A versatile ratiometric fluorescent probe based on two-isophorone fluorophore for sensing of nitroxyl

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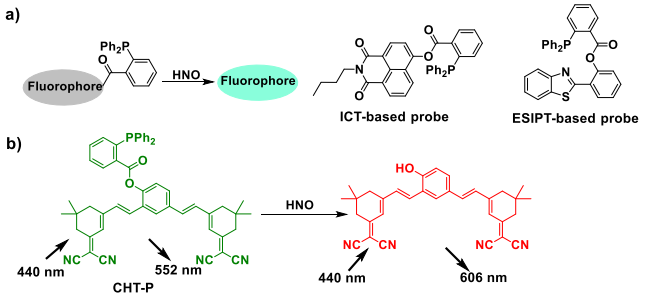
ABSTRACT: Nitroxyl (HNO) is closely linked with numerous biological processes. Fluorescent probes provide a visual tool for determining HNO. Due to fluorescence quenching by HNO-responsive recognition groups most of the current fluorescent probes exhibit an "off-on" fluorescence response. As such, the single fluorescence signal of these probes is easily affected by external factors such as the microenvironment, sensor concentration, and photobleaching. Herein, we have developed a ratiometric fluorescent probe (**CHT-P**) based on our previously developed two-isophorone fluorophore. **CHT-P** could be used to determine HNO through ratiometric signal readout with high selectivity and sensitivity, ensuring the accurate quantitative detection of HNO. Additionally, the probe exhibited low cytotoxicity, was cell permeable and could be used for ratiometric imaging of HNO in cells. Finally, **CHT-P** coated portable test strips were used to determine HNO using solid-state fluorescence signal readout.

Nitroxyl (HNO) is a biologically important reactive species that is a one-electron reduction and protonated derivative of nitric oxide (NO), which is closely linked with many physiological and pathological processes.[1,2] For example, HNO can enhance the myocardial contractility and inhibit platelet aggregation.[3] In addition, HNO has certain pharmacological applications, for example the treatment of heart failure and has certain anti-inflammatory capabilities. Other significant roles of HNO include interaction with thiols, inhibition of the activity of glyoxal dehydrogenase in cells, and it can be used to treat alcoholism, and alleviate ischemia-reperfusion injury.[2-5] Therefore, the development of a convenient and efficient method to detect HNO, especially the quantitative analysis, is of great significance in order to understand the underlying mechanism of HNO-related diseases in living organisms and in addition could aid the development of rapid and accurate diagnostic methods.

So far, numerous methods for HNO detection have been reported, such as EPR,[6] ^{19}FNMR,[7] MS,[8] HPLC.[9] colorimetry[10] and electrochemical analysis[11]. However, limitations still exist, especially for use in living biological samples, where systems require difficult instrumental operation, complex sample preparation and cause damage to living organisms and cells. As such, organic small-molecule fluorescent probes which exhibit high selectivity, sensitivity, and temporal resolution, for the specific detection of HNO have been applied to living biological systems.[12-17] Among these organic small-molecule fluorescent probes for HNO, most of them are constructed by introducing responsive groups including copper (II) complexes, triphenylphosphine, nitroxide and 2-mercapto-2-methylpropionic acid as the specific recognition site for HNO[12]. The quenching efficiency of the responsive groups attached to the core fluorophore makes most of these probes emit very weak fluorescence before they interact with HNO. Thus, these fluorescent probes commonly exhibit “off-on” response towards HNO, such single signal response systems can exhibit reduced accuracy for quantitative detection of HNO since the measurement of one fluorescence emission is easily affected by the microenvironment, photobleaching and excitation intensity. To address this problem, significant effort has been devoted to the development of ratiometric fluorescent probes, which could provide a direct relationship between the concentration of the target analytes and the ratiometric

signal without calibration.[18-20] Some recently reported dual-emissive fluorescence probes for HNO have been based on excited state intramolecular proton transfer (ESIPT),[21] intramolecular charge transfer (ICT),[22] and Förster resonance energy transfer (FRET),[23-25] using the 2-(diphenylphosphino)-benzoate moiety as the responsive group towards HNO.[21-30] However, for these dual-emissive systems the shorter wavelength fluorescence emission either increases or is not reduced significantly when the 2-(diphenylphosphino) benzoate has been cleaved by HNO, making these ESIPT and ICT-based probes unsuitable for accurate ratiometric dual emission monitoring (Scheme 1a).[21,22,26,27] Additionally, other probes (*e.g.* FRET-based fluorescence probes) need complex design and involve the multi-step synthesis of the two fluorescent dyes and incorporation of them into a single probe.[23-25,30]

