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Short Communication

An isophorone-fused near-infrared fluorescent probe with a large Stokes shift for imaging endogenous nitroxyl in living cells and zebrafish

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ABSTRACT

Nitroxyl (HNO) plays an important role in multiple physiological and pathological processes, but the detailed generation mechanism of the endogenous HNO still remained to explore and perfect further. There is an urgent need to develop an excellent fluorescent probe for selective recognition and sensitive detection of HNO in biological systems. Near-infrared (NIR) fluorescent probes with a large Stokes shift are an ideal tool for bioimaging applications. Here, we have developed a NIR fluorescent probe with a large Stokes shift, namely, **NIR-HNO**, to monitor HNO in cells and zebrafish. **NIR-HNO** consists of an isophorone-fused NIR fluorescence reporter and a diphenylphosphinobenzoyl HNO-responsive unit. Based on an aza-ylide intramolecular ester aminolysis reaction, **NIR-HNO** showed a rapid selective NIR fluorescent turn-on response for HNO, high sensitivity (detection limit was 39.6 nM), and large Stokes shift (265 nm). The biological imaging results indicate that **NIR-HNO** is a good candidate for imaging of endogenous HNO in living systems.

1. Introduction

Reactive nitrogen species (RNS) is one class of highly chemical active species in living systems. Nitroxyl (HNO) is a well-known member of RNS, which is the one-electron reduced and further protonated form of nitric oxide (NO). HNO plays important roles during a series of physiological and pathological processes [1]. Endogenous HNO can come from the reaction of *S*-nitrosoglutathione with glutathione [2], the reduction of L-arginine by nitric oxide synthase [3], and oxidation of hydroxylamine by peroxidases. In addition [4], it is also reported that HNO has unique biological activity and medicinal potential. For example, HNO was used in research to treat heart failure because it could enhance the myocardial contractile force and promote the contraction of heart cells [5,6]. HNO could also inhibit the activity of acetaldehyde dehydrogenase to be used, as a potential drug for treating alcoholism [7]. Recently, HNO has been reported to possess anti-inflammatory effects [8–10]. Although HNO has the above versatile roles, its specific mechanism is still unclear. As a result, it is very necessary to develop a reliable method for selective recognition of HNO in biological systems.

At present, fluorescence probe assay is a fast-growing technique for the recognition and detection of different analysts because of its unique characteristics, such as high selectivity, good sensitivity, non-invasion, and spatiotemporal resolution, and has been basically mature and used in the fields such as life sciences, biotechnology, drugs analysis, *etc.* [11–18]. To date, based on the redox reaction and Staudinger reaction, multiple HNO fluorescent probes have been reported [19–21]. Among them, the Staudinger reaction has been widely applied for developing HNO fluorescent probes because of its better biocompatibility [22–40]. However, many reported fluorescent probes with short excitation and emission wavelengths, suffer from the potential photobleaching, background fluorescence interference, and photo-damage to cells and tissues, which could be used for detecting HNO in living cells, but not suitable for *in vivo* applications [22,32–37]. To make up for these deficiencies, many near-infrared (NIR, >650 nm) fluorescent probes have been developed for imaging HNO in biological specimens [23–28,30,31]. As is well-known, fluorescent probes with large Stokes shift could be free from interference from excited light scattering, which leads to more suitable for practical application *in vivo*. Thus,

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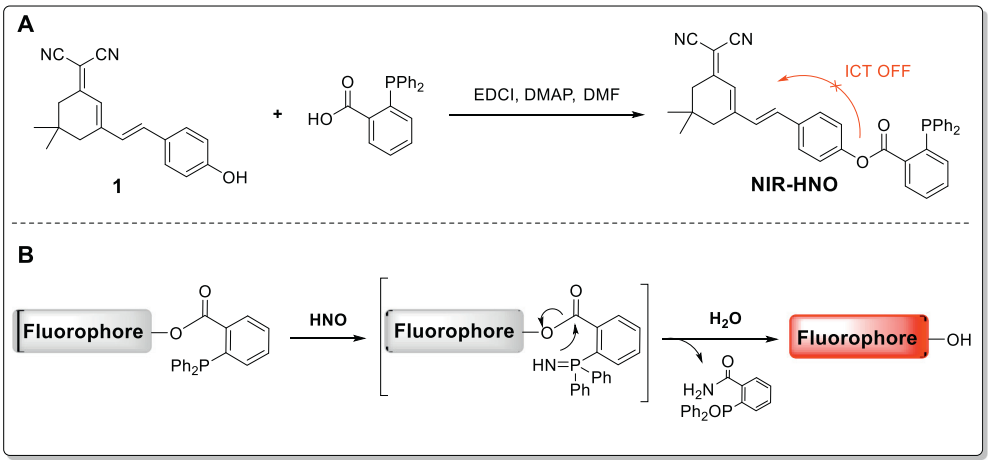

