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A FRET-based Ratiometric Fluorescent Probe for Nitroxyl Detection in Living Cells

Huatang Zhang,[†,‡] Ruochuan Liu,[†] Yi Tan,[†] William Haowei Xie,[†] Haipeng Lei,[†] Hon-Yeung Cheung,[‡,§] and Hongyan Sun*[,†,‡]

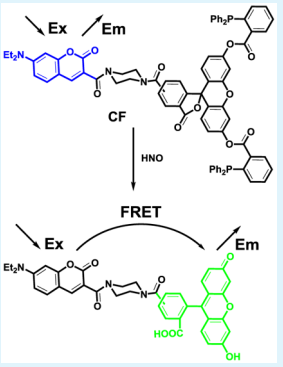
[†]Department of Biology and Chemistry, City University of Hong Kong, 83 Tat Chee Avenue, Kowloon, Hong Kong, People's Republic of China

[‡]Key Laboratory of Biochip Technology, Biotech and Health Centre, Shenzhen Research Institute of City University of Hong Kong, Shenzhen, People's Republic of China

[§]Department of Biomedical Sciences, City University of Hong Kong, 83 Tat Chee Avenue, Kowloon, Hong Kong, People's Republic of China

Supporting Information

ABSTRACT: HNO has recently been found to possess distinct biological functions from NO. Studying the biological functions of HNO calls for the development of sensitive and selective fluorescent probes. Herein, we designed and synthesized a FRET-based ratiometric probe to detect HNO in living cells. Our studies revealed that the probe is capable of detecting HNO in a rapid and ratiometric manner under physiological conditions. In bioimaging studies, the probe displayed a clear color change from blue to green when treated with HNO.



KEYWORDS: *FRET probe, HNO, ratiometric, bioimaging, fluorescent probe*

1. INTRODUCTION

Nitric oxide (NO) is well-known to play an important role in regulating many physiological processes. Its one-electron reduced and protonated product, nitroxyl (HNO), has recently been found to exhibit chemical properties and biological functions distinct from those of NO.[1,2] HNO has recently attracted substantial interest from biologists. A number of studies have suggested that it may confer important biological functions in various physiological processes. For example, HNO was found to react with thiols in aldehyde dehydrogenase, leading to the inhibition of the enzyme's activity.[3] HNO could mediate relaxation of resistant-like arteries by activating voltage-dependent K^+ channel.[4] Furthermore, HNO is implicated in regulating cardiovascular functions and may provide useful tools for treating cardiovascular diseases such as heart failure.[5,6] Despite the aforementioned progress, the biological roles of HNO still remain largely unknown. Therefore, chemical tools that allow for sensitive and selective detection of HNO are highly needed to further investigate the functions of HNO.

Fluorescence-based method has served as a popular and robust tool for detecting biologically active species in living cells due to its simplicity, excellent sensitivity, and well-defined spatiotemporal resolutions.[7−9] The HNO fluorescent probes developed in previous studies were mostly based on the reduction of (1) Cu(II) to Cu(I),[10−16] or (2) nitroxide to hydroxylamine[17] using HNO. These probes were shown to be capable of detecting HNO in living cells. However, it was also noted that the probes might be interfered by other biologically abundant reductants in the living cells, such as glutathione and ascorbate.

Recently, Nakagawa's group developed a novel fluorescent probe for HNO detection based on the reaction of HNO and phosphine.[18] The method has the advantage of being able to resist cellular reductant, thereby improving the probe's selectivity. Based on this method, Tan's group and Zhang's group further developed different fluorescent probes with good solubility, high fluorescence quantum yield, and large Stokes shift.[19,20] Nevertheless, one of the major limitations associated with most of these probes is that the fluorescence intensity measurement may be interfered by various external factors, including variations in excitation intensity, emission collection efficiency, sample thickness, and probe concentration and environment (pH and polarity). Ratiometric probes, which provide built-in correction by simultaneously measuring two different emission signals, will in principle minimize these

