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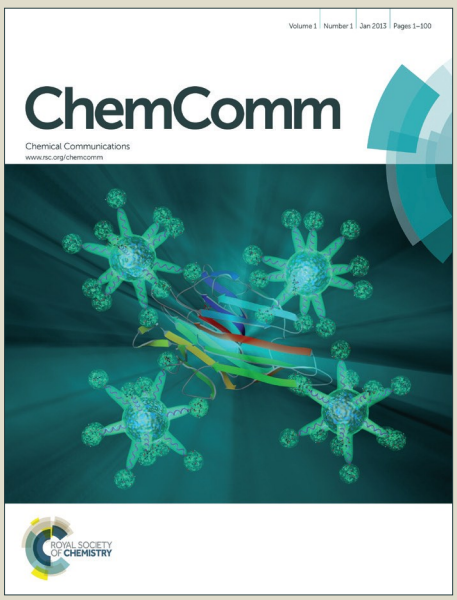

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A FRET-based ratiometric two-photon fluorescent probe for dual-channel imaging of nitroxyl in living cells and tissues

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A FRET-based two-photon fluorescent probe, P-Np-Rhod, which exhibited a fast and high selective ratiometric response to nitroxyl, was first proposed. P-Np-Rhod was successfully applied to two-photon dual-channel imaging of nitroxyl in living cells and tissues with less cross-talk between channels and satisfactory deep-tissue imaging depth.

Nitroxyl (HNO), the one-electron reduced form of NO, has recently been found to possess unique biological activity and pharmacology applications.[1] For example, HNO can inhibit the activity of aldehyde dehydrogenase by interacting with its protein thiols.[2] And it has been demonstrated to exhibit the potential for treating heart failure through upregulating the calcitonin gene-related peptide which can induce vasorelaxation.[3] Moreover, HNO could also be used to treat alcoholism, mitigate ischemia-reperfusion injury and functionalize as a potential anti-cancer drug.[4] In this light, the development of a reliable assay method that can monitor HNO in biosystems is highly desired.

Until recently, several analytical methods including colorimetric approach,[5] electron paramagnetic resonance spectroscopy,[6] high performance liquid chromatography,[7] mass spectrometry[8] and electrochemical analysis[9] have been developed for HNO detection. In despite of appropriate sensitivity, these methods are unsuitable for detection of HNO in living systems, due to the complicated pretreatment and potential destruction of biological samples. In recent years, fluorescent probes have been widely applied for bio-imaging of various analytes in living cells, tissues and organisms by virtue of their high sensitivity, fast response time, and little bio-damage.[10] Recently, based on the reaction of HNO with triarylphosphine, various fluorescent probes were rationally designed to discriminate HNO from other biological reductants.[11] Nevertheless, the biological applications of these fluorescent probes are limited by one-photon excitation with short wavelengths, which could lead to photobleaching of probes, restricted tissue-penetration depth, and high cellular autofluorescence. In sharp contrast, two-photon microscopy (TPM) approach, which excites a two-photon (TP) fluorophore with near-infrared (NIR) laser pulses, can overcome these shortages and provide improved three-dimensional spatial localization with reduced photodamage to biosamples and increased tissue penetration depth.[12] Despite all these advantages, only few TP fluorescent turn-on probe has been reported for the detection of HNO.[11k] Moreover, this turn-on probe was limited for practical applications, due to the fact that the fluorescence intensity measurement within a single detection window may vary with the experimental conditions, such as the incident laser power and the distribution of probes. Ratiometric probes, by measuring the changes of the intensity ratio at two different emission peaks, can provide a built-in correction to minimize these interferences, resulting in a more effective system for imaging living cells and tissues.[13] It is worth pointing out that, although several one-photon excited ratiometric fluorescent probes have been developed for the detection of HNO[11d, e, g, j], to our best knowledge, no ratiometric TP fluorescent probes for detecting HNO has been reported to date.

