A 4-HYDROXY-1,8-NAPHTHALIMIDE-BASED TURN-ON TWO-PHOTON FLUORESCENT PROBE FOR HYDROGEN POLYSULFIDE SENSING

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In order to quantitatively detect hydrogen polysulfides (H_2S_n, $n > 1$) in biosystems, we propose a new two-photon turn-on fluorescent probe, named N_p-S_n, based on the D-π-A skeleton two-photon fluorophore of 4-hydroxy-1,8-naphthalimide derivative and deprotection of 2-fluoro-5-nitrobenzoic ester by H_2S_n. N_p-S_n displayed a more than 80-fold enhancement towards H_2S_n in 550 nm and high sensitivity with a detection limit as low as 33 nM. Additionally, the probe N_p-S_n was further used for fluorescence imaging of H_2S_n in living cells under two-photon excitation (820 nm), which showed a high-resolution imaging, thus demonstrating its practical application in biological systems for the study of physiological and pathological functions of H_2S_n.

Keywords: *hydrogen polysulfides, two-photon fluorescent imaging.*

ДВУХФОТОННЫЙ ФЛУОРЕСЦЕНТНЫЙ ЗОНД НА ОСНОВЕ 4-ГИДРОКСИ-1,8-НАФТАЛИМИДА ДЛЯ ОПРЕДЕЛЕНИЯ ПОЛИСУЛЬФИДА ВОДОРОДА

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Для количественного обнаружения полисульфида водорода (H_2S_n, $n > 1$) в биосистемах предложен двухфотонный флуоресцентный зонд N_p-S_n на основе структуры D-π-A двухфотонного флуорофора — производного 4-гидрокси-1,8-нафталимида и 2-фтор-5-нитробензойной кислоты H_2S_n. Зонд N_p-S_n продемонстрировал более чем 80-кратное усиление флуоресценции при 550 нм по сравнению с H_2S_n и высокую чувствительность с пределом обнаружения 33 нМ. Он используется для флуоресцентной визуализации H_2S_n в живых клетках при двухфотонном возбуждении (820 нм)

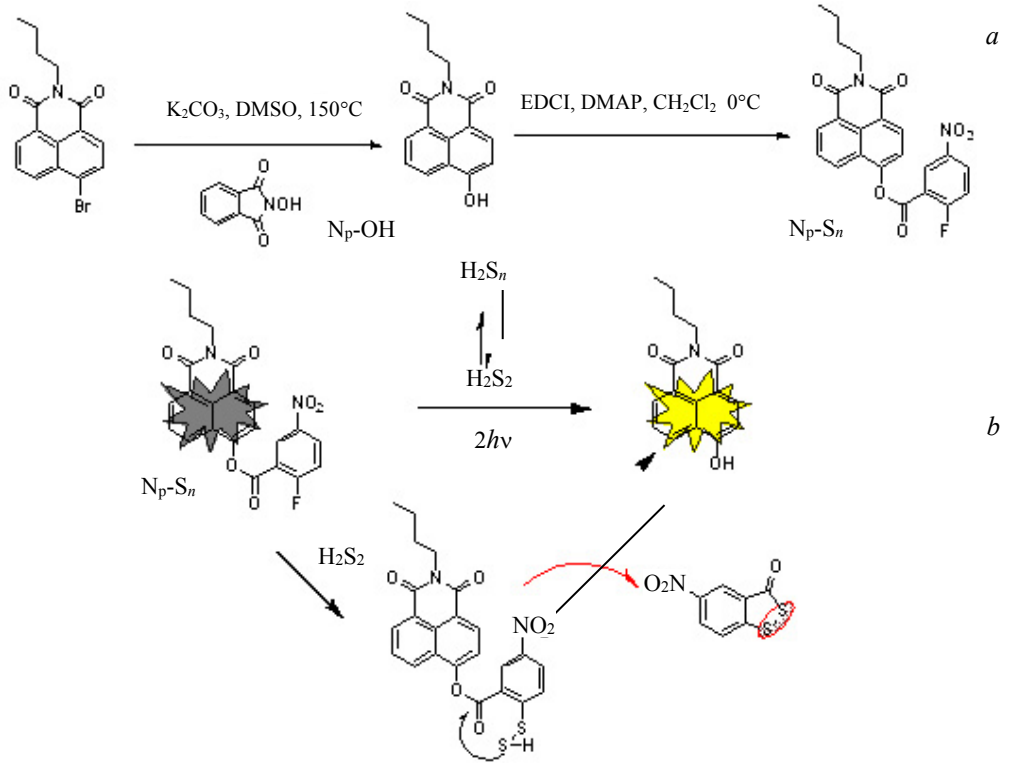
Ключевые слова: *полисульфиды водорода, двухфотонная флуоресцентная визуализация.*

