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Short Communication

Selective detection of peroxynitrite in living cells by a near-infrared diphenyl phosphinate-based dicyanoisophorone probe

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ABSTRACT

A new NIR fluorescent probe for detection of $ONOO^-$ has been developed, which possesses a large Stokes shift, good selectivity and low cytotoxicity. This NR-ONOO probe exhibits a strong turn-on near-infrared fluorescence response toward $ONOO^-$ ion under excitation at 560 nm and has been successfully applied in detecting $ONOO^-$ in living HeLa cells.

1. Introduction

Peroxynitrite ($ONOO^-$) acts as a reactive oxygen species (ROS) in various pathological and physiological processes, which was endogenously formed by the combination reaction of superoxide ($O_2^{\bullet -}$) and nitric oxide (NO•) [1–3]. In living biosystem, low level of peroxynitrite plays a critical role in signal transduction, but high level of peroxynitrite could induce damage of some certain biomolecules, such as lipids, proteins, and nucleic acids, *etc* [4,5]. The abnormal fluctuations of the peroxynitrite concentration eventually cause various diseases including inflammation, cancers, cardiovascular diseases, atherosclerosis, ischemia-reperfusion injury, and diabetes [6]. Thus, the development of peroxynitrite detection methods in living biosystem has become urgently needed.

Compared with other techniques, fluorescent probe-based imaging technology has higher sensitivity, less invasiveness and greater convenience, thus it attracted a great deal of attention [7,8]. However, the excitation and emission wavelengths of most previously reported probes for detection of peroxynitrite just cover ranges within 650 nm, which render them not suitable for detecting peroxynitrite *in vivo* due to the autofluorescence interferences and insufficient penetration depth in the visible region [9–12]. In contrast, the use of near-infrared fluorescence (650–900 nm) avoids the influence of background bio-autofluorescence and allows deep penetration into tissues [13–15]. However, to date, only a few probes of NIR fluorescence for detecting peroxynitrite have been reported [16–27], and it is severely desired to develop new fluorescent probes for the detection of peroxynitrite with NIR emission.

Thus, we herein developed a new NIR florescent probe NR-ONOO for detection of peroxynitrite by using dicyanoisophorone as fluorophore, which was widely utilized in NIR fluorescence because of its excellent photophysical property including large Stokes shift, good photostability and lower biotoxicity. Besides, diphenylphosphinic group was chosen as a sensitive and selective recognition group for the detection of peroxynitrite. The fluorescence of probe NR-ONOO was inhibited by the blocking of the intramolecular charge transfer (ICT). After reaction with peroxynitrite, phosphinate group departed and released the free phenolic hydroxyl group as a strong electron-donating group, which recovers the ICT effect and the generated dicyanoisophorone exhibits the NIR fluorescence emission (Scheme 1). The probe NR-ONOO could selectively and sensitively detect peroxynitrite with a large Stokes shift (133 nm) and a low detection limit (78.70 nM). Besides, this probe also has good membrane penetrability as well as low cell toxicity, making it suitable for the detection of peroxynitrite in living HeLa cells by using confocal microscope.

2. Material preparation and methods

2.1. Materials and instruments

Unless specifically mentioned, all used chemical reagents were obtained from Sigma-Aldrich chemical company without further purification. Deionized water was used for preparing phosphoric acid buffer solution. UV–Vis-NIR absorption spectra and fluorescence spectra

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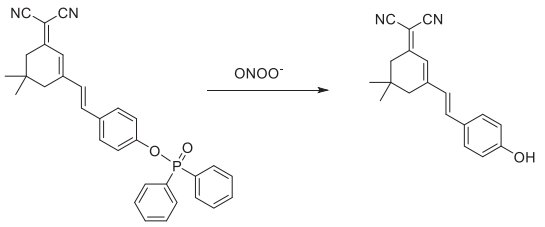


Scheme 1. Detecting peroxynitrite by probe NR-ONOO.

were obtained with LAMBDA 1050+ UV/Vis/NIR and Horiba Scientific Spectrofluorometer. Bruker 400 MHz spectrometer is used for ^{1}H NMR and ^{13}C NMR experiments. Nikon A1MP confocal laser scanning microscope was used for living cell imaging.