Our research group is particularly interested in developing ratiometric fluorescent probes for the quantitative detection of biological relevant analytes. Previously, we reported a fluorophore bearing two isophorone malononitrile structures (**CHT-OH**) that could be used to construct a ratiometric fluorescent probe for biothiols[31]. We realized that our fluorophore (**CHT-OH**) could provide a core on which to develop a range of ratiometric fluorescence based probes (Fig. S5). In this work, we incorporate a HNO-response group 2-(diphenylphosphino)-benzoate moiety into the **CHT-OH** core in order to develop a ratiometric fluorescent probe that could be used as a chemosensor (**CHT-P**, Scheme 1b) for the quantitative detection of HNO. Herein, the 2-(diphenylphosphine)benzoic acid group limits delocalization within **CHT-P** and as such the probe exhibits a maximum emission at 552 nm when excited at 440 nm. When HNO interacts with **CHT-P**, the recognition unit 2-(diphenylphosphino)benzoate moiety is cleaved. The maximum fluorescence emission of **CHT-P** is then red-shifted due to the redistribution of the electrons throughout the whole conjugated system of **CHT-P**, and ratiometric detection could be achieved.



Scheme 1. (a) Commonly used design strategy for ICT and ESIPT-based dual-emissive fluorescent probes for the detection of HNO. (b) Our design strategy for the ratiometric detection of HNO.

EXPERIMENTAL SECTION

Materials and Methods. All experimental reagents are commercially available and were used directly without further purification. Angeli's salt (AS) was used as the donor of nitrosyl hydrogen in the experimente. Reagents such as absolute ethanol (EtOH), piperidine, potassium carbonate (K_2CO_3), 4-hydroxyisophthalaldehyde, malononitrile, isophorone, dichloromethane (DCM), 4-dimethylaminopyridine (DMAP), dicyclohexylcarbodiimide (DCC) and 2-(diphenylphosphine)benzoic acid were purchased from Energy Chemical and Macklin Chemical. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and various amino acids were purchased from Sigma-Aldrich. Column chromatograpic separation was performed using silica gel of 200-300 Mesh (Shanghai, Qingdao). Thin layer chromatography (TLC) was performed on silica gel plates, and the product visualized using a 365 nm ultraviolet lamp. The absorption measurement of the target probe was obtained on a Hitachi U-3900 UV-Vis spectrometer. The fluorescence spectra were measured on a Hitachi f-7000 fluorescence spectrometer. An appropriate fluorescence intensity range was determined by adjusting the excitation and emission slits. The pH of all solutions used in the experiments were measured using a Leici phs-3e pH meter, calibrated using pH 4.00 and pH 6.86 buffer. ^{1}H and ^{13}C NMR spectra were obtained using a Bruker AV-400 spectrometer. The chemical shifts are expressed in parts per million (Me_4Si as internal standard). The probe **CHT-P** (1×10^{-3} M) stock solution was prepared in dimethyl sulfoxide (DMSO) and diluted with PBS (pH 8.0) buffer during measurement in order to keep the final probe concentration at 10 μM. All spectroscopic experiments were performed using PBS buffer, and 3 μM of HSA was added to the test system to mimic the real cell microenvironment. AS, inorganic salts and various amino acid were prepared as stock solutions in water.

