Application of a Colorimetric and Near-Infrared Fluorescent Probe in Peroxynitrite Detection and Imaging in Living Cells

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ABSTRACT: Peroxynitrite (ONOO\(^-\)) plays a vital role in pathological and physiological processes, and an excessive amount of ONOO\(^-\) causes various diseases. Developing a specific and sensitive method for the detection of ONOO\(^-\) in biological systems is significant. Herein, we reported a novel colorimetric and near-infrared fluorescent probe (pyridin-4-ylmethyl (Z)-2-cyano-2-(3-((E)-4-hydroxystyryl)-5,5-dimethylcyclohex-2-en-1-ylidene)acetate diphenyl phosphinate group (AN-DP)) based on isophorone and phosphinate groups for ONOO\(^-\) detection. The probe displayed excellent selectivity toward ONOO\(^-\) compared with other relevant analytes. It showed a good linear relationship between the fluorescence intensity at 670 nm and ONOO\(^-\) concentration (0–10 μM) with a low detection limit (53 nM). Importantly, the probe was a colorimetric and near-infrared fluorescent probe suitable for ONOO\(^-\) detection. Furthermore, the probe could be used for imaging ONOO\(^-\) in HepG2 cells.

1. INTRODUCTION

As one of the vital reactive nitrogen species, peroxynitrite (ONOO\(^-\)) is produced by the coupling reaction of superoxide radicals and nitric oxide.\(^1,2\) It serves crucial roles in signal transduction and antimicrobial activities.\(^3,4\) ONOO\(^-\) can react with many biomolecules including nucleic acids, proteins, and lipids because of its high oxidizable and nucleophilic characteristics.\(^5,6\) As a result, an excessive amount of ONOO\(^-\) is associated with various diseases, such as inflammatory diseases, autoimmune neurodegenerative diseases, metabolic diseases, cardiovascular diseases, inflammation, and cancers.\(^7,8\) Thus, development of effective methods for measuring ONOO\(^-\) is urgently required.

Currently, some techniques have been reported for detecting ONOO\(^-\): electrochemistry,\(^9\) electron spin resonance,\(^10\) UV–vis absorbance spectroscopy,\(^11\) high-performance liquid chromatography,\(^12\) and electro paramagnetic resonance spectroscopy.\(^13\) Even though these methods worked well in the detection of ONOO\(^-\), it is difficult to trace the dynamic changes of ONOO\(^-\) levels in organisms using these methods. As a promising method, fluorescent probes have attracted wide attention owing to their superior real-time visualization, fast response, nondestructive detection, and excellent sensitivity, which are advantageous for detecting active species in biosystems.\(^14,15\) Thus, development of reliable fluorescent probes for detecting ONOO\(^-\) is of great importance. To date, many fluorescent probes for ONOO\(^-\) detection have been constructed based on hydrazides,\(^29-32\) aryboronic esters,\(^33,34\) tellurium or selenium,\(^35,36\) and C=C double bonds.\(^37,38\) However, for some probes, it is difficult to discriminate ONOO\(^-\) from other relative species including HClO and H\(_2\)O\(_2\),\(^39,40\) which remains a challenge for selective detection of ONOO\(^-\). Additionally, most of the reported fluorescent probes had a short emission wavelength (300–650 nm) resulting in photodamage and excitation interference, which limits their biological applications. Near-infrared (> 650 nm) fluorescence probes can avoid the abovementioned problems because of their unique, superior low autofluorescence interference, deeper tissue penetration, and minimum photodamage.\(^37,41,42\) Therefore, it is wise to construct novel near-infrared fluorescent probes with high specificity for the detection of ONOO\(^-\) in vitro and in vivo.

