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A fast-response, highly sensitive and selective fluorescent probe for the ratiometric imaging of nitroxyl in living cells†

Caiyun Liu,[ab] Huifang Wu,[b] Zuokai Wang,[b] Changxiang Shao,[b] Baocun Zhu*[b] and Xiaoling Zhang*[a]

A fast-response, highly sensitive and selective fluorescent probe with the 2-(diphenylphosphino)benzoate moiety as a recognition receptor for the ratiometric imaging of nitroxyl in living cells was first developed.

Nitroxyl (HNO) is the one-electron reduced or protonated form of the well-known signaling agent nitric oxide (NO), and can be formed directly from nitric oxide synthase under the appropriate conditions.[1] However, recent investigations demonstrate that HNO displays important biological roles with potential pharmacological applications distinct from those of NO.[2] For example, HNO has been shown to possess the potential for therapeutic applications in a variety of diseases including treatments for heart failure and alcohol abuse.[3] Moreover, HNO reacts as an electrophile with thiols to resist superoxide infraction in mammalian vascular systems.[4] Unfortunately, studies on the elucidation of HNO mechanisms *in vivo* and the identification of endogenous sources are hampered by a lack of reliable detection methods.

Among the various available methods, the fluorescence technique with the help of fluorescent probes is the preferred method for *in situ* visualization of biologically important species in living systems owing to its various advantages such as high sensitivity, non-invasiveness, and high spatiotemporal resolution.[5] Therefore, the development of fluorescent probes for the determination of HNO has attracted intense interest.[5,6] Lippard *et al.* pioneered a series of fluorescent probes for HNO based on the design strategy of HNO-induced reduction of Cu(II) to Cu(I).[6a–f] Subsequently, Yao *et al.* developed two fluorescent probes for imaging HNO in living cells employing the same strategy.[6g,h] Despite advances in the development of fluorescent probes for HNO, the above-mentioned fluorescent probes might be prone to be disturbed in bioimaging applications by abundant biological reductants including glutathione (GSH) and ascorbate in living matrices.[6] Additionally, as far as we know, all of the current fluorescent probes respond to HNO with changes only in fluorescence intensity, which might be influenced in quantitative detection by many factors, such as variabilities in probe distribution, excitation and emission efficiency, the environment around the probe (pH, polarity, temperature, *etc.*), and effective cell thickness in the optical beam.[7] So, ratiometric fluorescent probes for HNO are urgently needed because they can eliminate numerous ambiguities by self-calibration of two emission bands. On the other hand, fast-response and sensitive fluorescent probes for HNO are also in high demand as HNO and NO may be able to interconvert in the presence of superoxide dismutase (SOD).[8] Based on the above considerations, in this communication, we developed the first fluorescent probe for the ratiometric imaging of HNO levels in living systems, which features fast-response, high sensitivity, the important properties of a large emission shift for enhancing resolution in the ratiometric bioimaging, and especially, excellent selectivity even at high concentrations of GSH and ascorbate.

The 1,8-naphthalimide fluorophore containing an electron donor and an acceptor has been continually used in ratiometric fluorescent probes owing to its outstanding internal charge transfer (ICT) structure and desirable photophysical properties, such as a large Stokes shift and insensitivity to pH.[9] Recently, we successfully developed a series of 4-hydroxynaphthalimide-derived ratiometric fluorescent probes.[10] In connection with our continuing research, we herein describe the design and synthesis of a simple 4-hydroxynaphthalimide-based ratiometric fluorescent probe (Scheme 1, **1**) employing the 2-(diphenylphosphino)benzoate moiety as a recognition receptor for the detection of HNO. Probe **1** possesses a compromised ICT structure due to the opposite electron-withdrawing carbonyl group of the 2-(diphenylphosphino)-benzoate moiety. HNO-mediated cleavage of the 2-(diphenyl-phosphino)benzoate moiety shows long-wavelength absorption