In this work, by combining the advantages of TPM technique with ratiometric probe, we designed and synthesized, for the first time, a ratiometric TP fluorescent probe (**P-Np-Rhod**, Scheme 1) for HNO based on a FRET strategy. In this FRET system, a TP naphthalene derivative served as the energy donor and a rhodol fluorophore was chosen as the energy acceptor which was modified with 2-(diphenylphosphino)-benzoate moiety as a recognition unit for HNO. In the absence of HNO, the rhodol existed in a non-fluorescent lactone form and the FRET is off. As reported by previous literatures, HNO can react with 2-(diphenylphosphino)-benzoate moiety to give the corresponding aza-ylide, which can attack the adjacent ester linker in an intramolecular manner to release hydroxyl. And then the closed spirolactone form was converted to a conjugated fluorescein xanthene form to induce the occurrence of FRET (Scheme S2, ESI†). The sensing process was confirmed by both NMR spectroscopy and mass spectrometry (Fig. S9 and Fig. S10, ESI†). The detailed synthesis procedures and characterization of all the new compounds

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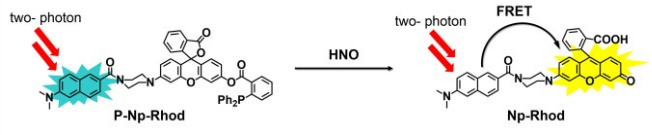
are described in the ESI†.



Scheme 1 Chemical structure and the response mechanism of probe **P-Np-Rhodol** to HNO.

The spectroscopic properties of the probe **P-Np-Rhod** were firstly investigated in buffered (10 mM PBS, pH = 7.4) aqueous CH_3CH_2OH solution (H_2O/CH_3CH_2OH = 1:1, v/v). In the absence of HNO, the free probe **P-Np-Rhod** displayed a cyan-colored fluorescence with an emission peak at 448 nm (Fig. 1). After treatment with enhanced concentrations of AS (from 0 to 100 μM, Angeli's salt (AS) acted as the source of HNO in our experiments), a new fluorescence emission peak at 541 nm appeared and increased gradually, while the donor's characteristic emission peak (448 nm) showed a decreased tendency (Fig. 1), allowing a ratiometric fluorescence response for the detection of HNO. It is noteworthy that the large wavelength difference (93 nm) between the two emissions leads to two well-resolved emission bands for the probe, which is favourable for dual-channel imaging of HNO in biological samples with less cross-talk observed. We proceeded to investigate the UV-absorption properties of the probe. Upon addition of AS, no obvious absorption changes of the donor occurred, while a new absorption peak (at 511 nm) belonging to the conjugated xanthene form of the acceptor increased with an AS dose-dependent pattern. Meanwhile, the solution changed from colorless to orange, allowing colorimetric detection of HNO by naked eyes (Fig. S1, ESI†). The remarkable changes in absorption and fluorescence spectra might be attributed to the HNO-induced occurrence of FRET between the donor naphthalene and acceptor rhodol. The Förster energy transfer efficiency between the donor and acceptor rhodol was calculated to be 88.6% (Fig. S2, ESI†). The fluorescence intensity ratio value between the two wavelengths ($I_{541\ nm}/I_{448\ nm}$) varied from 0.13 to 4.67 with the concentration of HNO changed from 0 to 100 μM (Fig. 1). The detection limit for HNO was calculated to be 5.9×10^{-7} M (3σ/slope, Fig. S3, ESI†), indicating that the probe **P-Np-Rhod** exhibited a high sensitivity for HNO.