Scheme 1. (A) The synthetic scheme of **NIR-HNO**; (B) the possible mechanism of sensing HNO by **NIR-HNO**.

new NIR fluorescent HNO probes with large Stokes shifts are urgently needed.

Herein, we report a NIR fluorescent probe with a large Stokes shift, namely, **NIR-HNO**, for detection HNO in living systems (Scheme 1A). **NIR-HNO** consists of an isophorone-fused dye **1** as a NIR fluorophore and a diphenylphosphinobenzoyl group as the HNO responsive group. The fluorescence of **NIR-HNO** was inhibited by disrupting the intramolecular charge transfer (ICT) effect. Upon reaction with HNO, aza-ylide and further intramolecular aminolysis reaction occur, and the resulting fluorophore **1** emits NIR fluorescence (Scheme 1B). **NIR-HNO** could selectively and sensitively detect HNO with a rapid fluorescent response, a low detection limit (39.6 nM), and a remarkable large Stokes shift (265 nm). The results for the detection of the endogenous generation of HNO demonstrate the suitability of the probe in practical applications in living systems.

2. Materials and methods

Unless especially noted, all chemical agents were purchased from J&K Scientific. NMR spectra were determined using Advance 600 MHz (Bruker, Germany). High-Resolution Mass Spectrum (HRMS) were collected using Varian 7.0 T FTICR-MS. UV–Vis absorption spectra and fluorescence spectra were recorded on Cary 5000 Bio (Agilent, USA) and F-7000 (Hitachi, Japan) spectrophotometer, respectively. Bioimaging of cells and zebrafish were acquired on a confocal microscope (Olympus Fluoview FV1000 and Zeiss LSM 880).

2.1. Synthesis

A mixture of 2-(diphenylphosphanyl)benzoic acid (230 mg, 0.75 mmol), *N*-(3-dimethylaminopropyl)-*N′*-ethyl carbodiimide hydrochloride (EDCI) (289 mg, 1.50 mmol) and 4-dimethylamino pyridine (DMAP) (367 mg, 3.00 mmol) was dissolved in dry *N*,*N*-dimethylformamide (DMF) (10 mL). After stirring for 30 min, compound **1** [41] (145 mg, 0.50 mmol) was added into the above solution and further stirred overnight at room temperature. After the reaction completely, the solvent was poured into ice water (50 mL), the forming precipitation was filtered, and the crude product was purified by silica gel column chromatography to give **NIR-HNO** as a light yellow solid (217 mg, 75%). m.p.: 186–188 °C; ^{1}H NMR (600 MHz, $CDCl_3$): δ = 8.27–8.24 (m, 1H), 7.51–7.45 (m, 4H), 7.38–7.31 (m, 11H), 7.04–6.90 (m, 5H), 6.84 (s, 1H), 2.60 (s, 2H), 2.46 (s, 2H),1.08 (s, 6H). ^{13}C NMR (151 MHz, $CDCl_3$): 169.16, 164.91, 153.60, 151.54, 141.42, 141.15, 137.40, 1.37.30, 135.93, 134.49, 134.13, 133.92, 133.28, 133.10, 132.67, 131.32, 129.17, 128.83, 128.71, 128.60, 128.53, 128.45, 128.38, 123.63, 122.29, 113.43, 112.64, 78.82, 42.97, 39.19, 31.99, 27.99. HRMS calculated for $C_{38}H_{32}N_2O_2P$ $[M + H]^+$ 579.2201, found: 579.2198.