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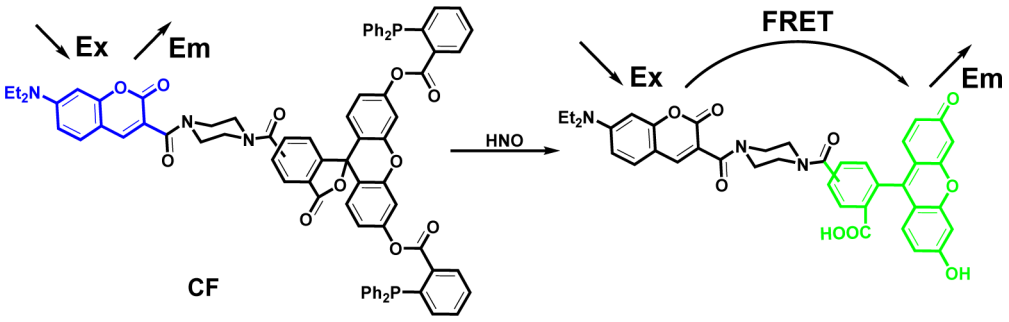
Scheme 1. Chemical Structure and "Turn on" Mechanism of Probe CF



interferences and improve the accuracy of fluorescence readouts. A strategy to design ratiometric probes can be based on Förster resonance energy transfer (FRET) approach, in which a FRET donor and a FRET acceptor are connected through a rigid linker (1−10 nm).[7−9,21−25] The energy of the excited donor fluorophore can be transferred to the acceptor through nonradiative dipole−dipole coupling.[26−33] To our best knowledge, no FRET-based ratiometric probes for detecting HNO has been reported to date. Herein, we have designed and synthesized the first FRET-based ratiometric probe for HNO and applied the probe for living cell imaging studies.

2. EXPERIMENTAL SECTION

2.1. Materials and Chemicals. All chemicals used for synthesis were purchased from commercial suppliers and applied directly without purification. Anhydrous dichloromethane (DCM) and ethanol were purchased from commercial suppliers and used in all reactions as solvent. Angeli's salt (AS) and S-Nitrosoglutathione (GSNO) were obtained from Cayman. All reactions that utilize air- or moisture-sensitive reagents were performed in dried glassware under dry N_2 atmosphere. All spectroscopic measurements were performed in phosphate-buffered saline (PBS; 10 mM, pH 7.4). The progress of the reaction was monitored by thin-layer chromatography (TLC; Merck 60F-254). Merck silica gel 60 (70−200 mesh) was used for general column chromatography purification purpose. ^{1}H NMR, ^{13}C NMR, and ^{31}P NMR spectra were taken on a Bruker 300 MHz or Bruker 400 MHz NMR spectrometer. For ^{1}H NMR, coupling constants (J) are reported in Hertz (Hz), and multiplicity is indicated as follows: s (singlet), d (doublet), t (triplet), m (multiplet), dd (doublet of doublets), and bs (broad singlet). Mass spectra were obtained using PC Sciex API 150 EX ESI-MS system. High-resolution mass spectrometry was performed with ABI Qstar Elite Q-TOF. UV absorption spectra were obtained on Shimadzu 1700 UV/vis Spectrometer. Fluorescence spectra were acquired with a Fluoro-Max-4 fluorescence photometer. Fluorescence images were captured using a Leica TCS SPE Confocal Scanning Microscope. pH value was recorded with a FiveEasy FE20 pH meter.

2.2. Synthesis of Compounds 4, 5, and 6. Compounds 4, 5, and 6 were synthesized according to literature.[21,34] Details of the synthesis and the corresponding characterization data are provided in the Supporting Information.

