response for the detection of hydrogen sulfide in living cells and mice, thus demonstrating its potential for visualizing hydrogen sulfide in living cells, which can probably be extended to assays involving biological fluids, such as serum, blood, or tissue homogenates.

Competing financial interests

The authors declare no competing financial interests.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bios.2016.09.093.
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