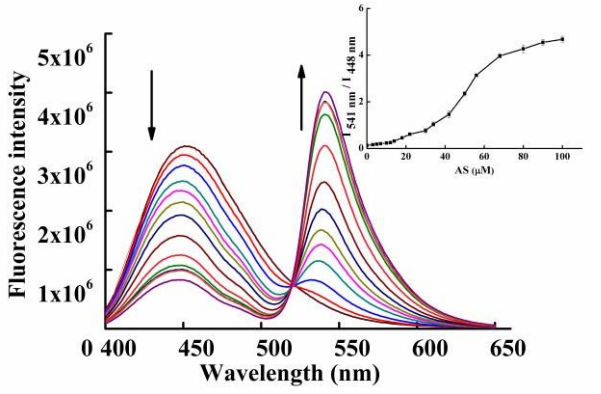


Fig. 1 Fluorescence emission spectra of probe **P-Np-Rhod** (15 μM) in the presence of various concentrations of AS (0-100 μM) in PBS buffered (10 mM, pH = 7.4) aqueous EtOH solution (1:1, v/v). Inset: The fluorescence intensity ratio ($I_{541\ nm}/I_{448\ nm}$) of probe **P-Np-Rhod** (15 μM) versus AS concentration after incubation of the probe with AS for 30 min. λ_{ex} = 370 nm.

The time-dependent fluorescence response of probe **P-Np-Rhod** to different concentrations of AS (5, 35, 55 or 100 μM) were examined by monitoring the change of fluorescent intensity ratio between the two wavelengths ($I_{541\ nm}/I_{448\ nm}$) after the addition of AS in PBS buffer. As shown in Fig. S4 (ESI†), the fluorescent intensity ratio ($I_{541\ nm}/I_{448\ nm}$) obviously increased and reached a plateau at around 25 min after the addition of AS, demonstrated that the probe **P-Np-Rhod** can provide a rapid response for HNO.

A highly selective response to the target molecule over other potentially competing molecules is of great significance for a bioimaging probe with potential application in the complex biological system. Therefore, the probe **P-Np-Rhod** was treated with various biologically relevant analytes to evaluate its selectivity, including reactive oxygen species, reactive nitrogen species, reducing agents, small molecule thiols, and biologically related metal ions. As shown in Fig. 2, no obvious changes of the fluorescence intensity ratio ($I_{541\ nm}/I_{448\ nm}$) were observed in the presence of these interference species. In contrast, the introduction of AS could trigger remarkable change of the fluorescence intensity ratio at $I_{541\ nm}/I_{448\ nm}$. This result indicated that the probe **P-Np-Rhod** shows a high selectivity for HNO and possesses the ability for detecting HNO in complex biological samples.

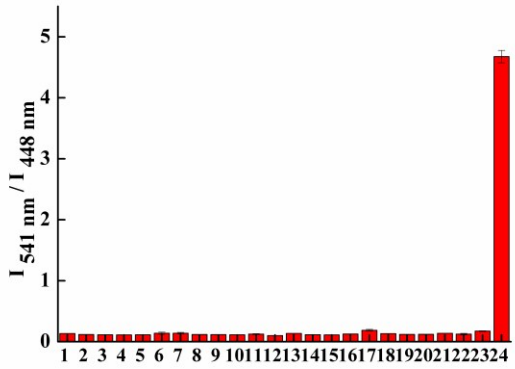


Fig. 2 Fluorescence response of the probe **P-Np−Rhod** (15 μM) toward various analytes in PBS buffered (10 mM, pH = 7.4) aqueous EtOH solution (1:1, v/v) at room temperature for 30 min. The fluorescence intensity ratio ($I_{541\ nm}/I_{448\ nm}$) was plotted versus substances: 1. Probe only (15 μM); 2-12. Ca^{2+}, Ascorbic Acid, NO_2^-, N_3^-, S^{2-}, K^+, Zn^{2+}, NO_3^-, Na^+, Fe^{2+}, Mg^{2+} (1 mM); 13. Fe^{3+} (100 μM); 14-15. Cys, GSH (3 mM); 16-23. H_2O_2, ClO^-, NO, O_2^-, •OH, TBHP, •^{t}OBu, $ONOO^-$ (500 μM); 24. AS (100 μM). λ_{ex} = 370 nm.