2.2. Synthesis of probe

Compound 1 was synthesized according to previous literature [28]. Compound 1 (580 mg, 2 mmol), Compound 2 (472 mg, 2 mmol) and 0.1 mL triethylamine was added in 10 mL dichloromethane and stirring at room temperature for 8 h. After the reaction was finished monitored by TLC, 10 mL water was added into the crude mixture. The organic layer was extracted with dichloromethane (5mL*3), dried with rotary evaporator and then purified by silica gel flash chromatography (eluted with petroleum ether/dichloromethane 5:1, v/v) to afford the desired product as a yellow solid (900 mg, 92% yield). (Scheme 2A).

2.3. Spectra measurements

All fluorescence measurement was performed in PBS (10 mM, pH 7.4, 10% DMSO, 25 °C) with excitation at 560 nm and slit width of 5.0 nm. The stock solution of $ONOO^-$ was dissolved in deionized water to concentration of 2 mM. And the stock solution of probe NR-ONOO was prepared according to previous literature and dissolved with deionized water to concentration of 10 mM. Other analytes with a concentration of 100 mM were prepared in PBS solution including $O_2•^-$, H_2O_2, NO, ClO^-, •OH, tBuO•, H_2S, SO_3^{2-}, Cys, Hcy and GSH.

2.4. Cell maintenance and fluorescence imaging

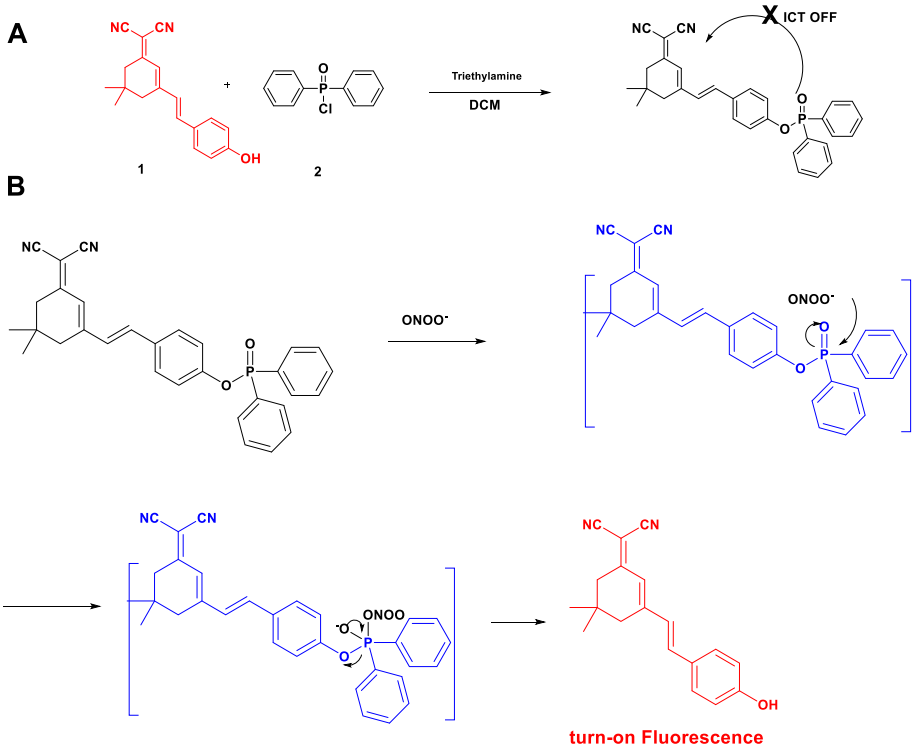
Dulbecco's Modified Eagle's Medium (DMEM) (Gibco) with fetal bovine serum (Gibco 10%), penicillin and streptomycin (100 μg/mL) were used for HeLa cells culture at 37 °C in 5% CO_2. Cell imaging of HeLa: The HeLa cells were incubated with probe NR-ONOO for 30 min at 37 °C, then incubated with peroxynitrite donor SIN-1 (a peroxynitrite generator) or treated with the $ONOO^-$ scavenger, ebselen. Cell imaging in HepG2: pretreated with PMA and LPS followed by incubating with the probe. Next, the cells were washed with D-PBS two times and then were observed by Nikon A1MP confocal laser scanning microscope for cell imaging. Cell viability assays of probe were determined using MTT methanol method.