2.3. Synthesis of Compound 2. EDCI (0.288 g, 1.5 mnol), DMAP (0.024 g, 0.2 mmol) and NHS (0.138 g, 1.2 mmol) were added to a solution of 2-(diphenylphosphanyl)benzoic acid (0.376 g, 1 mmol) in 20 mL of dry DCM. The reaction solution was stirred at room temperature for 6 h. 5(6)-Carboxyfluorescein (0.151 g, 0.4 mmol) was then added. The solution was stirred for another 12 h, and 50 mL of DCM was added. The solution was then washed with water and brine and dried with anhydrous $MgSO_4$. The organic solvent was subsequently evaporated under reduced pressure. The crude product was purified by silica gel column chromatography with DCM/MeOH = 10/1 to obtain 0.164 g of white solid **2** with 43% yield. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 8.90 (s, 1H), 8.77 (d, J = 11.6 Hz, 1H), 8.52 (d, J = 10.8 Hz, 1H), 8.41 (d, J = 11.2 Hz, 1H), 8.29 (m, 4H), 8.09 (s, 1H), 7.48 (m, 8H), 7.36 (bs, 40H), 7.24 (d, J = 10.4 Hz, 1H), 7.04 (bs, 4H), 6.98 (d, J = 10.4 Hz, 4H), 6.81 (m, 4H), 6.69 (m, 4H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 168.35, 168.31, 168.07, 167.94, 164.70, 164.68, 163.24, 162.94, 157.51, 157.44, 156.47, 156.38, 152.22, 152.16, 152.08, 151.64, 151.39, 141.79, 141.51, 137.47, 137.36, 134.52, 134.48, 134.16, 134.07, 133.96, 139.86, 132.97, 132.90, 132.83, 132.79, 132.73, 131.48, 128.94, 128.70, 128.63, 128.50, 128.43, 127.60, 127.19, 126.79, 126.43, 124.83, 124.28, 118.10, 117.97, 115.80, 115.43, 110.83, 110.68, 82.08, 81.87. ^{31}P NMR ($CDCl_3$, 162 MHz) δ: −4.33. ESI-MS: calcd for $C_{59}H_{38}O_9P_2Na$ $[M + Na]^+$, 975.2; found, 975.8.

2.4. Synthesis of Compound CF. EDCI (0.192 g, 0.1 mnol) and DMAP (0.006 g, 0.05 mmol) were added to a solution of compound **2** (0.078 g, 0.082 mmol) in 10 mL of DCM. The reaction mixture was stirred in an ice−water bath. After 30 min, compound **6** (0.033 g, 0.1 mmol) was added, and the solution was stirred at room temperature overnight. Subsequently, 30 mL of DCM was added to the reaction solution. The solution was washed with water and brine and dried with anhydrous $MgSO_4$. The organic solvent was then evaporated under reduced pressure. The crude product was purified by silica gel column chromatography with EA/Hexene =2/1 to obtain 0.053 g of solid **CF** with 51% yield. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.85 (s, 1H), 8.48 (d, 1H, J = 8.0), 8.25 (m, 4H), 8.08 (s, 1H), 8.05 (s, 1H), 7.93 (d, J = 2.8 Hz, 1H), 7.70 (d, J = 7.2 Hz, 1H), 7.47 (m, 8H), 7.31 (m, 40H), 7.19 (d, 1H, J = 7.2), 7.00 (m, 4H), 6.94 (dd, J = 2.0, 7.2 Hz, 4H), 6.76 (d, J = 8.0 Hz, 4H), 6.65 (m, 4H), 6.60 (m, 2H), 6.48 (s, 2H), 3.94 (bs, 6H), 3.57 (bs, 6H), 3.43 (d, J = 6.8 Hz, 8H), 1.64 (s, 8H), 1.22 (m, 12H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 168.71, 168.67, 168.37, 168.02, 165.54, 164.80, 164.78, 163.05, 159.39, 157.59, 154.57, 154.45, 152.32, 152.26, 152.17, 152.11, 151.98, 151.50, 151.42, 146.14, 141.87, 141.64, 137.59, 137.48, 134.63, 134.61, 134.28, 134.25, 134.07, 134.04, 133.09, 132.96, 132.93, 131.58, 130.19, 130.08, 129.04, 128.80, 128.73, 128.50, 127.73, 126.89, 126.36, 126.22, 124.92, 124.04, 118.18, 118.09, 115.82, 115.58, 115.48, 110.92, 110.78, 109.65, 109.57, 107.90, 97.08, 82.16, 81.95, 45.12, 12.35. ^{31}P NMR ($CDCl_3$, 162 MHz) δ: −3.87. ESI-MS: calcd for $C_{77}H_{60}N_3O_{11}P_2$ $[M + H]^+$, 1264.4; found, 1264.9.