Introducation. It is essential to detect endogenous substances in diagnosing disease. The performance of reactive sulfur species (RSS), including bio-thiols, hydrogen sulfide (H_2S), hydrogen polysulfides (H_2S_n, $n > 1$), persulfides, and S-modified cysteine adducts (i.e., S-nitroso-thiols, sulfenic acids, etc.), is involved in every aspect of cell biology, from protein function to redox signal transduction [1, 2]. Among them, H_2S_n, which can be derived from endogenous H_2S by the action of reactive oxygen species, have recently received

particular attention since they are believed to be involved in H_2S-mediated signaling transduction [3]. As the redox partners of H_2S, H_2S_n very likely coexist with H_2S *in vivo*, and they work together to regulate the sulfur redox balance. Recent studies suggested that H_2S_n might act as the real regulators in cellular signaling transduction. Some biological mechanisms previously attributed to H_2S may actually be mediated by H_2S_n. For instance, H_2S_n has recently been found to be more effective than H_2S in the conversion of protein cysteine residues (-SH) to persulfides (-S-SH) (S-sulfhydration) [4–6].

The investigation of H_2S_n is now rapidly increasing, and the biological activity mediated by H_2S_n is yet to be discovered [7–9]. In order to better understand the roles of H_2S_n in biosystems, it is critical to develop efficient methods that can distinguish H_2S_n from other RSS, especially H_2S and biothiols. The traditional method for detecting H_2S_n is to measure UV absorption peaks at 290–300 and 370 nm [10]. However, this detection method requires the reduction of polysulfides to H_2S. Due to this limitation, the traditional method cannot meet the demands of biological *in situ* detection in sensitivity and selectivity. Fluorescence-based methods could be ideal due to their rapid, sensitive fluorescent responses and spatiotemporal resolution capability. The unique chemical characteristics of sulfur species have been utilized in fluorescent probe design. For example, H_2S_n with the estimated pK_a values in the range from 3 to 5, are more acidic and nucleophilic than the corresponding H_2S (7.0) and thiols (Cys, 8.30; GSH, 9.20) due to the α-effect under physiological pH. Compared with them, fluorescence-based methods could maintain comparable efficiency and accuracy, offer convenience and high sensitivity, as well as obtain noninvasive spatial temporal resolution imaging. Especially, two-photon (TP) probe-based fluorescent imaging, which is an emerging technique employing near-infrared (NIR) light source excitation that can provide improved spatial resolution and theoretically remarkably increased imaging depth in comparison with traditional one-photon (OP) imaging, might be the most attractive one for *in vivo* detection of bio-related species [11, 12]. Recently, some types of H_2S_n fluorescent probes have been developed by Xian [13–16] and others [17–21]. However, most of these fluorescent probes are OP fluorescent probes, and an effective two-photon fluorescent probe to specifically detect H_2S_n is very rare. Therefore, developing a simple and reliable TP fluorescent probe for the quantitative detection of H_2S_n concentration in living cells is of great interest.