3. Results and discussion

3.1. Design and synthesis

Fluorophore is an important member of fluorescent probes. In recent years, isophorone-fused fluorophore (compound 1) has attracted wide attention due to its excellent optical property such as near-infrared fluorescence emission [29], large Stokes shift and the convenience in modifying phenolic hydroxyl group. Besides, diphenylphosphinate group has been reported as $ONOO^-$-specific reaction model [30].Thus, it is highly probable to regulate the switching of fluorescence of probe NR-ONOO by the protection and deprotection of phenolic hydroxyl group with diphenyl phosphinate group.

The detailed synthetic procedures of the probe and the sensing mechanism toward detecting $ONOO^-$ were outlined in Scheme 2. Firstly, compound 1 was readily synthesized according to previous literature. Then, the probe was prepared by the reaction of compound 1



Scheme 2. (A) The synthetic route of probe NR-ONOO; (B) the possible mechanism of sensing peroxynitrite by probe NR-ONOO.

with compound 2 in the presence of triethylamine at room temperature *via* a one-step reaction, with dichloromethane as solvent. The molecule structures of the probe were characterized and confirmed by ^{1}H NMR, ^{13}C NMR, and MALDI-TOF-MS, as shown in SI. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.93–7.79 (m, 5H), 7.54 (d, J = 8.6 Hz, 2H), 7.46 (d, J = 7.0 Hz, 4H), 7.40–7.36 (m, 2H), 7.24–7.19 (m, 2H), 6.96 (s, 1H), 6.86 (s, 1H), 6.78 (s, 1H), 2.57 (s, 2H), 2.41 (s, 2H), 1.04 (s, 6H). ^{13}C NMR (100 MHz, Chloroform-*d*) δ 169.34, 153.81, 152.10, 136.05, 132.82, 131.96, 131.85, 130.03, 129.11, 128.98, 123.69, 121.52, 113.68, 112.89, 78.90, 43.26, 39.44, 32.34, 28.32. MALDI-TOF mass spectrometry:491.33.

After obtaining the probe, we first evaluated the possibility of the probe to detect $ONOO^-$ by UV–Vis absorption and fluorescence-emission spectra under physiological conditions. As shown in Fig. 1 (left), the UV-absorption peak at 400 nm decreased accompanied with an obviously increasing signal at 545 nm in the presence of $ONOO^-$. As shown in Fig. 1 (right), probe NR-ONOO displayed negligible fluorescence emission owing to the blocked ICT effect, because the electron-donating effect of the phenolic hydroxyl group was suppressed by diphenyl phosphinate protecting group. However, the fluorescence peak around 678 nm was gradually enhanced and reached saturation under 560 nm excitation in the presence of $ONOO^-$. Noteworthy, probe NR-ONOO shows a large Stokes shift (133 nm) in response to $ONOO^-$, which is much larger than other reported probes. This large Stokes shift is beneficial to minimize the interferences originated from self-absorption. Besides, it can be observed that the fluorescence spectrum of probe NR-ONOO after reaction with $ONOO^-$ was almost consistent to that of compound 1. MALDI-TOF mass spectrometry was applied to investigate the recognition mechanism and the peak at m/z 291.59 $[M + H]^+$ corresponding to compound 1 was observed after mixing probe NR-ONOO with $ONOO^-$. Additionally, the reaction product of mixing probe and $ONOO^-$ was monitored by TLC plate (Fig. S9), and was fully characterized by ^{1}H NMR and ^{13}C NMR (Figs. S10–11) confirming that the product is compound 1. The above data testifies that the significantly increased fluorescence signal was induced by the removal of the $ONOO^-$ responsive diphenyl phosphinate group (see Scheme 1) and the reaction mechanism of probe NR-ONOO accorded with the previous literature [31–42].