Herein, we developed a near-infrared fluorescent probe (pyridin-4-ylmethyl (Z)-2-cyano-2-(3-((E)-4-hydroxystyryl)-5,5-dimethylcyclohex-2-en-1-ylidene)acetate diphenyl phosphinate group (AN-DP)), for selective detection of ONOO\(^-\) in which isophorone is selected as the fluorophore and the phosphinate group is used as a triggered moiety for ONOO\(^-\) as well as a fluorescence quencher (Scheme 1). An isophorone derivative was chosen as the chromophore due to its eminent properties including long emission wavelengths in the near-infrared region, high photostability, predictable color change, and effective intramolecular charge transfer (ICT).\(^43-46\) The probe AN-DP displayed weak fluorescence due to the protection of hydroxyl groups with phosphinate. The addition—elimination of ONOO\(^-\) resulted in detachment of
Scheme 1. Probable Response Mechanism of AN-DP with ONOO^−

phosphinate groups of AN-DP, and thus the ICT process took place, releasing a red fluorescence signal. The probe AN-DP showed many salient features. First, AN-DP displayed a long emission wavelength at 670 nm, which was valuable in bioimaging. In addition, AN-DP can act as a colorimetric probe with high selectivity for ONOO^− detection, which could help distinguish ONOO^− with the naked eye. Moreover, AN-DP could be applied for imaging ONOO^− in living cells.

2. RESULTS AND DISCUSSION

2.1. Spectral Properties. To elucidate the optical response of AN-DP toward ONOO^−, we studied the absorption and fluorescence spectra of AN-DP in the absence and presence of ONOO^− in 20 mM phosphate-buffered saline (PBS) buffer solutions (pH 7.4, containing 30% DMSO). The free probe AN-DP solution had a maximum absorption band at 404 nm. However, upon addition of ONOO^−, a new absorption peak at 454 nm was observed and the color of the solution changed from light yellow to red (Figure S1), suggesting that the probe AN-DP possessed a distinct colorimetric response for ONOO^− detection. As displayed in Figure 1, the probe AN-DP showed weak fluorescence (Φ = 0.006) before adding ONOO^−. However, the emission peak of the probe AN-DP enhanced at 670 nm (Φ = 0.142) upon treatment with 1 equiv. of ONOO^−. The absorption changes and fluorescence enhancement caused by ONOO^− due to the addition−elimination of phosphinate resulted in the formation of phenolate groups, which had a strong electron-donating ability to lead the ICT process. Subsequently, the fluorescence titration experiments for the probe AN-DP with ONOO^− were carried out (Figure 2). With the increase of ONOO^− concentration, the emission intensity of the probe AN-DP at 670 nm increased gradually. When 10 μM ONOO^− was added, the emission intensity reached the platform. A good linear relationship between the fluorescence intensity and ONOO^− (0−10 μM) was observed. Based on 3σ/K, the detection limit of the probe AN-DP toward ONOO^− was calculated to be 53 nM. These results demonstrated that AN-DP can be applied for the quantitative detection of ONOO^− with high sensitivity.

2.2. Response Time. The time courses of the fluorescence intensities of the probe AN-DP in the absence and presence of ONOO^− were investigated in 20 mM PBS buffer solution (pH 7.4, containing 30% DMSO). As shown in Figure 3, the probe AN-DP itself was nonfluorescent during the whole test time. However, after the addition of ONOO^−, the fluorescence intensity at 670 nm increased gradually and a plateau was reached within 20 min, indicating that the probe AN-DP possesses the ability of quick response to ONOO^−.

2.3. Influence of pH. To evaluate the effect of pH on emission properties of the probe AN-DP and its responses to ONOO^−, the fluorescence spectra of the probe AN-DP in the absence and presence of ONOO^− were measured at different pH values (Figure 4). As displayed in Figure 4, the probe AN-DP showed little fluorescence change at 670 nm at pH 2.0−10.0, which suggested that the probe AN-DP is not affected by wide pH values. However, in the presence of ONOO^−, the probe AN-DP displayed obvious fluorescence enhancement at pH 2.0−3.0 and maintained a platform at pH 3.0−10.0, which indicated that AN-DP can act as a near-infrared fluorescent probe for monitoring ONOO^− under physiological conditions.