2.2. Procedure of fluorescence measurement

The stock solution of **NIR-HNO** (2 mM) was prepared using DMSO and was used with a concentration of 10 μM. Different analytes with a concentration of 1 M were prepared in PBS solution. These analytes include NaF, NaCl, NaBr, NaI, KCl, $MgCl_2$, $CuCl_2$, $ZnCl_2$, $FeCl_3$, Na_2SO_4, Na_2SO_3, $NaNO_3$, $NaNO_2$, NaClO, H_2O_2, KO_2, Sodium nitroprusside (source of NO), $ONOO^-$, CySNO, GSNO, Na_2S (source of H_2S), cysteine (Cys), homocysteine (Hcy), glutathione (GSH) and Angeli's salt (source of HNO, AS). All spectroscopic measurement was performed in PBS (10 mM, pH 7.4, 50% DMF, 25 °C) with excitation at 556 nm and slit width of 5.0 nm.

2.3. Cell culture and fluorescence microscopy imaging

HeLa cells were cultured in DMEM supplemented with 10% fetal calf serum at 37 °C in 5% CO_2. The cytotoxicity was measured

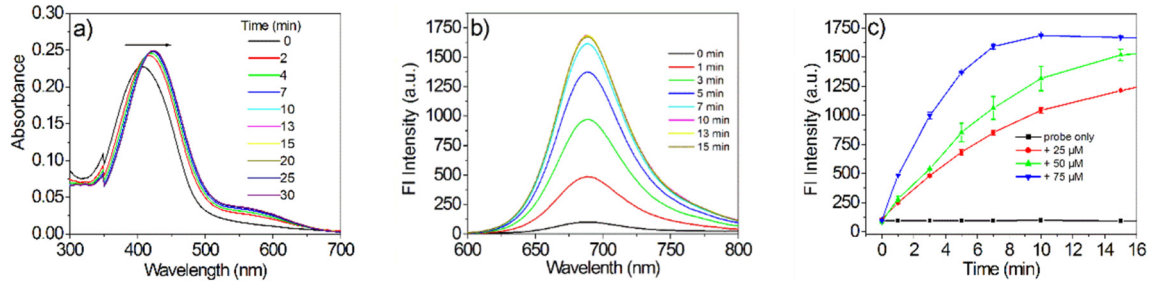

Fig. 1. Time-dependent optical changes in absorption (a) and emission (b) of **NIR-HNO** (10 μM) in the presence of AS (75 μM) in PBS solution (10 mM, pH = 7.4, 50% DMF) at 25 °C. (c) Time dependence of emission intensity at 688 nm for **NIR-HNO** (10 μM) in the absence and presence of AS (75 μM).

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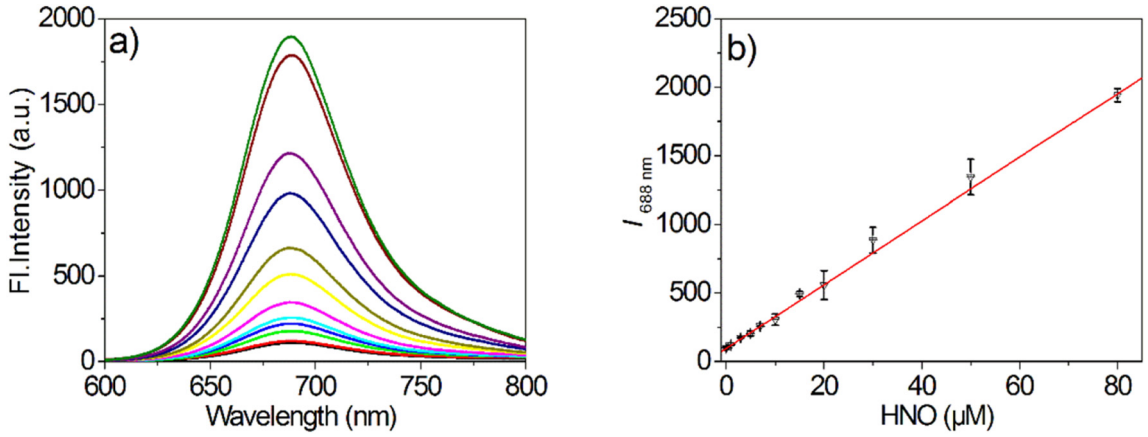

Fig. 2. (a) Emission spectra of **NIR-HNO** (10 μM) upon addition of AS (0–80 μM). (b) The linearity of fluorescent response at 688 nm between 0 and 80 μM AS concentration.

through MTT assays [36]. Cells were incubated with different concentrations of **NIR-HNO** for 12 h in a 96-well plate than remove the medium, MTT (0.5 mg/mL) media was added to cells. After incubation for 4 h, the produced formazan was dissolved in DMSO (150 μL) and read at OD 490 nm with a Spectramax microwell plate reader.