Synthesis of CHT-P. 2-(Diphenylphosphine)benzoic acid (0.600 g, 1.96 mmol), **CHT-OH** (0.477 g, 0.98 mmol), 2- DMAP (0.239 g, 1.96 mmol), DCC (0.404 g, 1.96 mmol) were mixed in DCM (15 mL). The reaction mixture was then heated under a nitrogen atmosphere at 40 °C for about 2 h. The solvent was removed under reduced pressure, and the product was purified by flash column chromatography to provide **CHT-P** as areddish brown solid (0.319 g, yield 42%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.30–8.27 (m, 1H), 8.18–8.15 (m, 1H), 7.74 (d, J = 1.2 Hz, 1H), 7.55-7.53 (m, 2H), 7.48-7.43 (m, 2H), 7.35-7.33 (m, 9H), 7.22-7.18 (m, 1H), 7.11-7.00 (m, 4H), 6.96-6.89 (m, 2H), 6.84 (s, 1H), 2.63 (s, 2H), 2.58 (s, 2H), 2.49 (s, 2H), 2.41 (s, 2H), 1.10 (s, 6H), 1.03 (s, 6H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 190.51, 169.05, 153.30, 153.20, 149.60, 137.17, 137.07, 135.22, 134.85, 134.02, 133.81, 133.07, 131.91, 130.06, 129.36, 128.67, 128.60, 128.48, 113.32, 112.44, 42.97, 39.29, 39.20, 32.03, 31.99, 28.02, 27.96. HRMS (ES^+): calc. for $[M+H]^+$ 775.3196, found 775.3193, M represents $C_{51}H_{43}N_4O_2P$.

Fluorescence Imaging using CHT-P. Fluorescence imaging of exogenous HNO in live cells. HeLa cells were cultured in a confocal culture dish with the complete medium (90% MEM and 10% FBS) in a standard culture condition (5% CO_2 moist sterilization incubator at 37°C) for 15 hours to make the cells adhere to the confocal culture dish. Then the cells were washed with MEM medium three times, 2 mL fresh MEM was finally loaded with the

cells. Then the probe (10 μM) was added and incubated for 30 min under standard culture conditions to allow the probe to enter the cells. After that, AS (200 μM) was added and incubated for a further 30 min under standard culture conditions. After that, the cells were washed three times with fresh MEM and 1.5 mL of fresh MEM was added for fluorescence imaging of the cells.

The fluorescence imaging of the cells was performed using a Leica TCS SP5 II confocal laser scanning microscope using an HC × plapo 63x oil objective (NA: 1.40). The probe was excited at 405 nm and the emission collection range was 520 to 590 nm (channel 1, green) and 610 to 700 nm (channel 2, red).

RESULTS AND DISCUSSION

Design strategy for the CHT-P probe for ratiometric detection. We previously synthesized a fluorescent probe for determining biothiols using **CHT-OH** as the core structure[31]. To further extend the application of the core fluorophore **CHT-OH** using a different sensing mechanism, we prepared a ratiometric fluorescent probe (**CHT-P**) for the detection of HNO by anchoring the 2-(diphenylphosphino)benzoate moiety to the two-isophorone fluorophore. As indicated in Fig. S1, the absorption and fluorescence emission spectra of **CHT-P** and **CHT-OH** indicated that **CHT-P** could be used as a ratiometric probe. Additionally, the quantum yield of **CHT-P** was determined to be 0.28 (Fig. S2) and molar absorptivity was 56330 M^{-1} cm^{-1} (Fig. S3).