2.5. Absoprtion and Fluorescence Measurement. CF was dissolved in an appropriate amount of DMSO to obtain 10 mM stock solution. It was then diluted in PBS buffer (10 mM, pH 7.4) to afford a final concentration of 10 μM. Angeli's salt (AS) and other biological analytes were prepared as 10 mM stock solutions in PBS buffer. Specifically, hydrogen peroxide and *tert*-butylhydroperoxide (TBHP) were delivered in 30 and 70% aqueous solutions, respectively. Hydroxyl radical (·OH) and *tert*-butoxy radical (·O^{t}Bu) were generated by reacting 1 mM Fe^{2+} with 1 mM H_2O_2 and 1 mM TBHP, respectively. To perform selectivity studies, we added the appropriate amounts of AS and other analytes to separate portions of the probe solution (10 μM) and mixed the solution thoroughly. The reaction mixture was shaken uniformly before emission spectra were measured. Measurement of the fluorescence emission spectra was

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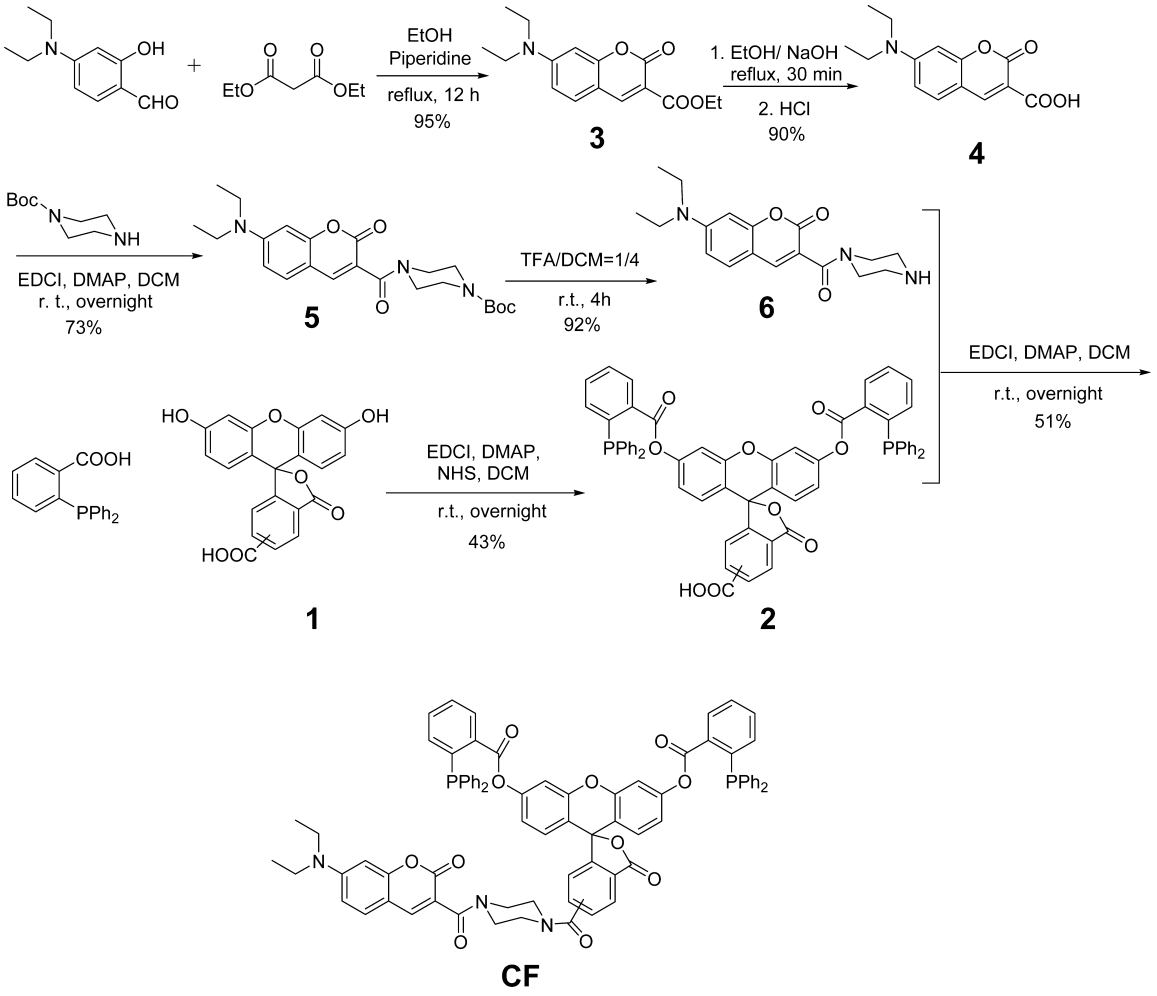
Scheme 2. Synthetic Route of CF