For imaging exogenous HNO, cells were firstly incubated with **NIR-HNO** (5 μM) for 30 min, washed with PBS, and then treated with HNO (20 μM and 50 μM) for another 30 min. For imaging of NaASc and SNP-induced endogenous HNO, cells were first co-incubated with NaASc (2 mM) and SNP (2 mM) for 2 h, washed with PBS, and then treated with **NIR-HNO** (5 μM) for another 1 h. The above-treated cells were imaged on a confocal microscope (Zeiss LSM 880). Emission was collected at the red channel (662–737 nm) with 561 nm excitation and a 20× objective lens.

2.4. Fluorescence imaging of zebrafish

Zebrafish (3 days postfertilization) were purchased from Eze-Rinka Company (Nanjing, China). The zebrafish were treated with **NIR-HNO** (10 μM) for 4 h. After removed the embryo medium and washed with PBS (10 mM, pH 7.4) three times, the zebrafish were further incubated with HNO (50 μM) for 1 h at 28 °C. The imaging was collected on a confocal microscope (Olympus Fluoview FV1000). Emission was collected at the red channel (570–670 nm) with 559 nm excitation and a 10× objective lens.

3. Results and discussion

3.1. Design and synthesis

The fluorophore is a crucial component of a fluorescent probe. Recently, isophorone-fused fluorophore **1** has received extensive attention due to its excellent NIR emission fluorescence and large Stokes shift, and the protection and deprotection of hydroxyl group of **1** can regulate the switch of fluorescence [41,42]. Thus, it was expected that probe **NIR-HNO** could be a NIR HNO fluorescent probe with large Stokes shift by the protection of the hydroxyl group with triarylphosphine. **NIR-HNO** was prepared by only one step with high yield, and its structure was characterized by NMR spectroscopy and HRMS in Supporting information (Figs. S1–3).

3.2. Spectral response

The absorption spectra of **NIR-HNO** and its reaction with HNO was firstly measured in PBS buffer solution (10 mM, pH 7.4, 50% DMF) at 25 °C. Angeli's salt (AS), a water-soluble HNO releasing molecule, was used as the source of HNO. The probe displayed a maximal absorption band at 406 nm. After incubating with AS, two absorption peaks appeared at 423 nm and 556 nm, which is because of the phenolic hydroxyl group of compound **1** could partly form a deprotonated phenate anion in DMF/PBS (1:1) solution (Fig. 1a) [43]. The fluorescence spectra of **NIR-HNO** were then tested. The probe itself exhibited weak fluorescence ($\Phi <$ 0.0006). After reaction with AS, an obvious NIR fluorescence with the