Time-dependent fluorescence changes of CHT-P for nitroxyl. The probe **CHT-P** was synthesized in two simple steps (Scheme S1). Then, we evaluated the photophysical properties of **CHT-P**. The absorption and fluorescence intensity of **CHT-P** did not change significantly over a pH range between 6.5 and 9.0 (Fig. S4). The fluorescence behavior of **CHT-P** was subsequently monitored in PBS buffer (with 5% DMSO and 3 μM HSA as cosolvent). Herein, HSA was used because in most assays in live organisms, serum albumin is an essential compound that should not be excluded and will coexist with the target compound in the assay system. Importantly, the HSA did not affect the UV absorption and fluorescence of **CHT-P** (Fig. S5). Then, we explored the response of **CHT-P** towards AS (a HNO donor) over time. When AS (200 μM) was added to a solution containing **CHT-P** (10 μM), an isosbestic point at 440 nm appeared (Fig. S6). Then under the excitation at 440 nm, the fluorescence intensity at 552 nm gradually decreased followed by a gradual increase of fluorescence intensity at 606 nm, and an iso-emissive point was observed at 574 nm (Fig. 1a and 1b). This phenomenon could be ascribed to the 2-(diphenylphosphino)benzoate moiety of **CHT-P** which quenches the long wavelength fluorescence of **CHT-P**, while the short-wavelength fluorescence of **CHT-P** is retained. The addition of HNO cleaves the 2-(diphenylphosphino)-benzoate resulting in restoration of the long wavelength fluorescence, facilitating the ratiometric detection of HNO. The response time of **CHT-P** towards HNO is comparable to most reported probes.[15,16,32] Actually, the probe **CHT-P** exhibits a fast response towards the addition of HNO, but requires about 30 min to complete the reaction. This phenomenon could be ascribed to that the time for reaction between the triphenylphosphine based group and Angeli's salt (to generate HNO) is more than 20 min.[33] Thus, the probe can response towards HNO fast (within 2 min) but it may need a relatively long time (about 30 min) to complete the interaction between the triphenylphosphine based fluorescent probe and Angeli's salt. The pH effect on the fluorescence response of **CHT-P** towards HNO was also investigated (Fig. S7). A high ratiometric signal was obtained when for buffer in the pH range of 6 to 9. While a remarkable decreased in the ratiometric signal with pH values above 9 or below 5.

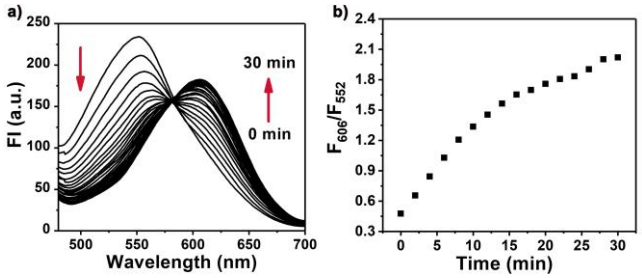


Figure 1. (a) Time-dependent fluorescence changes of **CHT-P** (10 μM) with the addition of AS (200 μM) in PBS/DMSO (v / v, 20: 1; pH 8.0; 3 μM HSA) at 25°C. (b) A plot of the ratiometric fluorescence intensity changes (based on fluorescence intensity at 552 nm and 606 nm, respectively). The assay was conducted in PBS/DMSO (v/v, 20:1; pH 8.0; 3 μM HSA) at 25°C, λ_{ex} = 440 nm.

Study of the reaction mechanism. We further explored the reaction mechanism of **CHT-P** using ^{1}H NMR and mass spectroscopic analysis. Comparing a **CHT-P** solution with and without AS, revealed the appearance of a phenol proton peak at about 9.85, and at the same time, changes of proton peaks at 8.33-8.27, 7.61-7.48, 7.32-7.28 which appeared similar to the peaks of **CHT-OH** (Fig. 2). Thus, the addition of AS converts probe **CHT-P** into **CHT-OH**. In addition, the MS analysis further confirmed that the addition of AS results in a mass peak due to **CHT-OH** (Fig. S8).

To further explore the sensing mechanism, we performed DFT calculations of **CHT-P** and **CHT-OH**. From the optimized S1 geometries, the HOMO-LUMO gap of **CHT-OH** was about 2.420 ev, while that for **CHT-P** was 2.450 ev (Fig. S9). The computational analysis indicates that cleavage of the 2-(diphenylphosphino)-benzoate of **CHT-P** by HNO results in the redistribution of electrons throughout the whole conjugated system of the product **CHT-OH**, resulting in a red-shift of the fluorescence.