Herein, we employed a large TP action absorption cross-section, high fluorescence quantum yield, excellent biocompatibility, and low fluorescence background fluorophore (4-hydroxy-1,8-naphthalimide) to design and synthesize a new TP turn-on fluorescent probe, N_p-S_n, for detecting H_2S_n (Scheme 1).



Scheme 1. The synthetic route of the fluorescent probe N_p-S_n and its reaction mechanism with H_2S_2.

The fluorescence intensity was quenched by the 2-fluoro-5-nitrobenzoic ester moiety through suppressing the intramolecular charge transfer (ICT) effect. Upon addition of H_2S_n, the 2-fluoro-5-nitrobenzoic ester moiety was cut off to form the OH group, resulting in the recovery of the ICT effect to turn on the fluorescence signal. N_p-S_n, exhibited a more than 80-fold fluorescence intensity enhancement at an emission wavelength of 550 nm after reaction with H_2S_n. Most importantly, the fluorescent probe exhibited high sensitivity and high selectivity toward H_2S_n over other analytes. Subsequently the probe was successfully applied to the detection of H_2S_n for bioimaging with a high-resolution imaging.

Experiment. Unless otherwise noted, all chemical reagents are supplied by commercial suppliers and used without further purication. All solvents were dried in a routine way and redistilled before use, and all water used in all experiments was secondarily distilled and pun fied by a Milli-Q system (Millipore, USA). Mass spectra were carried out by an LCQ Advantage ion trap mass spectrometer (Thermo Finnigan). NMR spectrum were recorded on a Bruker DRX-400 spectrometer in DMSO-d_6 with TMS as the internal reference. All chemical shifts are reported in the standard δ notation of parts per million. Absorption spectra were recorded in 1.0 cm path length quartz cuvettes on a Hitachi U-4100 UV-vis spectrophotometer (Kyoto, Japan). All fluorescence measurements were carried out on a F-4600 fluorescence spectrometer with both excitation and emission slits fixed at 2.5 nm. Fluorescence imaging of HeLa cells and tissues was conducted on a confocal laser scanning microscope (Olympus, Japan) with 820 nm excitation. Reactions were followed by thin-layer chromatography (TLC) on Merck (0.25 mm) glass-packed precoated silica gel plates (60 F254) and then visualized with a UV lamp. All chemical yields are unoptimized and generally represent the result of a single experiment. The pH was measured with a Mettler-Toledo Delta 320 pH meter.

Synthesis and characterization of 2-butyl-6-hydroxy-1H-benzo[de]isoquinoline-1,3(2H)-dione (compound 2). A 0.332 g (1.00 mmol) portion of 6-bromo-2-butyl-1H-benzo[de]isoquinoline-1,3(2H)-dione (**1**), 0.326 g N-Hydroxyphthalimide (2.00 mmol), 0.556 g (4 mmol) K_2CO_3, and 50 mL DMSO were added into a 100 mL flask with a reflux condenser, and the mixture was stirred at 150°C for 4 h under argon protection. Then the mixture was poured into ice water and filtered. Finally, the filtrate was dried in a vacuum oven to yield **2** as a yellow solid (0.243g, 90.1%). ^{1}H NMR (400 MHz, d_6-DMSO) δ: 8.55–8.53 (d, J = 8Hz, 1H), 8.45–8.43 (d, J = 8 Hz, 1H), 8.31–8.23 (m, 1H), 7.71–7.67 (t, J = 8 Hz, 1H), 7.03–7.01 (d, J = 8 Hz, 1H), 5.76 (s, 1H), 4.04–4.0 (t, J = 8 Hz, 2H), 1.63–1.29 (m, 4H), 0.94-0.91 (t, J = 6 Hz, 3H); ^{13}C NMR (100 MHz, d_6-DMSO) δ*:* 164.36, 163.35, 134.38, 131.38, 130.20, 129.79, 125.20, 123.84, 122.21, 111.17, 55.27, 30.28, 20.47, 14.28; ESI-MS: $[M]^+$ calcd: 269.30, found: 269.4.