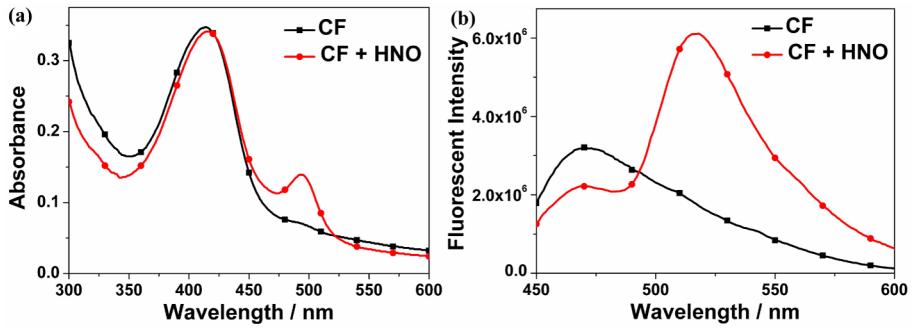


Figure 1. (a) Absorption spectra and (b) emission spectra of **CF** (10 μM) in the absence and in the presence of AS in PBS buffer (e.g., 415 nm).

conducted using a FluoroMax-4 fluorescence photometer with a 10 mm quartz cuvette. The excitation wavelength was set at 415 nm and the emission wavelength was set in the range of 430−600 nm. The slit widths of excitation and emission wavelength were both set at 5 nm.

2.6. Fluorescence Microscope Experiment. HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and appropriate amounts of antibiotics (penicillin and streptomycin). Approximately 1×10^5 cells were seeded in a confocal dish (20 mm) with 2 mL of medium at 37 °C. Before the probe was added, the cells were allowed to adhere to the dish for 24 h. They were then incubated with probe **CF** (5 μM) at 37 °C for 30 min, and incubated with 100 μM AS for another 30 min. Fluorescence images were then taken using a Leica TCS SPE Confocal Scanning Microscope.

2.7. Detection Limit Studies. Detection limit or limit of detection (LOD) is calculated from the mean of the blank, the standard deviation of the blank, and the corresponding linear regression equation. In this manuscript, we use the following formula:

$$\mathrm{LOD} = 3\sigma/s$$

$$\sigma = \sqrt{\frac{\sum (\bar{x} - xi)^2}{n - 1}}$$

$\bar{x}$ is the mean of the blank measures; xi is the values of blank measures; n is the tested number of blank measure (n = 11); and s is the slope of the linear regression equation.