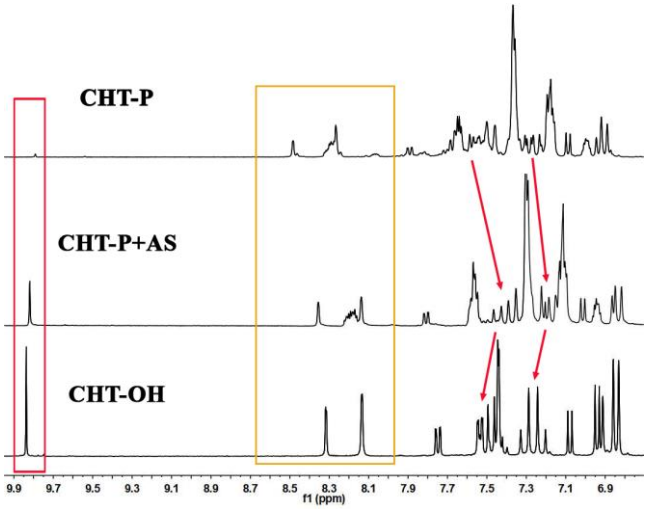


Figure 2. ^{1}H-NMR analysis of **CHT-P** (35 mM, up), **CHT-P** reacted with AS (middle) for 30 min, and **CHT-OH** (35 mM). The titration assay was conducted in DMSO-d_6, with addition of 1 equivalent of AS for 30 min.

Quantitative detection of HNO by CHT-P. In order to prove that **CHT-P** can quantitatively detect HNO, a gradual increase in concentration of AS (0-200 μM) was added into a solution of **CHT-P** and an isosbestic point at 440 nm appeared (Fig. S10). Excitation at 440 nm, with gradual addition of AS induced a decrease in the emission intensity at 552 nm, and increase of fluorescence intensity at 606 nm, with an iso-emissive point at 570 nm (Fig. 3a). A linear relationship between the fluorescence intensity ratio (F_{606} / F_{552}) of **CHT-P** (10 μM) and the concentration of AS (0 to 120 μM) was observed (Fig. 3b). The fluorescence ratio changes reached a plateau and the limit of detection (LoD) was determined to be 1.01 μM. These results indicate that **CHT-P** can be used for the quantitative detection of HNO using ratiometric fluorescence signal changes.

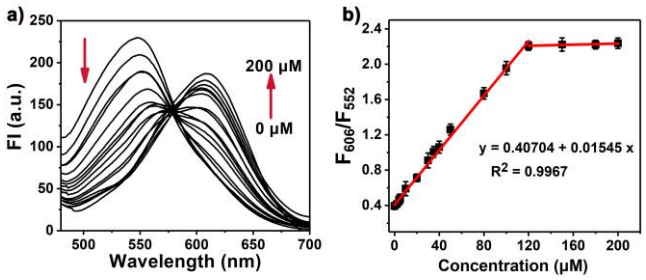


Figure 3. (a) Changes in fluorescence intensity of **CHT-P** (10 μM) with increasing concentration of AS (from 0 to 200 μM) in PBS/DMSO (v/v, 20:1; pH 8.0; 3 μM HSA) after 30 min. λ_{ex} = 440nm. (b) Linear relationship between fluorescence intensity ratio changes and concentration of HNO over a range from 0 to 200 μM (based on the fluorescence intensity at 552 and 606 nm, respectively).

Selectivity of CHT-P for HNO. We then investigated the selectivity of **CHT-P** towards HNO in the presence of other interfering substances. **CHT-P** exhibited remarkable selectivity towards HNO compared to other anions, cations, reactive oxygen species and amino acids. Because the fluorescence emission peak of the probe does not change significantly in the presence of these interferences. However, addition of AS induced a clear fluorescence change of **CHT-P** from 552 nm to 606 nm (Fig. 4). In addition, fluorescence color changes could be conveniently observed under a hand-held laser lamp (405 nm, inset in Fig. 4b). In detail, the probe solution treated with just AS appears orange-red, while the probe solution treated with other analytes appears pale yellow, indicating that **CHT-P** has the potential to be used as a fast, convenient, and portable method for the measurement of HNO concentrations. To explore the selectivity of **CHT-P**, we carried out a competition assay for the response of **CHT-P** to AS in the presence of other analytes. From Fig. S11, it is clear that **CHT-P** exhibits no response towards other analytes, but that the addition of AS could significantly induce a red shift of the fluorescence emission of **CHT-P**, clearly demonstrating the high selectivity of **CHT-P** towards HNO.