3.2. Selectivity and pH value effect of probe NR-ONOO toward $ONOO^-$

The selectivity experiments of probe NR-ONOO toward other ROS/RNS such as $O_2^{\bullet-}$, H_2O_2, NO, ClO^-, •OH, tBuO•, H_2S, SO_3^{2-}, Cys, Hcy and GSH were conducted with fluorescence spectrometry. As shown in Fig. 2, upon the addition of $ONOO^-$, an obvious signal change in the fluorescence spectrum of probe NR-ONOO was observed under 560 nm excitation. In contrast, even with the addition of 10 equivalent

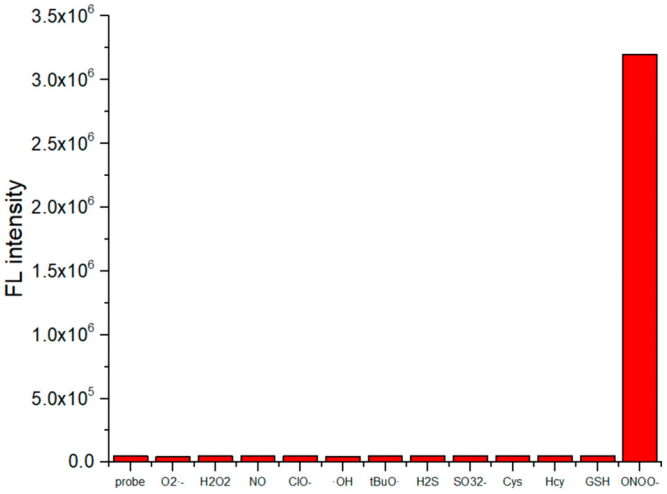


Fig. 2. The fluorescent recognition (slit: 5 nm) of probe NR-ONOO (10 μM) to $ONOO^-$ (100 μM) and other various species (100 μM).

of other analytes made negligible changes in the emission spectra. These results suggested that the probe NR-ONOO has high selectivity for $ONOO^-$. Besides, the competing experiments in the presence of other ROS/RNS were also conducted and there are no obvious fluorescence fluctuations with other analytes (see Fig. S5), and the negligible effect of physiological concentrations (GSH and Cys) at 2 mM level was shown in Fig. S12. In short, all these results indicate that the diphenyl phosphinate group of this probe was specifically reactive to $ONOO^-$ over other analytes, causing the excellent selectivity for $ONOO^-$. Because the normal physiological pH value is about 7.4, we further discussed the effect of pH on the fluorescence response of NR-ONOO to $ONOO^-$ in the revised manuscript. The results are shown in Fig. S8. The probe had great change on the performance with pH changes (lower than 6.5 and higher than 8.5), but slight effect at physiological pH (6.5–8.5). Those experiments results demonstrated that the probe showed high prospect for biological utilization.

3.3. Time-dependent fluorescence response

After the selectivity study was completed, time-dependent fluorescence experiments of probe for $ONOO^-$ were carried out. As shown in Fig. S6, the fluorescence intensity changing at 678 nm was saturated within ~25 min when 10.0 equiv. of $ONOO^-$ was added to 10 μM probe, ~40 min when 5.0 equiv. of $ONOO^-$, and ~60 min when 1.0 equiv. of $ONOO^-$ was added, respectively (Fig. S6). These data show that although the probe needs a certain period to respond to $ONOO^-$,

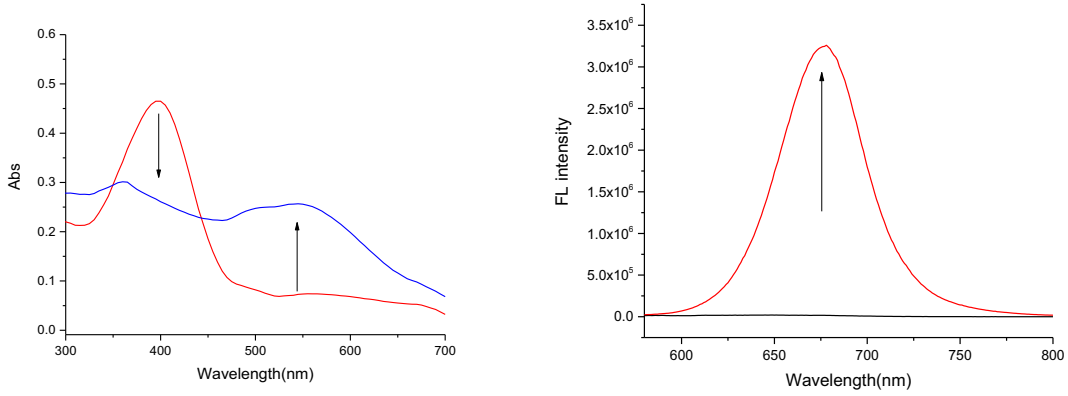


Fig. 1. (left) The UV–vis spectra of probe NR-ONOO (10.0 μM) in the absence (red line) and presence of $ONOO^-$ (20 equiv.) (blue line). (right) The fluorescence emission spectra of probe NR-ONOO (10.0 μM) in the absence (black line) and presence of $ONOO^-$ (20 equiv.) (red line).

but less than an hour recognition time is acceptable for monitoring $ONOO^-$ *in vivo*.