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3. RESULTS AND DISCUSSION

3.1. Design and Synthesis of Probe CF. Scheme 1 describes the chemical structure and fluorescence turn-on mechanism of our probe **CF**. The probe consists of a FRET pair (coumarin/fluorescein), in which the fluorescein molecule is protected by a functional group called diphenylphosphino benzoate. As reported by other literature,[18−20] HNO could react with phosphine moiety to produce an aza-ylide intermediate. Aza-ylide will attack the adjacent electrophilic ester in an intramolecular manner, leading to the release of alcohol and "turn-on" of fluorescence (Scheme S1, Supporting Information).[35−37] To synthesize the probe, 5(6)-carboxyfluorescein **1** was first synthesized according to the previous procedure. 2-(Diphenylphosphanyl) benzoic acid was then covalently linked to the fluorescein molecule to obtain compound **2**. Concurrently, a piperazine linker was installed onto coumarin by standard coupling method. The obtained coumarine-piperazine molecule was then coupled to compound **2** with moderate yield and finally afforded the desired probe **CF** (Scheme 2). The chemical structure of the probe and intermediates were characterized by ^{1}H NMR, ^{13}C NMR, ^{31}P NMR and ESI-MS (Supporting Information).

3.2. Optical Response of CF to HNO. For preliminary studies, we first investigated the optical properties of the probe in PBS buffer (10 mM, pH 7.4). As shown in Figure 1a and Figure S2 (Supporting Information), the UV−vis spectra of **CF** (10 μM) displayed the characteristic spectra of coumarin with a maximum absorption peak at 415 nm. After treatment with 10 equiv of Angeli's salt (AS, a chemical reagent for producing HNO), a new absorption peak at 495 nm appeared and corresponds to the absorption peak of fluorescein. At the same time, the absorption signals at 415 nm decreased a little (Figure 1a). For fluorescence property studies, it was found that the probe itself exhibited a fluorescence emission peak at 470 nm, which is characteristic of coumarin. After the addition of 10 equiv of AS, a fluorescence increase at 517 nm could be observed when the probe was excited at 415 nm. At the same time, a fluorescence decrease at 470 nm was observed. Together, these results clearly suggested that FRET occurs efficiently between coumarin and fluorescein with HNO treatment (Figure 1b, S3 and S4 in Supporting Information).

3.3. Kinetic Study of CF to HNO. Next, we moved on to investigate the kinetic property of **CF** when reacted with HNO under physiological condition. Time dependent fluorescence responses were obtained by monitoring the change of fluorescent intensity ratio between the two wavelengths ($I_{517\ nm}/I_{470\ nm}$) of the probe after adding AS in PBS buffer. As shown in Figure 2 and Figure S5 (Supporting Information), **CF** (10 μM) displayed a quick response toward AS (100 μM). The reaction took around 15 min to reach plateau. In addition, **CF** was found to be stable in PBS buffer even after incubation at 37 °C for 30 min (Figure 2).

3.4. Selectivity test of CF. To examine whether **CF** can selectively detect HNO, we treated **CF** with various biologically relevant analytes in PBS buffer (10 mM, pH 7.4) and incubated the solution for 30 min. A total of 15 different biologically relevant species were screened in the selectivity assays. As shown in Figure 3, **CF** showed excellent selectivity toward HNO over reactive oxygen species (TBHP, H_2O_2, ·OH, ·O^{t}Bu, O_2^-, ClO^-, benzoyl peroxide), reactive nitrogen species (NO_3^-, NO_2^-), reactive sulfur species (S^{2-}, Cys, GSH, Hcy, GSNO) and biologically related metal ions (Fe^{2+}, Fe^{3+}, Mg^{2+}, Zn^{2+}, Ca^{2+}). Among all the tested species, only H_2O_2, cysteine and GSH showed slight increment in fluorescence intensity even though their concentrations were 200 equiv of the probe.