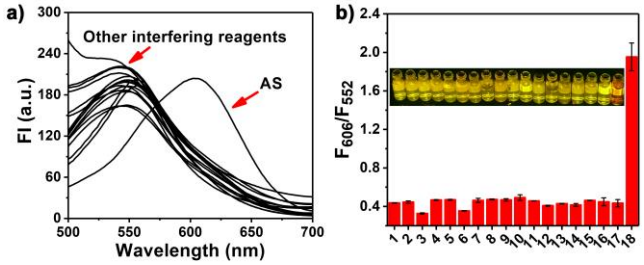


Figure 4. (a) Fluorescence responses of **CHT-P** (10 μM) towards different species: AS (200 μM) and other interfering reagents within 30 min (1. K^+; 2. Cu^{2+}; 3. Na^+; 4. Zn^{2+}; 5. F^-; 6. S^{2-}; 7. Mn^{2+}; 8. NO_2^-; 9. NO_3^-; 10. GSH; 11. Cys; 12. Sodium ascorbate (NaAsc); 13. DL-Homocysteine (Hcy); 14. H_2O_2; 15. ClO^-; 16. $ONOO^-$; 17. $ROO^•$). Herein, species 1-15 are 3 mM, and 16, 17 are 200 μM. b) Fluorescence intensity ratio changes (based on the peak heights at the maxima, 552 nm and 606 nm respectively) according to a). The inset is a photograph of the species under the irradiation using a hand-held laser lamp (405 nm).

Fluorescence imaging in living cells. Having established the ratiometric detection of HNO using **CHT-P**, we then explored the fluorescence imaging ability of **CHT-P** in live cells. Initially, cytotoxicity experiment was conducted and indicated that **CHT-P** has low toxicity, permitting its use in live cell imaging experiments (Fig. S12). Then the photostability of **CHT-P** was evaluated. Both **CHT-P** and **CHT-OH** exhibited high photostability during the continuous irradiation under the Xe lamp (Fig. S13). **CHT-P** also exhibited photostable in living cells (Fig. S14). Then, the fluorescence images of the cells with **CHT-P** were obtained using a confocal laser scanning microscope. From Fig. 5a, a strong green fluorescence (collected at channel 1: 520 to 590 nm), and no red (collected at channel 2: 610 to 700 nm) was observed (**CHT-P** panel in Fig. 5a).