[a] *Key Laboratory of Cluster Science of Ministry of Education, Beijing Key Laboratory of Photoelectronic/Electrophotonic Conversion Materials, School of Chemistry, Beijing Institute of Technology, Beijing 100081, China. E-mail: zhangxl@bit.edu.cn; Fax: +86-10-88875298; Tel: +86-10-88875298*

[b] *School of Resources and Environment, University of Jinan, Jinan 250022, China. E-mail: lcyzbc@163.com*

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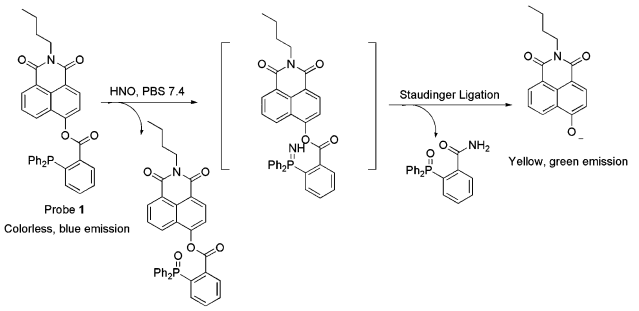

Scheme 1 Proposed reaction mechanism of **1** and HNO.

and fluorescence of 4-hydroxy-1,8-naphthalimide owing to the stronger electron donating ability of oxygen anions.[11] The detailed recognition mechanism was further confirmed by the identification of resultant products 4-hydroxy-1,8-naphthalimide and (diphenylphosphonyl)benzamide. The recognition mechanism of probe **1** for HNO is shown in Scheme 1. Probe **1** was prepared in a satisfactory yield from the corresponding 4-hydroxy-1,8-naphthalimide and 2-(diphenylphosphino)benzoic acid. Detailed procedures and characterization are described in the ESI.†

We firstly investigated the spectroscopic properties of probe **1** under physiological conditions (5 mM PBS, pH 7.4). In the absence of HNO, probe **1** displays one major absorption band centered at 350 nm with a corresponding blue-colored fluorescence maximum at 418 nm (Φ = 0.18) (Fig. 1 and Fig. S1, ESI†). The relative blue shift of these absorption and emission spectra compared to other 4-alkoxylnaphthalimide dyes is ascribed to the introduction of opposite electron-withdrawing carbonyl groups, and is of significant importance for the construction of ratiometric fluorescent probes with a large emission shift. As expected, upon addition of HNO (Angeli's salt (AS), a commonly employed HNO donor), the maximum emission peak exhibited a 128 nm red shift, which makes probe **1** possess the potential of enhancing resolution in the ratiometric bioimaging (Fig. 1). At the same time, the maximum absorption peak underwent a red shift to 454 nm accompanied by the color change of the solution from colorless to yellow, and thus **1** can serve as a "naked-eye" probe for HNO (Fig. S1, ESI†). The remarkable changes in absorption and fluorescence spectra might be attributed to the cleavage of the opposite electron-withdrawing carbonyl group of the 2-(diphenylphosphino)benzoate moiety and the coinstantaneous production of stronger electron-push capacity of the oxygen anion. Additionally, there was a good linearity between the fluorescence intensity ratio, R (F_{546}/F_{418}), and the concentrations of AS in the range of 2 to 35 μM with a detection limit of 0.5 μM[12] (Fig. 1). These results demonstrated that probe **1** could detect HNO qualitatively and quantitatively by the ratiometric fluorescence method with excellent sensitivity.[5,6]

Response time is a fundamental parameter for most reaction-based probes, and the kinetic profile of the reaction of probe **1** and HNO at room temperature was examined (Fig. S2–S5, ESI†). Upon addition of 100 μM AS, the *pseudo*-first-order rate constant was determined to be k_{obs} = 0.2759 min^{-1}. With the various concentrations of AS (40, 50 and 100 μM), the second-order rate constant for the reaction of probe **1** and AS was also calculated as k' = 3.3 × 10^{-3} μM^{-1} min^{-1}. These results implied that our proposed probe would provide a rapid analytical method for the detection of HNO.