3.4. Linear relationship between the fluorescence response of NR-ONOO and total $ONOO^-$ ion concentration

Next, the fluorescence titration studies were carried out to investigate the sensitivity of probe NR-ONOO toward $ONOO^-$. As shown in Fig. 3 (top), the increasing concentration of $ONOO^-$ caused an obvious fluorescence turn-on at 678 nm in a concentration-dependent manner. Fig. 3 (down) shows that the fluorescence response has a good linear relationship between the emission intensity and $ONOO^-$ concentration ($R^2 = 0.99293$). Then, we use the formula DL = 3 s/k (s for the standard deviation of the fluorescence spectrometer and k for the slope of the correction curve) to get the detection limit as low as 78.70 nM, indicating that probe NR-ONOO has excellent sensitivity for the quantitative detection of $ONOO^-$.

3.5. Cytotoxicity of probe NR-ONOO

To validate the cytotoxicity of the probe NR-ONOO, MTT method was used in living HeLa cells. As shown in Fig. S7, HeLa cells remained high activity after incubating with various concentrations of probe NR-ONOO over 24 h (10, 20, 30, 40, 50 μM), and the highest viability about 80% can be found even at 50 μM concentration, which means that this probe has slight cytotoxicity and satisfactory bio-

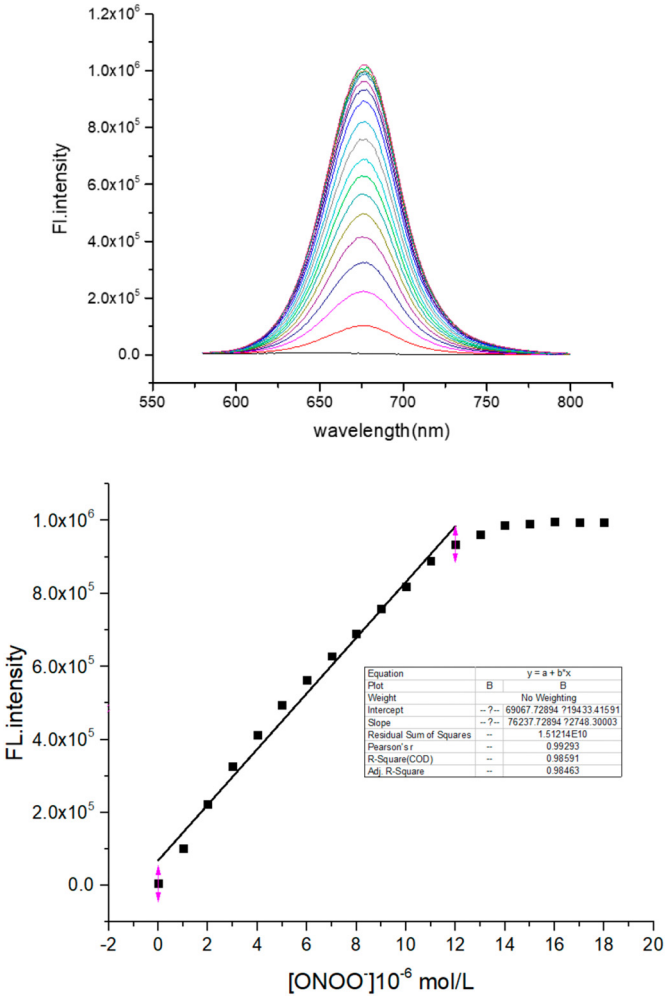


Fig. 3. (top) Fluorescence response of probe (10 μM) to various concentrations of $ONOO^-$ (reaction time is 1 h) (down) Linear correlation between the enhancement of emission intensity at 638 nm. $\lambda_{ex} = 560$ nm, $\lambda_{em} = 678$ nm.