Synthesis and characterization of 2-butyl-1,3-dioxo-2,3-dihydro-1H-benzo[de]isoquinolin-6-yl 2-fluoro-5-nitrobenzoate (N_p-S_n). Compound **2** (0.135 g, 0.5 mmol), 2-fluoro-5-nitrobenzoic acid (0.0930 g, 0.5 mmol), 1-ethyl-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCI) (0.0960 g, 0.5 mmol), and 4-dimethylaminopyridine (DMAP) (0.0244 g, 0.2 mmol) were dissolved in dichloromethane (DCM) (40 mL) under argon protection at 25°C for 12 h. Then, the mixture was poured into ice water and extracted three times by DCM (15×). Then, the solvent was evaporated in vacuum, and the crude solid was purified by column chromatography on silica gel eluting (CH_2Cl_2/MeOH = 100:1, v/v) to afford a 0.198g yellow solid in 90.4% yield. ^{1}H NMR (400 MHz, d_6-DMSO) δ: 8.95 (s, 1H), 8.88 (s, 1H), 8.69–8.67 (t, J = 4 Hz, 1H), 8.57–8.49 (m, 3H), 7.93–7.88 (q, J = 10 Hz, 2H), 7.84–7.79 (t, J = 10 Hz, 1H), 4.06–4.02 (t, J = 8 Hz, 2H), 1.64–1.320 (m, 4H), 0.95–0.92 (t, J = 6 Hz, 3H); ^{13}C NMR (100 MHz, d_6-DMSO) δ*:* 166.62, 163.91, 163.64, 163.12, 160.43, 151.09, 144.31, 131.76, 128.98, 128.64, 128.40, 125.04, 122.88, 121.01, 120.64, 119.92, 119.67, 118.68, 118.57, 30.08, 20.27, 14.18; ESI-MS: $[M]^+$ calcd: 436.40, found: 436.4.

The fluorescence was measured in a 10 mM phosphate buffer solution (containing 1% DMSO as a co-solvent). The pH value of the PBS solution used was from 4.0 to 9.0, which was achieved by adding minimal volumes of the HCl solution or the NaOH solution. The fluorescent emission spectra were recorded at an excitation wavelength of 400 nm with emission wavelength range from 520 to 630 nm. A 1×10^{-3} mol/L stock solution of the probe was prepared by dissolving N_p-S_n in DMSO. The procedure of calibration measurements with the probe in the buffer with different H_2S_n followed: 2 μL stock solution of the probe and 1998 μL of the PBS buffer solution with different H_2S_n were combined to afford a test solution, which contained 1×10^{-6} mol/L of probe. The solutions of various testing species were prepared from 0.5 mM Cys, 0.5 mM Hcy, 0.5 mM GSH, 0.5 mM CysSSCys, 0.5 mM GSSG, 0.5 mM Cys-poly-sulfide, 0.5 mM S_8, 20 μM Na_2S_2, 0.5 mM $NaHSO_3$, 0.5 mM ascorbic acid, 0.5 mM tocopherol, 0.5 mM NaHS, and 0.5 mM $Na_2S_2O_3$. The procedure of the selectivity experiments consists of 1×10^{-6} mol/L of the probe and different concentrations of analytes. Na_2S_2 was prepared using the reported procedures [14]. The mixture was equilibrated for 5 min before measurement.

Prior to the image experiments, the HeLa cells were washed with phosphate-buffered saline (PBS), and incubated with 1 μM probe N_P-S_n for 30 min at 37°C, then washed with PBS three times and incubated with 15 μM Na_2S_2 for another 30 min at 37°C. Finally, the Hela cells were washed with PBS three times again before imaging. A confocal fluorescence image of H_2S_n in HeLa cells was observed under an Olympus FV 1000 laser confocal microscope. Two-photon image: λ_{ex} = 820 nm, λ_{em} = 500–570 nm. All images were acquired with a 40× oil immersion objective, scale bar 10 μm.