The influence of pH on the fluorescence response of **P-Np-Rhod** to HNO was further investigated. As shown in Fig. S5 (ESI†), in the absence of HNO, the probe **P-Np-Rhod** was pH insensitive over a pH range of 5.0-10.0, indicating that the ester group was stable in this pH range. Upon addition of AS, the maximal fluorescence intensity ratio ($I_{541\ nm}/I_{448\ nm}$) appeared in the pH range of 6.0-7.8, which demonstrated that probe **P-Np-Rhod** can be employed to detect HNO in physiological system. With the pH value larger than 8.0, fluorescence intensity

($I_{541\ nm}/I_{448\ nm}$) showed a decreased tendency due to the decreased decomposition rate of AS into nitroxyl under the strong basic conditions.[14]

The TP active cross-section (δΦ) value of **P-Np-Rhod** and **Np-Rhod** were respectively estimated to be 48 GM and 27 GM at 740 nm (Fig. S6, ESI†), indicating high capability of the probe **P-Np-Rhod** for detecting HNO with TP mode. In the absence of HNO, **P-Np-Rhod** showed a fluorescence emission peak at 448 nm under excitation with 740 nm femtosecond pulses. However, a new fluorescence emission peak at 541 nm appeared while the donor's emission peak at 448 nm vanished in the presence of HNO (Fig. 3). The probe **P-Np-Rhod** was demonstrated to possess potentail ability for ratiometric TP fluorescent imaging of HNO.

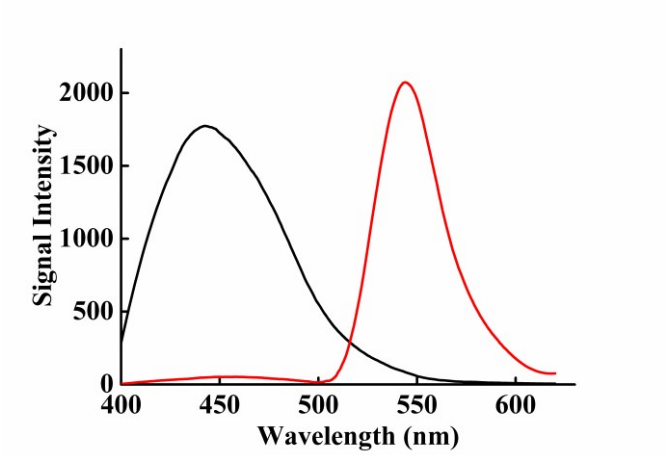


Fig. 3 TP excited emission spectra of **P-Np-Rhod** (black line) and **Np-Rhod** (red line). TP excitation at 740 nm in PBS/EtOH (1:1, v/v, 10 mM, pH=7.4).

Encouraged by the remarkable features of the probe **P-Np-Rhod** including high selectivity and sensitivity, rapid response and good TP fluorescence properties, we next investigated whether this probe can be applied in TP excited dual-channel imaging of HNO in living cells. MTT assays were first performed to evaluate the potential cytotoxicity of **P-Np-Rhod** and **Np-Rhod** at various concentrations against HeLa cells. The results indicate that both **P-Np-Rhod** and **Np-Rhod** have no obvious cytotoxicity to live cells under the experimental conditions. (Fig. S7, ESI†). We then examined the applicability of the probe for detecting HNO in living cells, with results shown in Fig. 4. The HeLa cells incubated with only the probe **P-Np-Rhod** (15 μM) displayed a strong TP fluorescence signal in the cyan channel but weak fluorescence signal in the yellow channel, corresponding to strong fluorescence of the donor and weak fluorescence of the acceptor due to its spirolactone form. In contrast, HeLa cells incubated with **P-Np-Rhod** (15 μM) for 30 min, and then treated with AS (100 μM or 200 μM) exhibited a significant fluorescence enhancement in the yellow channel and a concomitant decrease in cyan channel, corresponding to strong fluorescence from the open-ring acceptor and the weak fluorescence from the donor due to the effective FRET process triggered by HNO. All above results showed that **P-Np-Rhod** was cell membrane permeable and could be successfully applied to TP-excited ratiometric dual-channel imaging of HNO in living cells.