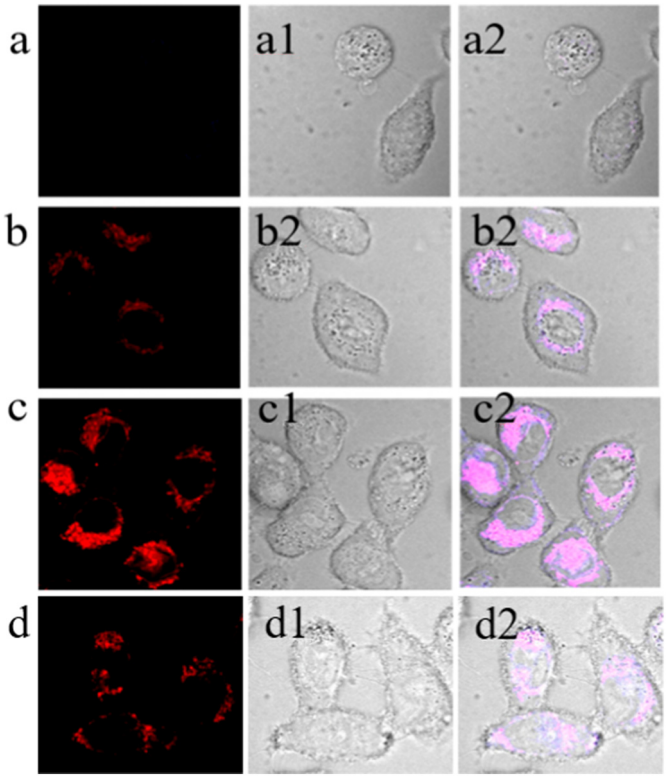


Fig. 4. Fluorescence images for exogenous $ONOO^-$ in HeLa cells. a: HeLa cells were incubated with 10 μM probe, control group; b: HeLa cells were incubated with 5 μM probe, then incubated by SIN-1 (100 μM, 0.5 h); c: HeLa cells were incubated with 10 μM probe, then incubated by SIN-1 (100 μM, 0.5 h); d: HeLa cells were incubated with 10 μM probe, pretreated with 100 μM ebselen, and then treated with 100 μM SIN-1 for 30 min.

compatibility. The low cytotoxicity is in favor of gaining a better understanding for biological utilization.

3.6. Imaging $ONOO^-$ in living cells

Encouraged by the selective and sensitive characteristic of probe NR-ONOO toward $ONOO^-$, we evaluated the bioimaging applications of this probe for detection of $ONOO^-$ in living HeLa cells. First, HeLa cells were treated with probe, and further incubated with $ONOO^-$ peroxynitrite donor SIN-1 (a peroxynitrite generator), as shown in Fig. 4b and c, a strong fluorescence can be observed in red channel. To verify if the fluorescence increase is resulting from $ONOO^-$, ebselen, an $ONOO^-$ scavenger, was applied in a blocking experiment, the red fluorescence is significantly reduced by the pretreatment with the ebselen (see Fig. 4d). Additionally, HepG2 cells was used to monitor the generation of endogenous $ONOO^-$. HepG2 cells were pretreated with phorbol 12-myristate 13-acetate (PMA) and lipopolysaccharide (LPS) for 1 h to produce endogenous $ONOO^-$ followed by incubating probe. As shown in Fig. S13, the HepG2 cells stimulated with PMA and LPS also exhibited intense fluorescence. This result indicated that the probe could achieve visually tracing the changes of endogenous or exogenous $ONOO^-$ levels in living cells.

Determination of $ONOO^-$ in living cells.

4. Conclusion

A new NIR fluorescent probe for detection of $ONOO^-$ through an ICT process has been developed and successful applied in detecting $ONOO^-$ in living HeLa cells. This probe has a large Stokes shift, good selectivity and low cytotoxicity. The addition-elimination reaction process by nucleophilic attack of $ONOO^-$ to diphenyl phosphinate motif of the

probe yielding free phenolic hydroxyl-containing compound 1 generated the fluorescence emission locating at NIR region. We anticipate that the probe might be useful to offer a new way to track $ONOO^-$ in living cells.

CRediT authorship contribution statement

Yibin Zhang: Conceptualization, Methodology, Software, Data curation, Writing - original draft. **Dongge Ma:** Visualization, Investigation, Supervision, Validation, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.saa.2020.118890.

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