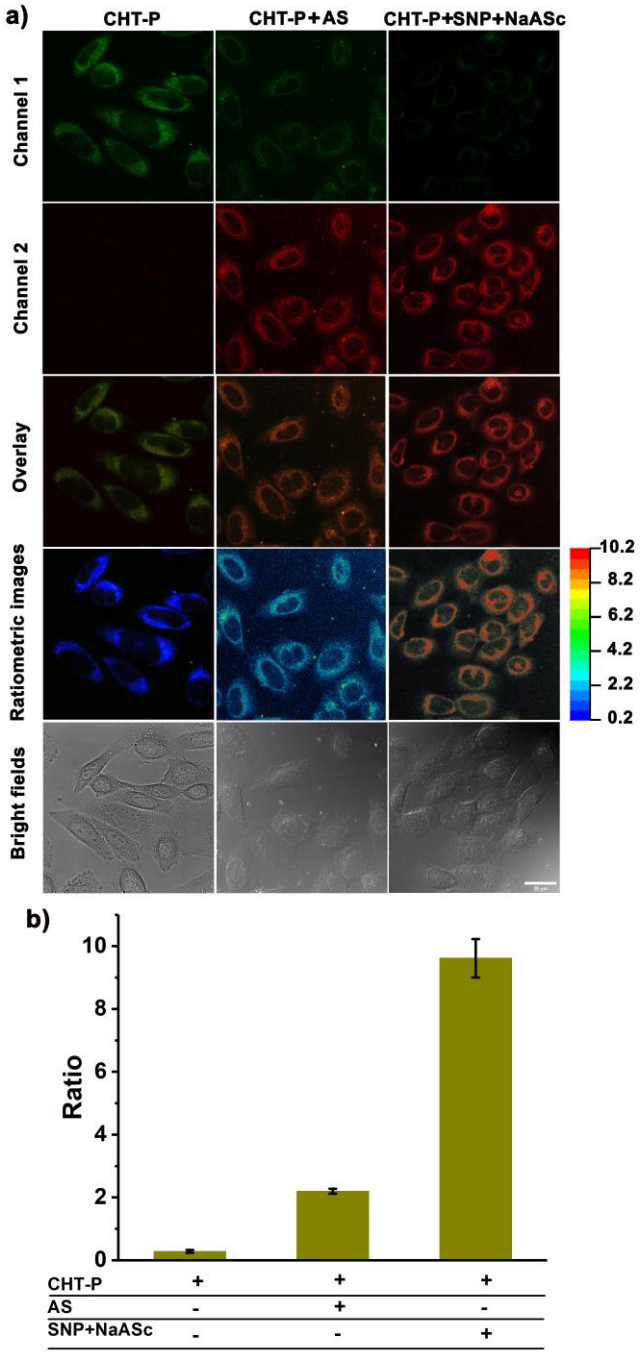


Figure 5. (a) Fluorescence imaging of HeLa cells with probe **CHT-P** (10 μM). Channel 1 (green): 520 to 590 nm. Channel 2 (red): 610 to 700 nm. (**CHT-P**): confocal images of HeLa cells pretreated with **CHT-P** (10 μM) for 30 min; (**CHT-P** + AS): confocal images of HeLa cells pretreated with **CHT-P** (10 μM) for 30 min and then incubated with AS (200 μM) for 30 min. (**CHT-P** + SNP + NaAsc): confocal images of HeLa cells pretreated with **CHT-P** (10 μM) for 30 min and then incubated with SNP (2 mM) and NaAsc (2 mM) for 2 h. Overlay: merge channel of channels 1 and 2. Ratiometric images were obtained with ImageJ software. Scale bar, 25 μm. (b) Semi-quantitative determination of HNO in live cells with the ratiometric fluorescence signal readout. Ratio is obtained according to the ratio values of fluorescence intensity of channel 1 (from 520 to 590 nm) to channel 2 (from 610 to 700 nm). The semiquantitative calculation was conducted by ImageJ software. Error bars represent SD. "+" represents the addition of compounds, "-" represents no addition of compounds.

Then, AS (for generating the exogenous HNO) was added to the cells leading to a ratiometric fluorescence change. The fluorescence signal collected from channel 1 decreased significantly, whereas a strong fluorescence signal appears in the red fluorescence channel (collected at channel 2: 610 to 700 nm) (**CHT-P** + AS panel in Fig. 5a). Based on the above results, we evaluated whether **CHT-P** can be used to monitor endogenous HNO in cells. According to literature reports, L-ascorbate (NaAsc) reacts with NO to form intracellular HNO in the cellular environment[34,35]. Therefore, live HeLa cells were treated with **CHT-P** (10 μM) for 30 minutes, and then 2.0 mM NaAsc and 2.0 mM sodium nitroprusside (SNP, NO donor) were added to pretreat the HeLa cells for another 2 h. In detail, no significant fluorescence signal was collected in channel 1, while a strong fluorescence signal appears in the red fluorescence channel (collected at channel 2: from 610 to 700 nm) (**CHT-P** + SNP + NaAsc panel in Fig 5a). The merged channel and ratiometric images from channel 1 and channel 2 were obtained. In addition, the semi-quantitative determination of HNO in cells was possible using the ratio values of the averaged fluorescence intensity of channel 1 to channel 2 (Fig. 5b). These results indicated that **CHT-P** was a cell-permeable fluorescent probe, that could be used for the ratiometric imaging of exogenous and endogenous HNO in cells. In addition, the ratiometric signal could be used for semi-quantitative determination of HNO.