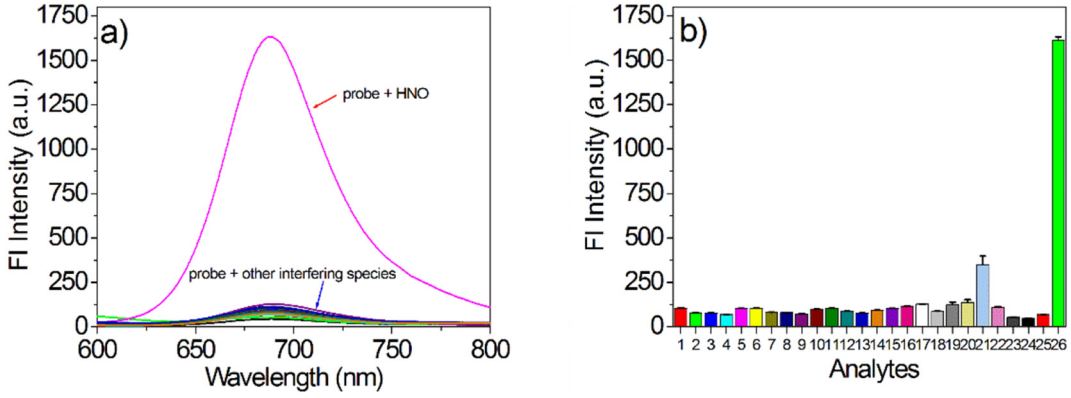

Fig. 3. Fluorescence responses of **NIR-HNO** (10 μM) in the presence of HNO (75 μM) and various relevant analytes (200 μM) in PBS solution (10 mM, pH = 7.4, 50% DMF) at 25 °C for 15 min, λ_{ex} = 556 nm. (1–18: NIR-HNO, NaF, NaCl, NaBr, NaI, KCl, $MgCl_2$, $CuCl_2$, $ZnCl_2$, $FeCl_3$, Na_2SO_4, Na_2SO_3, $NaNO_3$, $NaNO_2$, NaClO, H_2O_2, KO_2, NO, 200 μM; 19: $ONOO^-$, 100 μM; 20–21: CySNO, GSNO, 50 μM; 22: H_2S, 200 μM; 23–25: GSH, Cys, Hcy, 1 mM; 26: HNO, 75 μM).

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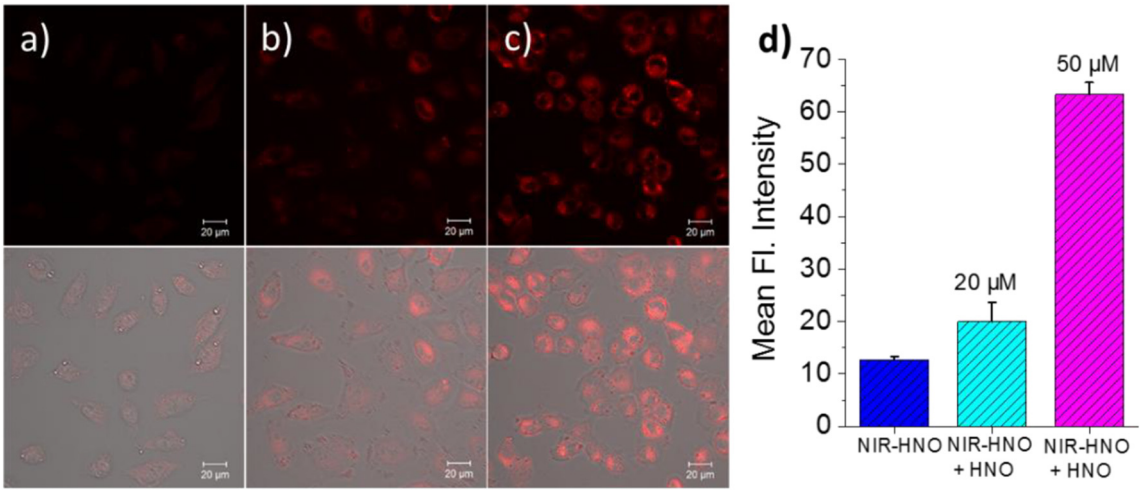

Fig. 4. Fluorescent imaging of exogenous HNO in HeLa cells. Cells were incubated with **NIR-HNO** (5 μM) for 30 min (a), and then treated with 20 μM (b) and 50 μM (c) AS for another 30 min. (Up) Red fluorescence channels. (Down) Merged fields. Scale bars = 20 μm. (d) Average fluorescence intensity of a–c. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

maximal value at 688 nm appeared with the excitation at 556 nm. To our knowledge, the remarkable Stokes shift for **NIR-HNO** is up to 265 nm, which is the largest among the NIR fluorescent probes reported so far for HNO (Table S1). The fluorescence quantum yield of compound **1** in the PBS and in PBS/DMF (1:1) solution is approximately 0.0095 and 0.0231, respectively, which indicate the potential applicability of the probe. HRMS of the reaction system was used to analyze the recognition mechanism. The peak at *m/z* 313.1318 $[M + Na]^+$ and 344.0811 $[M + Na]^+$ corresponding to compound **1** and amide derivative, respectively, was observed (Fig. S4). Therefore, **NIR-HNO** most likely undergoes the mechanism as the reported literature [32] and shown in Scheme 1B.

The time-dependent fluorescent response of the probe solution toward AS was performed. As shown in Fig. 1c, 75 μM AS could cause the reaction to be completed within 10 min. The pseudo-first-order rate constant (k_{obs}) was calculated as 0.29 min^{-1} and the reaction rate (k_2) was determined to be 64.78 $M^{-1}\cdot s^{-1}$, which is comparable with other phosphine-based HNO probes [37,39,44]. The rapid fluorescence response indicated that **NIR-HNO** was suitable for the detection of HNO. A time of 15 min after the addition of AS was selected in the subsequent experiments.