Results and discussion. As a proof of concept, a D-π-A-structured N_p-S_n was chosen as the fluorophore for its outstanding two-photon properties, while a 2-fluoro-5-nitrobenzoic ester moiety was used as the recognition moiety due to its rapid response to H_2S_n, according to some reports [17–21]. All compounds were characterized by ^1H and ^{13}C NMR and ESI-MS.

As expected, the spectroscopic properties of N_p-S_n (1 μM) were examined in a phosphate buffer solution (10 mM, pH 7.4) with different concentrations of Na_2S_2. The probe displays sensitive absorption and fluorescence responses to different concentrations of Na_2S_2 (Fig. 1). As shown in Fig. 1a, in the absence of Na_2S_2, an absorption peak was observed at the maximum absorption wavelengths λ = 350 nm. After Na_2S_2 (15 μM) was added, two absorption peaks were observed at the maximum absorption wavelengths λ = = 350 and 475 nm. Further fluorescence experiments showed that N_p-S_n is weakly-fluorescence (Fig. 1b) due to the strong electron-withdrawing 2-fluoro-5-nitrobenzoic ester moiety that prevented the N_p-S_n from changing to fluorophore N_p-OH. Upon reaction with Na_2S_2 from 0 to 25 μM, which results in converting into N_p-OH and a significant turn-on fluorescence enhancement at λ_{em} = 550 nm, the fluorescence ratio of F_{550}/F_0 gradually increased ~80-fold. Further detailed studies also revealed that there is an excellent linear relationship between F_{550} and different Na_2S_2 concentrations (0–5.0 μM) (Fig. 1c). The detection limit (utilizing the $3\sigma/k$ method) for H_2S_2 was determined to be as low as 33 nM, which is enough for direct detection of H_2S_2 *in vitro* and *in vivo*.

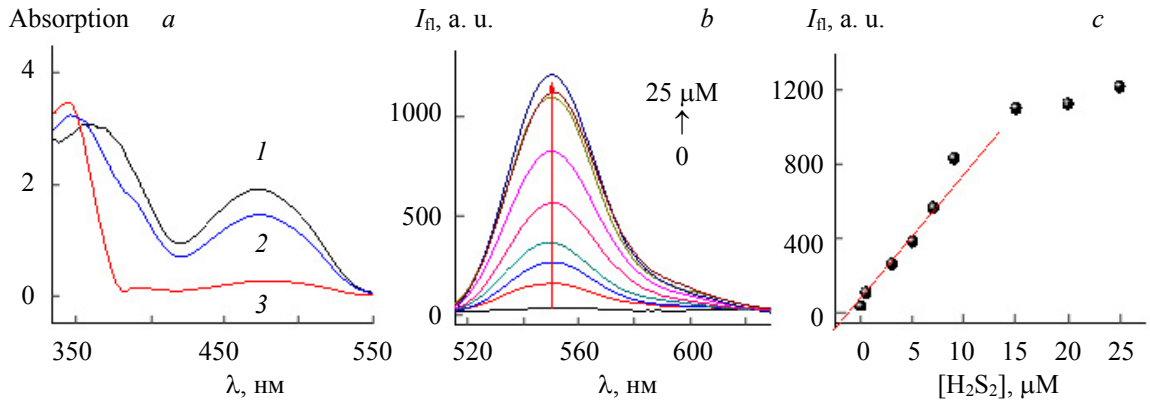


Fig. 1. (a) UV-vis absorption (1): 1 μM N_p-OH; (2): 1 μM N_p-S_n+15μM Na_2S_2; (3): 1μM N_p-S_n; (b) fluorescence spectra of N_p-S_n 1 μM in the presence of various concentrations of Na_2S_2 (0–25 μM); (c) calibration curve of N_p-S_n to Na_2S_2. The curve was plotted with fluorescence intensity *vs* Na_2S_2 concentration (0–25 μM).