Then we evaluated the selectivity of probe **1** towards HNO under the same analytical conditions. As shown in Fig. 2, nearly no fluorescence intensity changes were observed in the presence of NO_3^-, NO_2^-, O_2^-, *tert*-butylhydroperoxide (TBHP), ascorbate, H_2O_2, the *tert*-butoxy radical ($^{\bullet}O^tBu$), the hydroxyl radical ($^{\bullet}OH$),

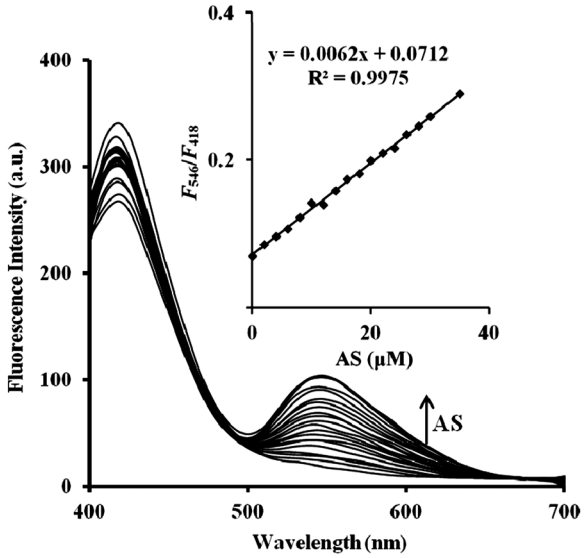

Fig. 1 Fluorescence responses of **1** (5 μM) toward different concentrations of AS (final concentration: 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 35, 40, 45, 50, 70, and 100 μM); inset: the fluorescence intensity ratio (F_{546}/F_{418}) of **1** *vs.* increasing concentrations of AS (final concentration: 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, and 35 μM). Each spectrum was acquired 20 min after AS addition at 25 °C.

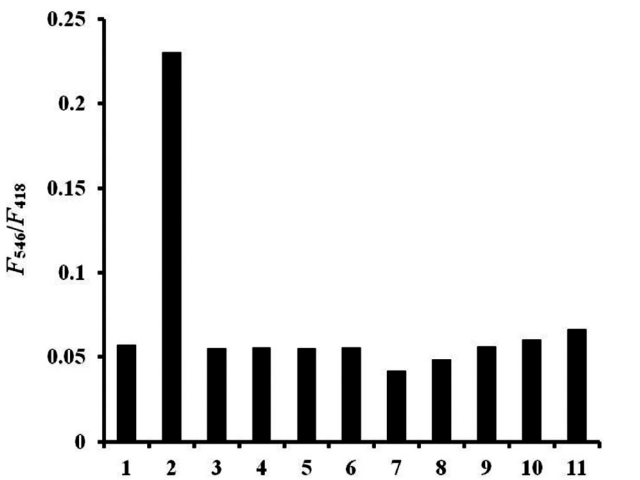

Fig. 2 Fluorescence responses of **1** (5 μM) toward various biospecies (50 μM except for notation). 1, NO_3^- and NO_2^-; 2, HNO; 3, O_2^-; 4, TBHP; 5, ascorbate (1 mM); 6, H_2O_2; 7, $^{\bullet}O^tBu$; 8, $^{\bullet}OH$; 9, Cys; 10, GSH; and 11, GSH (1 mM). Bars represent fluorescence intensity ratio F_{546}/F_{418}. Each spectrum was acquired 20 min after various analytes addition at 25 °C.