3.3. Sensitivity and detection limit

To evaluate the sensitivity of **NIR-HNO** to HNO, the fluorescence titration experiments were further carried out. As shown in Figs. 2 and S5, with the increase of AS concentration, the fluorescence signal gradually increased. The fluorescence signal at 688 nm was linearly related to the concentration of AS from 0 to 80 μM ($R = 0.9990$), indicating a large dynamic range for the detection of HNO. The detection limit was calculated to be 39.6 nM based on the $3\sigma/k$ method, where k is the slope between fluorescence intensity *versus* HNO concentration and σ is the relative standard deviation of the blank measurements. These results demonstrated that **NIR-HNO** is highly sensitive toward HNO in buffer solution.

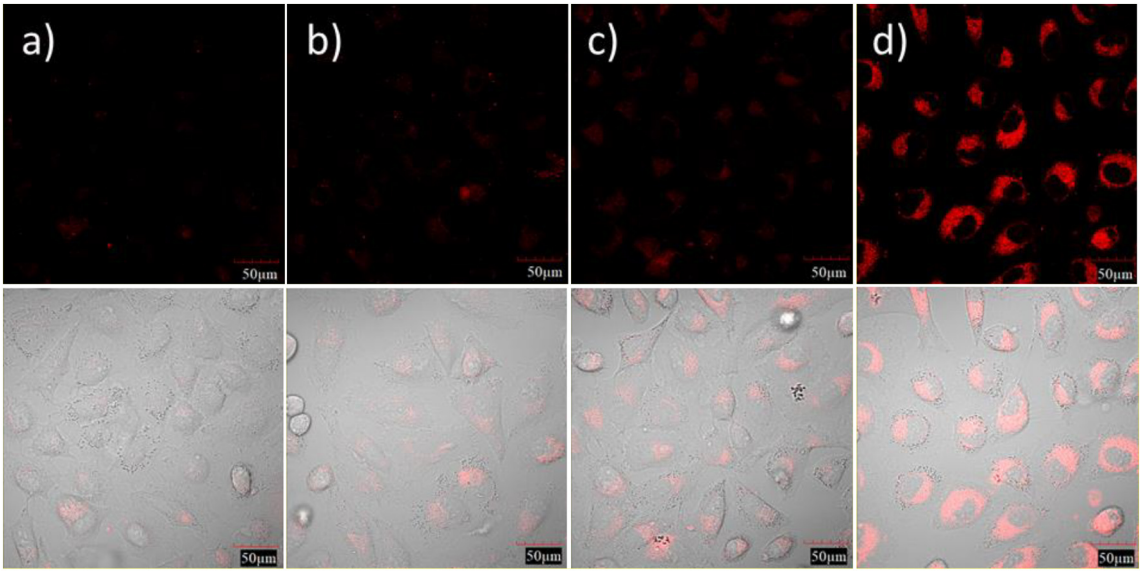

Fig. 5. Fluorescent imaging of NaASc and SNP-induced endogenous HNO in HeLa cells. Cells were incubated with **NIR-HNO** (5 μM) for 30 min (a), pre-treated with NaASc (2 mM) (b) or SNP (2 mM) (c) for 2 h, pre-treated with NaASc (2 mM) and SNP (2 mM) (d) for 2 h, and then treated with **NIR-HNO** (5 μM) for another 1 h. (Up) Red fluorescence channels. (Down) Merged fields. Scale bars = 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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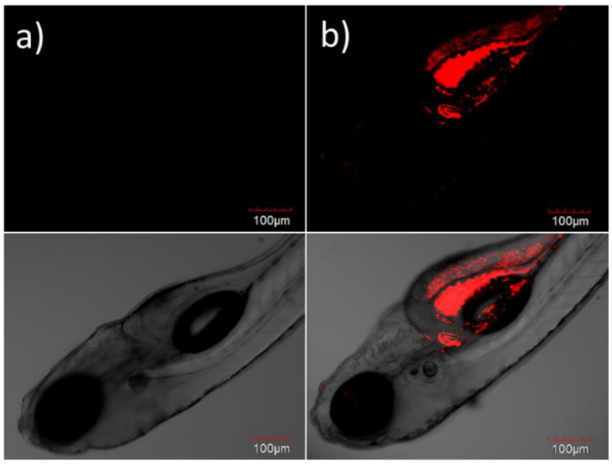