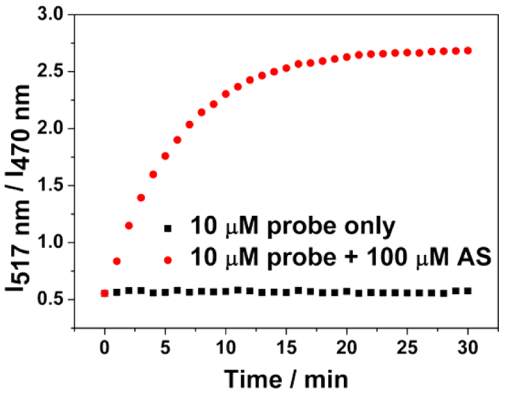

Figure 2. Time course experiment of **CF** (10 μM) reacting with AS (100 μM) in PBS buffer (10 mM, pH 7.4).

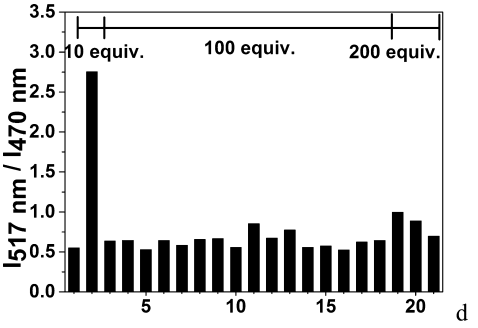

Figure 3. Fluorescence response of **CF** (10 μM) after 30 min of incubation with various analytes: 1. Probe only (10 μM); 2. AS (100 μM); 3−18. Fe^{2+}, Fe^{3+}, Mg^{2+}, Zn^{2+}, Ca^{2+}, NO_3^-, NO_2^-, TBHP, H_2O_2, ·OH, ·O^{t}Bu, O_2^-, ClO^-, benzoyl peroxide, GSNO and S^{2-} (1 mM); 19−21, Cys, GSH, and Hcy (2 mM).

3.5. Sensitivity and Detection Limit of CF. To determine the detection limit of our probe, we performed detailed titration experiment on the probe with HNO. When 10 μM of **CF** was treated with various concentrations of AS (from 0 μM to 100 μM), the fluorescence signal at 517 nm was observed to increase gradually (Figure 4a). On the other hand, the fluorescence intensity at 470 nm was concomitantly decreased. This clearly indicates that FRET signal increases with increasing concentration of HNO. To find out whether the probe can be used to detect HNO quantitatively, the fluorescence intensity ratio between the two wavelengths ($I_{517\ nm}/I_{470\ nm}$) was calculated, and the values were found to vary from 0.55 to 2.68. The fluorescence intensity ratio was subsequently plotted against the AS concentration, and it was found to be linearly related to the concentration in the range of 0−100 μM (Figure 4b). The regression equation was determined to be $I_{517\ nm}/I_{470\ nm} = 0.02193 \times [AS] + 0.55428$, with $R^2 = 0.9994$. The detection limit was calculated as 1.4 μM based on 3σ/slope method.[38,39] Compared with the existing probes, our probe has moderate sensitivity (Table S1, SI). However, it should be pointed out that some of the existing probes require the addition of organic solvent and surfactant, which may limit their biological applications. The results of our study have clearly shown that **CF** could be used to determine HNO concentration quantitatively and in a ratiometric manner.

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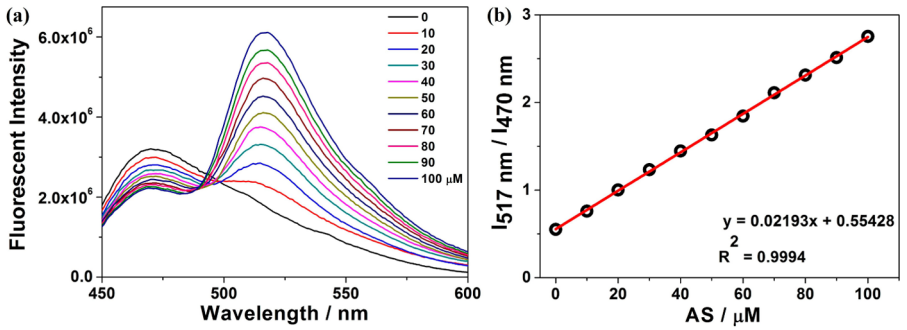

Figure 4. (a) Fluorescence spectra of **CF** (10 μM) with addition of various concentrations of AS. (b) Plot of fluorescent intensity ratio of the two wavelengths against the concentration of AS.