2.4. Selectivity. To explore the selectivity of the probe AN-DP toward ONOO^−, the fluorescence responses of AN-DP toward various analytes were investigated under the same conditions. As shown in Figure 5, only the addition of ONOO^− leads to a dramatic increase of fluorescence intensity of the probe AN-DP with distinct colorimetric changes. However, addition of other analytes (Zn^{2+}, Fe^{3+}, K^+, Al^{3+}, Cu^{2+}, NO_2^−, NO_3^−, F^−, Cl^−, Br^−, CO_3^{2−}, H_2PO_4^−, AcO^−, Cys, Hcy, GSH, H_2O_2, HClO, HO^•, BuOH^•, ROO^•, O_2^{1•}, and 1O_2) did not result in obvious fluorescence and color variations in the probe AN-DP solution. These results demonstrate that the probe AN-DP displayed superior selectivity toward ONOO^− over other interferents.

2.5. Proposed Mechanism. According to the above experimental results, we proposed that ONOO^− could attack phosphinate groups to give hydroxyl phenols (Scheme 2). To confirm the sensing mechanism, the reaction products of the probe AN-DP and ONOO^− were characterized using high-resolution mass spectrometry (HRMS) (Figure S5). The mass peak of the probe AN-DP at m/z 601.2249 [M + 1]^+ disappeared, and a mass peak at m/z 401.1869 [M + 1]^+ emerged, which agreed well with the compound AN (calcd m/z 401.1860 [M + 1]^+), directly demonstrating the correctness of our proposed mechanism.

2.6. Biological Applications. Before conducting cell imaging experiments, the cytotoxicity of the probe in living cells was evaluated using MTT assays. As displayed in Figure S6, the cell viabilities still remained above 90% after treatment with AN-DP (0−25 μM) for 12 h, which indicates that AN-DP is nontoxic to cultured cells and suitable for biological applications. To represent the application of the probe AN-DP, fluorescence imaging was performed. As shown in Figure 6, upon treatment with only the probe AN-DP solution, HepG2 cells gave almost no fluorescence. In contrast, the strong red fluorescence was observed when the HepG2 cells were
pretreated with AN-DP for 30 min and then further incubated with SIN-1 for another 30 min, demonstrating that the probe AN-DP can sense ONOO\(^-\) in living cells.

### 3. CONCLUSIONS

In summary, we have developed a novel colorimetric and near-infrared fluorescent probe for ONOO\(^-\) detection. In the probe, the ICT process was blocked by diphenyl phosphinate. Therefore, the probe itself displayed almost no fluorescence. In the presence of ONOO\(^-\), the ICT process was recovered due to the selective cleavage of phosphinate by ONOO\(^-\), which resulted in the fluorescence response. The probe showed excellent sensitivity (53 nM) and high selectivity for the colorimetric and near-infrared detection of ONOO\(^-\). In addition, the probe could be applied for monitoring ONOO\(^-\) in living cells. We expect that the probe is useful for the selective detection of ONOO\(^-\) in vivo despite the cleavage of diphenyl phosphinate.

### 4. EXPERIMENTAL SECTION

#### 4.1. Materials and Methods

The chemical reagents were purchased commercially. The nuclear magnetic resonance (NMR) was carried out using a Bruker Ascend 400 NMR spectrometer. Absorption spectra were recorded using a UV-2600 UV−vis spectrophotometer (Shimadzu). Fluorescence experiments were performed using a RF-5301PC spectrometer (Shimadzu). HRMS was carried out using an Agilent 6210 ESI/TOF/MS instrument. Confocal fluorescence images were collected using a Leica TCS SP8 confocal microscope.