Detection of HNO using CHT-P-based portable test papers. To further explore the function of the **CHT-P**, a portable test strip was prepared by immersing filter paper into **CHT-P** solution (detailed procedure is contained in the supporting information). Due to the solid-state fluorescence signal, the **CHT-P** coated test strips exhibited yellow fluorescence signal after it was exposed to HNO solution, while other analytes resulted in a green fluorescence signal, indicating the high selectivity of the test strips towards HNO (Figure 6a). Additionally, the test strips could be used for the detection of different concentrations of HNO solution. The fluorescence signal changes from the green to yellow when the concentration of HNO increased from 0 to 200 μM, suggesting the efficiency of the **CHT-P** coated test strips for monitoring HNO in solution over a concentration range from 0-200 μM. Thus, the use of the **CHT-P** as a fluorescent probe could be expanded for use as portable test strips, which facilitates the detection of HNO in various complex samples. In addition, this strategy may offer a new approach for constructing low-cost portable devices for the detection of other analytes.

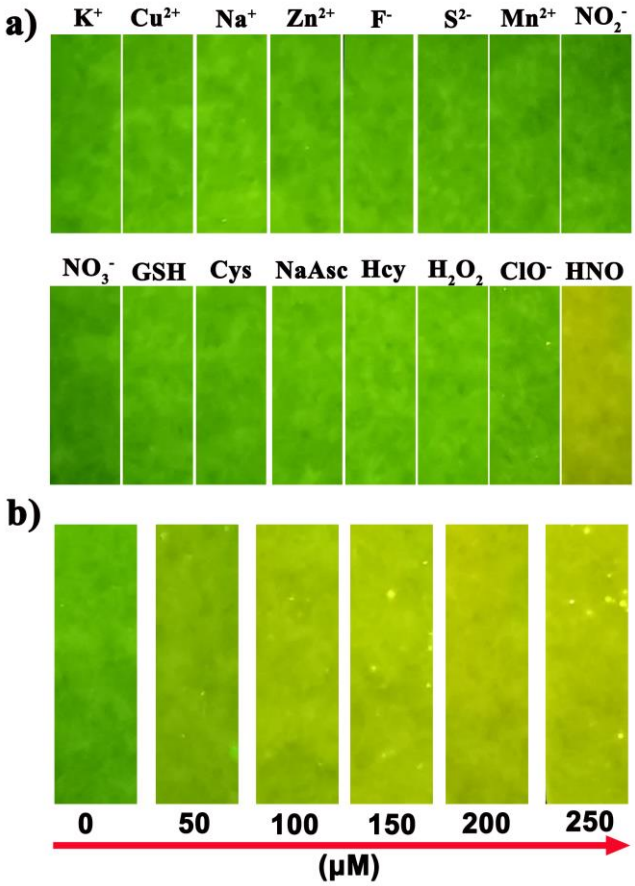


Figure 6. Portable test strips for the detection of HNO using solid-state fluorescence signal readout. (a) The photograph of the portable test strips after exposure to various analytes (3 mM) and AS solution (200 μM) under a hand-held UV light (365 nm). (b) The photograph of the portable test strip after exposure to various concentration of HNO solution.

CONCLUSION

In summary, a ratiometric fluorescent probe (**CHT-P**) for HNO has been developed using a novel fluorophore with two isophorone malononitrile groups that displays green to red fluorescence changes when an activatable group was removed from the fluorophore. Thus, **CHT-P** exhibited ratiometric fluorescence signal changes towards HNO, permitting the quantitative detection of HNO using the linear relationship between changes of the ratio of the fluorescence signal and concentration of HNO. The LoD was determined to be 1.01 μM. In addition, **CHT-P** exhibited a high selectivity, low cytotoxicity, and cell permeability. The probe could be used for the ratiometric imaging of exogenous and endogenous HNO in living HeLa cells under physiological conditions. Finally, **CHT-P** coated portable test strips exhibited high performance in detecting HNO using fluorescence signal readout.

ASSOCIATED CONTENT

Supporting Information. Additional materials and methods including detailed protocol for synthesis, characterization of target compounds, cell cultures, and detailed experiments for live cell imaging were in the supporting information.

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Notes

The authors declare no competing financial interest.

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