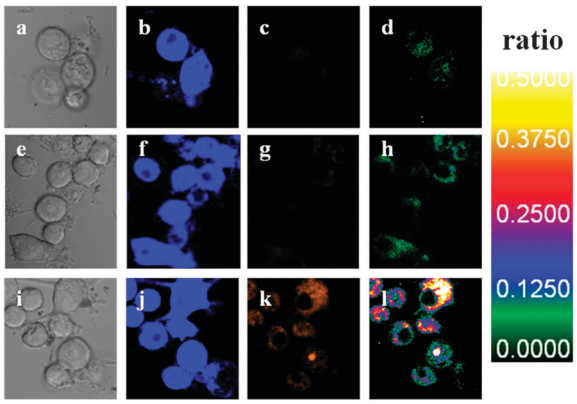

Fig. 3 Confocal fluorescence images of living RAW 264.7 macrophage cells: RAW 264.7 macrophage cells incubated with probe **1** (5 μM) for 20 min: (b) blue channel, (c) orange channel, (d) ratio image generated from (c) and (b), and (a) bright-field transmission image; RAW 264.7 macrophage cells incubated with probe **1** (5 μM) for 20 min were further treated with 50 μM AS for another 10 min: (f) blue channel, (g) orange channel, (h) ratio image generated from (g) and (f), and (e) bright-field transmission image; RAW 264.7 macrophage cells incubated with probe **1** (5 μM) for 20 min were further treated with 100 μM AS for another 10 min: (j) blue channel, (k) orange channel, (l) ratio image generated from (k) and (j), and (i) bright-field transmission image. Incubation was performed at 37 °C under a humidified atmosphere containing 5% CO_2.

cysteine (Cys) and GSH, which is ascribed to the adoption of a HNO-specific receptor of the 2-(diphenylphosphino)benzoate moiety. In addition, the effects of interference of the above-mentioned other analytes on monitoring HNO were investigated (Fig. S6, ESI†). Thus, these results demonstrated that probe **1** possesses high selectivity towards HNO even in the presence of high concentration of GSH and ascorbate. Additionally, probe **1** exhibited excellent photostability, which is very important to the reaction-based probes (Fig. S7, ESI†).

Next, we attempted to apply probe **1** for the ratiometric fluorescence imaging of HNO in living systems. Confocal fluorescence imaging in living RAW 264.7 macrophage cells was carried out (Fig. 3). The intense intracellular fluorescence of the cells incubated with probe **1** (5 μM) for 20 min demonstrated that probe **1** is cell-permeable (Fig. 3b). Furthermore, the cells incubated with probe **1** were treated with 50 μM and 100 μM AS (a commonly employed HNO donor) for another 10 min, respectively. As expected, distinct changes in ratiometric fluorescence responses generated from the orange channel and the blue channel in living cells were observed (Fig. 3d, h and l). Gratifyingly, the little changes in HNO levels were also clearly observed by the ratiometric fluorescence imaging, implying that our proposed probe **1** possesses high resolution in bioimaging.[5b,6f] These results revealed that probe **1** could be used for the ratiometric fluorescence imaging of HNO in living matrices.

To further estimate the cytotoxicity of probe **1**, we performed 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays in RAW 264.7 macrophage cells with 5, 10, 20 and 30 μM probe **1** for 24 h, respectively. The experimental results are shown in Fig. S8 (ESI†). The obtained results showed that our proposed probe exhibited low toxicity to cultured cells under the experimental conditions at the concentration of 5 μM for 30 min.

In conclusion, we have presented the design, synthesis and properties of an ICT-based ratiometric fluorescent probe **1** for HNO with the design platform of carbonyl protected 4-hydroxynaphthalimide. Probe **1** exhibits high HNO-selectivity even in the presence of high concentration of GSH and ascorbate, which is ascribed to the adoption of the 2-(diphenylphosphino)benzoate moiety. In addition, probe **1** displays a 104 nm red-shift of absorption spectra and the color changes from colorless to yellow upon addition of HNO, and thus can serve as a "naked-eye" probe for HNO. Importantly, probe **1** can detect HNO quantitatively by the ratiometric fluorescence method with a 128 nm red-shifted emission with excellent sensitivity. We highlight the simplicity of the design and synthesis of probe **1**, and its combined properties, such as high specificity and sensitivity, fast response, visual and ratiometric fluorescence determination with a large red-shifted emission and ratiometric bioimaging in living cells, and anticipate that this probe would be of great benefit to biological researchers for investigating the detailed function of HNO in living systems.

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12 See ESI† for the detailed calculation method.