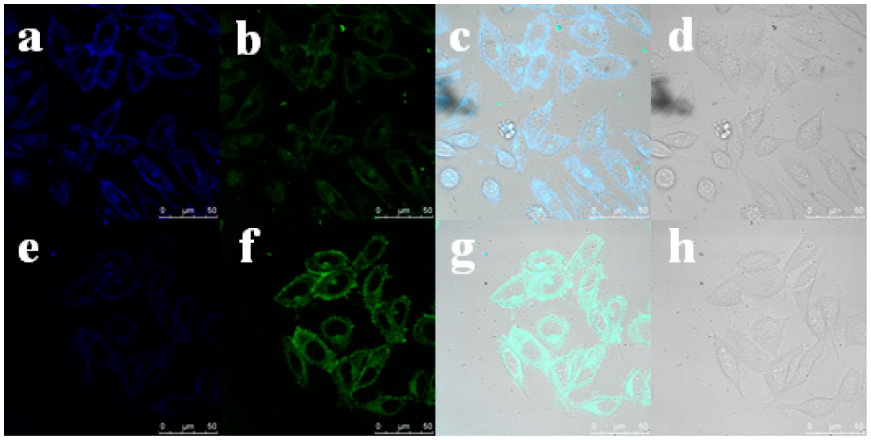

Figure 5. (a) Fluorescence image of HeLa cells incubated with **CF** (5 μM) from blue channel; (b) fluorescence image of image a from green channel; (c) overlay of images a and b; (d) bright field image of image a; (e) Fluorescence image of HeLa cells incubated with **CF** for 30 min and then incubated with AS (100 μM) for 30 min from blue channel; (f) fluorescence image of image e from green channel; (g) overlay of images e and f; (h) bright field image of image (e).

3.6. HNO Imaging in Living Cells. Encouraged by the above results, we next investigated whether **CF** can be applied in bioimaging studies with living cells. MTT experiment was first performed to evaluate the cytotoxicity of **CF**; 1−30 μM of probe were incubated with HeLa cells and cytotoxicity was measured (Figure S6, Supporting Information). The result indicated that 30 μM of **CF** was of low toxicity toward cell cultures under experimental conditions. In our confocal imaging studies, HeLa cells were first incubated with 5 μM of **CF** at 37 °C for 30 min. The cells were then incubated with and without adding AS for another 30 min before images were taken. As shown in Figure 5, the cells displayed strong blue fluorescence but not much green fluorescence in the absence of AS, suggesting that the probe is cell permeable and that there is not much endogenous HNO. In contrast, cells treated with AS displayed a significant fluorescence enhancement in green channel and a concomitant decrease in blue channel. The color change from blue to green with AS treatment clearly indicated the probe can be used to detect HNO in living cells.

4. CONCLUSION

In summary, we have developed the first FRET-based ratiometric probe to detect HNO in living cells. The probe reacted with HNO rapidly and displayed higher selectivity to HNO over other reactive biological species under physiological conditions. Importantly, the probe could detect HNO in a ratiometric manner, thereby minimizing environmental effect on the fluorescence. Furthermore, bioimaging studies also demonstrated that the probe can be used to detect HNO in living cells. We envisage that the probe can add useful tools for investigating the biological functions of HNO.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Detailed experimental procedures and characterization of compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: hongysun@cityu.edu.hk.

Notes

The authors declare no competing financial interest.

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