Naphthalimide derivatives exhibit excellent two-photon properties with a two-photon action cross-section, showing that this two-photon dye is potentially useful for bioimaging applications. The N_p-S_n, was calculated to have a two-photon absorption cross-section of 12 GM (1 GM = 10^{-50} $cm^4 \cdot s$/photon) at 550 nm upon excitation at 820 nm (Fig. 2a, curve 2). However, the N_p-OH was calculated to have a large two-photon absorption cross-section of 78 GM, as well as a new strong fluorescent peak at 550 nm (curve 1). High selectivity is an important parameter to evaluate a newly designed fluorescent probe. For this purpose, N_p-S_n was treated with a series of analytes such as Cys, Hcy, GSH, CysSSCys, GSSG, Cys-poly-sulfide, S_8, Na_2S_2, $NaHSO_3$, ascorbic acid, tocopherol, NaHS, and $Na_2S_2O_3$ to examine its selectivity. The results are shown in Fig. 2b. The probe demonstrated almost unchanged fluorescence intensity responses before and after addition of analytes to the N_p-S_n solution. N_p-S_n could meet the selective requirements for practical applications. Next, we studied the effect of the pH on N_p-S_n in the absence and presence of H_2S_2 (Fig. 2c). Without H_2S_2, no obvious characteristic fluorescence could be observed from pH 3.0–9.0 (Fig. 2c, curve 2). Upon reaction with H_2S_2, the best response towards H_2S_2 could be achieved with a pH range of 4.0–8.0 (Fig. 2c, curve 1).

Thus, the PBS solution (pH 7.4) was utilized throughout the experiment. These results indicated N_p-S_n was favorable for applications in practical samples at different pH values.

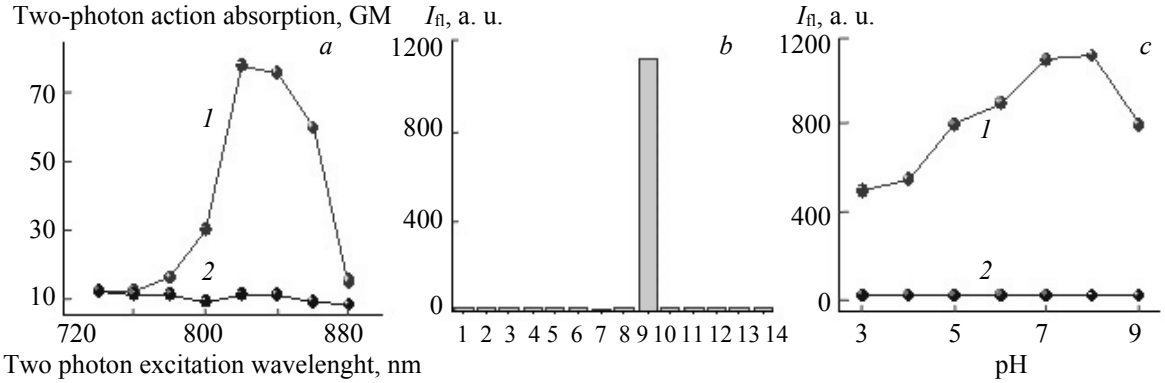


Fig. 2. (a) Two-photon absorption cross-section of N_p-OH (1) and N_p-S_n (2); (b) fluorescence responses of N_p-S_n (1 μM) to biologically relevant RSS. Numbers from 1 to 14 correspond to black to $Na_2S_2O_3$ (blank, 0.5 mM Cys, 0.5 mM Hcy, 0.5 mM GSH, 0.5 mM CysSSCys, 0.5 mM GSSG, 0.5 mM Cys-poly-sulfide, 0.5 mM S_8, 20 μM Na_2S_2, 0.5 mM $NaHSO_3$, 0.5 mM ascorbic acid, 0.5 mM tocopherol, 0.5 mM NaHS, and 0.5 mM $Na_2S_2O_3$); (c) pH effects on N_p-S_n (1) in the absence or presence of Na_2S_2 (2). Data were recorded in 10 mM PBS buffer (pH 7.40, containing 5% DMSO as a cosolvent) at room temperature for 5 min. λ_{ex} = 475 nm, λ_{em} = 550 nm.