#### 4.2. Synthesis of Probe AN-DP

Compounds AN (0.40 g, 1.0 mmol), diphenylphosphinic chloride (0.35 g, 1.5 mmol), and Et\(_3\)N (0.21 mL, 1.5 mmol) were dissolved in dry tetrahydrofuran (10 mL) and stirred at room temperature for 12 h. The resulting mixture was concentrated under a reduced pressure. The crude was purified using column chromatography (20:1, CH\(_2\)Cl\(_2\); CH\(_3\)OH, v/v) to afford the desired product AN-DP (0.28 g, 48%) (Scheme 2). \(^1\)H NMR (400 MHz, DMSO-\(d_6\)) \(\delta\) (ppm): 8.60 (d, \(J = 6.4\) Hz, 2H), 7.94−7.85 (m, 5H), 7.65−7.62 (m, 4H), 7.58−7.53 (m, 4H), 7.30 (d, \(J = 8.0\) Hz, 2H), 7.12 (d, \(J = 8.0\) Hz, 2H), 5.53 (s, 2H), 2.51 (s, 2H), 1.01 (s, 6H); \(^{13}\)C NMR (100 MHz, DMSO-\(d_6\)) \(\delta\) (ppm): 166.5, 162.0, 154.7, 154.1, 151.8, 150.3, 145.3, 135.5, 133.3, 132.0, 131.7, 130.6, 130.4, 129.7, 129.5, 125.9, 124.0, 122.1, 121.5, 121.4, 117.3, 116.5, 98.2, 97.5. HRMS calcd for C\(_{37}\)H\(_{34}\)N\(_2\)O\(_4\)P \([\text{M} + 1]\) \(801.2251\); found, 801.2249.

#### 4.3. Cell Imaging

HepG2 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) for 24 h and then seeded in a 96-well plate. For the control group, the cells were incubated with the 10 \(\mu\)M AN-DP solution dissolved in a culture medium (DMEM: DMEM = 2:98, v/v) for 30 min and then washed with PBS buffer for imaging (excitation 514 nm, emission 670 nm).
emission 600–700 nm). For the experimental group, the AN-DP-loaded cells were further incubated with SIN-1 (200 μM, a peroxynitrite generator) for another 30 min and washed with PBS buffer for imaging.

Figure 5. (a) Fluorescence spectra and (b) fluorescence responses of AN-DP (10 μM) toward different analytes in 20 mM PBS buffer (pH 7.4, containing 30% DMSO). (1) AN-DP; (2) Zn²⁺; (3) Fe³⁺; (4) K⁺; (5) Al³⁺; (6) Cu²⁺; (7) NO₂⁻; (8) NO₃⁻; (9) F⁻; (10) Cl⁻; (11) Br⁻; (12) CO₃²⁻; (13) H₂PO₄⁻; (14) AcO⁻; (15) Cys; (16) Hcy; (17) GSH; (18) H₂O₂; (19) HClO; (20) HO²⁻; (21) BuHO²⁺; (22) ROO⁻; (23) O₂⁻; (24) O₂⁻; and (25) ONOO⁻. 100 μM for metal ions (Zn²⁺, Fe³⁺, K⁺, Al³⁺, and Cu²⁺) and anions (NO₂⁻, NO₃⁻, F⁻, Cl⁻, Br⁻, CO₃²⁻, H₂PO₄⁻, and AcO⁻), 1 mM for biothiols (Cys, Hcy, and GSH), and 10 μM for other reactive oxygen/nitrogen species (H₂O₂, HClO, HO²⁻, BuHO²⁺, ROO⁻, O₂⁻, O₂⁻, and ONOO⁻).

Scheme 2. Synthesis of AN-DP

Figure 6. Fluorescence images of HepG2 cells. (a) Brightfield and (c) fluorescence image of HepG2 cells incubated with only AN-DP (10 μM) for 30 min. (b) Brightfield and (d) fluorescence image of AN-DP-loaded HepG2 cells incubated with SIN-1 (200 μM) for 30 min.
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