Fig. 6. Fluorescent imaging of exogenous HNO in zebrafish. Zebrafish were incubated with **NIR-HNO** (10 μM) for 4 h (a), and then treatment with AS (50 μM) for another 1 h (b). (Up) Red fluorescence channels. (Down) Merged fields. Scale bars = 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.4. Selectivity

Because of the complexity of biological systems, the selectivity is extremely important for the fluorescent probe. To test the selectivity of **NIR-HNO**, the reactivity of **NIR-HNO** toward different biological analytes was also carried. These biologically species include anions (F^-, Cl^-, Br^-, I^-, SO_4^{2-}, SO_3^{2-}, NO_3^-, NO_2^-), cations (K^+, Mg^{2+}, Cu^{2+}, Zn^{2+}, Fe^{3+}), reactive oxygen species (ClO^-, H_2O_2, KO_2), reactive sulphur species (Cys, Hcy, GSH, H_2S), and reactive nitrogen species (NO, $ONOO^-$, CySNO, GSNO). Various biological species were respectively added into **NIR-HNO** solutions to test their fluorescence response. As shown in Fig. 3, of all the tested species, GSNO only triggered small fluorescence enhancement, HNO caused an obvious increase of fluorescence, while other analysts did not trigger dramatic fluorescence enhancement. The excellent selectivity of **NIR-HNO** to HNO suggested it can be a powerful tool for specifically detecting HNO in biological environments. In addition, **NIR-HNO** could function over a wide range of pH from 6.5 to 10.0 (Fig. S7).

3.5. Bioimaging application in living cells

Before bioimaging, HeLa cells were chosen to evaluate the cytotoxicity of **NIR-HNO**. MTT assay results show **NIR-HNO** has only minimal cytotoxicity at 20 μM concentration, implying that the probe is suitable for imaging HNO in living cells (Fig. S7). HeLa cells were firstly incubated with **NIR-HNO** (5 μM) for 30 min, washed with PBS to remove the excess probe, and then treated with AS (20 and 50 μM) for another 30 min. As shown in Fig. 4, cells only treated with **NIR-HNO** displays very weak red fluorescence (Fig. 4a). For the cells treated with AS, a remarkable red fluorescence increase can be observed (Fig. 4b–d).

NIR-HNO was next used to imaging endogenous HNO. It is reported that intracellular reactions of L-ascorbate and NO could produce endogenous HNO [45]. HeLa cells were treated only with **NIR-HNO**, negligible fluorescence can be observed (Fig. 5a). By contrast, for HeLa cells were pretreated with L-ascorbate (NaASc, 2.0 mM) and sodium nitroprusside (SNP, NO donor, 2.0 mM) for 2 h, then incubated with **NIR-HNO** (5 μM) for another 1 h, the bright red fluorescence intensity was gathered (Fig. 5d). However, cells were pretreated with NaASc (2.0 mM) or SNP (2.0 mM) for 2 h and then incubated with **NIR-HNO** (5 μM) for 1 h, slightly brighter fluorescence imaging appeared (Fig. 5b and c). Whereafter, the photostability of compound 1 was also studied through fluorescence confocal measurements in living HeLa cells (Fig. S9), which indicated the possible capability of this probe for long time monitoring intracellular HNO changes in living HeLa cells.

3.6. Bioimaging of HNO in zebrafish

To further investigate the application of **NIR-HNO** in the living system, a zebrafish imaging experiment was also carried out. As shown in Fig. 6, zebrafish were first treated with **NIR-HNO** (10 μM) for 4 h, and almost no fluorescence could be observed. After further treated with AS (50 μM) for another 1 h, the red fluorescence increased dramatically. These results demonstrated that **NIR-HNO** can track HNO in a living system.

4. Conclusions

In summary, a new isophoron-fused NIR fluorescent probe was developed for the detection of HNO. This probe shows a fast response, good selectivity and high sensitivity. The remarkable large Stokes shift makes the probe more suitable for fluorescence detection of HNO in biological samples.

Declaration of competing interest

We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.saa.2019.117765.

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