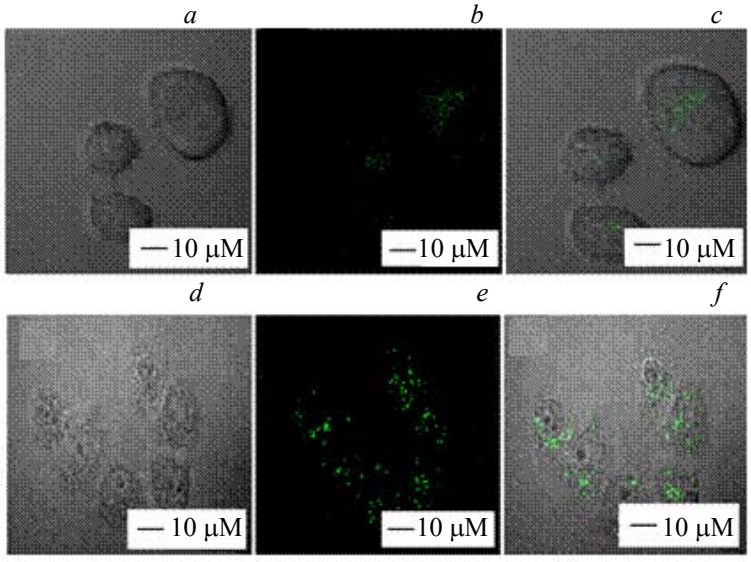


Fig. 3. TP fluorescence microscope (TPFM) images of HeLa cells: (a) bright field image; (b) HeLa cells incubated with 1μM N_p-S_n for 30 min; (c) merged image of (b) and bright field image (a); (d) bright field image; (e) HeLa cells pretreated with 10 μM Na_2S_2 for 15min and then incubated with 1 μM N_p-S_n for 30 min image; (f) merged image of (e) and bright field image (d). TP images: λ_{ex} = 820 nm, λ_{em} = 500–570 nm. All images were acquired with a 40× oil immersion objective, scale bar 10 μm.

In order to evaluate the imaging performance of N_p-S_n, we used this probe to detect H_2S_2 in live cells. HeLa cells were chosen as the model cell line. Before imaging, the cytotoxicity of the H_2S_2 was tested. The results showed that it was nearly nontoxic for living cells under experimental conditions. Then the HeLa cells were incubated with N_p-S_n (1 μM) at 37°C for 30 min, followed by excitation at 820 nm for TP image. The HeLa cells showed a weak fluorescence intensity in the green channel by TP image (Fig. 3b). In contrast, when treating N_p-S_n-incubated cultured cells with 10 μM Na_2S_2 for 30 min, the fluorescence intensity increased in the green channel, obviously (Fig. 3e) by TP image. Taken together, these results showed that N_p-S_n was cell membrane-penetrable and could be used for TP images in live cells.

Conclusion. We designed and synthesized a novel TP fluorescent probe N_p-S_n to detect H_2S_n in living cells and tissues. The probe is based on TP excitation of the D-π-A structure of 4-hydroxy-1,8-naphthalimide derivative fluorophore (N_p-OH), which is obtained upon removal of a trigger moiety by the H_2S_n of interest. The probe was demonstrated to efficiently image H_2S_n produced in live cells. We believe that the probe N_p-S_n will be a useful tool for the study of physiological and pathological functions of H_2S_n in biological systems.

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The authors declare no conflicts of interest.

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