To further investigate the applications of **P-Np-Rhod** in fluorescence imaging of living samples, TP excited dual-channel imaging of **P-Np-Rhod** for HNO in rat liver tissue slices was then performed (Fig. 5). The changes of fluorescence signal with scan depth were recorded by TPM in the Z-scan mode. In the absence of AS, the tissue slices incubated with only **P-Np-Rhod** exhibited obvious fluorescence at the cyan channel with an imaging depths of 40-150 μm. In contrast, when the tissue slices were incubated with **P-Np-Rhod** (50 μM **)** for 60 min, and then treated with 1 mM AS for another 60 min, relatively significant fluorescence was observed at the yellow channel with an imaging depths of 20-130 μm, followed with a remarkable decrease at the cyan channel. These results indicated that **P-Np-Rhod** possesses excellent tissue penetration and staining ability as well as ratiometric dual-colour (cyan and yellow) imaging performance with less cross-talk between dual emission channels.

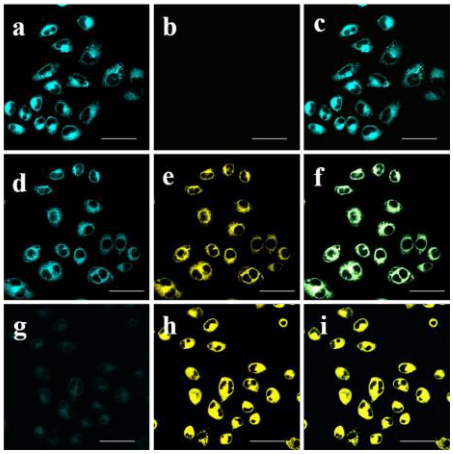


Fig. 4 TP fluorescence images of HNO in live HeLa cells with **P-Np-Rhod.** (a) TP fluorescence images HeLa cells incubated with only probe **P-Np-Rhod** (15 μM) for 30 min in the cyan channel; (b) images of (a) in the yellow channel; (c) Merged images of (a) and (b); (d, g) TP fluorescence images of HeLa cells incubated with **P-Np-Rhod** (15 μM) for 30 min, then with AS (100 μM or 200 μM) for 30 min in the cyan channel; (e, h) TP fluorescence images of (d, g) in the yellow channel; (f) Merged images of (d) and (e); (i) Merged images of (g) and (h). λ_{ex} = 740 nm, cyan channel: λ_{em} = 470-530 nm; yellow channel 550-650 nm. Scale bar: 50 μm.

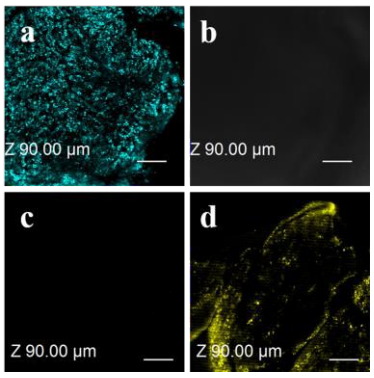


Fig. 5 TP fluorescence imaging of a rat liver frozen slice stained with **P-Np-Rhod** (50 μM) at ~90 μm for 60 min (a, b), followed by treatment with AS (1 mM) and incubated for another 60 min (c, d).

The images were collected at 450-530 nm (cyan channel, a, c) and 540-650 nm (yellow channel, b, d) upon excitation at 740 nm with femtosecond pulses. Scale bars: 200 μm

In summary, we have developed a ratiometric TP fluorescent probe **P-Np-Rhod** for detecting HNO for the first time. The diphenylphosphinobenzoyl group was employed as a recognition unit and a FRET cassette, termed **Np-Rhod**, acted as the fluorescent reporter. This ratiometric probe **P-Np-Rhod** can rapidly measure HNO with large emission shift (93 nm), high sensitivity and high selectivity. More importantly, **P-Np-Rhod** could be succesfully applied for TP fluorescence imaging of HNO in living cells and tissues with less cross-talk between dual emission channels. Far more than a promising tool to investigate the biological functions of HNO, this FRET-based TP strategy might also be used to design dual-channel imaging probes for other biological researches.

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