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Photocalibrated NO release from the zinc ion fluorescent probe based on naphthalimide and its application in living cells

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ABSTRACT

A highly sensitive zinc ion fluorescent probe NOD-NY with controlled release of nitric oxide was designed, synthesized and used for tracking intracellular zinc ions in living A549 cells. NOD-NY was prepared from naphthalimide as the fluorophore and *N*,*N*-bis(2-pyridylmethyl)amine as the zinc ion recognition receptor, the amide N atom of the naphthalimide was connected to *n*-butylamine. Under the irradiation of ultraviolet light, NOD-NY can quantitatively release nitric oxide and generate a highly sensitive zinc ion probe Zn-HN, accompanied by a red-shift process of maximum ultraviolet absorption from 350 nm to 450 nm. Upon addition of Zn^{2+} to the solutions of Zn-HN, a remarkable fluorescence enhancement was observed, which could be attributed to the photo-induced electron transfer (PET) mechanism. By replaced the *n*-butylamine on NOD-NY with diethylene glycolamine or triphenylphosphine structures, NOD-AY with good biocompatibility and NOD-BY that can target mitochondria were obtained respectively. In addition, the nitric oxide released by NOD-NY enriched in lysosome can diffuse into mitochondria. The released nitric oxide can stimulate metallothionein to release zinc ions, and the light-induced *in situ* generated zinc ion probe Zn-HN can have a highly sensitive fluorescence response to free zinc ions in living A549 cells.

As an endogenous signal gas small molecule, nitric oxide (NO) has various functions such as vasodilation, pathogen invasion defense, wound healing, neurotransmission, and inflammatory cell response [1,2]. It also plays a complex, diverse and indispensable important physiological role in mammalian nervous system [3], cardiovascular system, immune system, respiratory system, *etc*. Accordingly, NO donors (such as nitroglycerin, sodium nitrate) are frequently widely used in biomedical research [4-9]. On the other hand, zinc, as the second most abundant trace metal element in the human body [10,11], not only plays an important role in various enzymes and metal proteins, but also plays diverse functions in biological metabolic processes and signal transmission in the human body [12,13]. As an important trace metal element in the human body, Zn^{2+} has close and complex interactions and feedback mechanisms with NO in cardiovascular, nervous, immune and other systems [14-17]. In the nervous system, Zn^{2+} and NO are closely related to the induction and development of neurological diseases such as Alzheimer's disease [18]. High concentrations of NO can transfer Zn^{2+} from neuronal cytoplasm to the mitochondrial matrix, and high concentrations of zinc ions will induce the formation of O^{2-} and trigger mitochondrial dysfunction [19]. In the immune system, the deficiency of Zn^{2+} can damage immune function and increase infectivity, leading to the release of NO in inflammatory conditions [20]. In the cardiovascular system, NO, as a vasodilator, also has medicinal properties for the treatment of asthma and other diseases [21]. However, NO can release free Zn^{2+} through the nitrification of metal binding protein metallothionein (MT), resulting in vasoconstriction [22,23].

Currently, monomer that can achieve controlled liberation of NO and detect the release of zinc ions under NO stimulation has not been reported [19,22,24,25]. Therefore, the development of a powerful tool for the controlled quantitative release of nitric oxide while real time monitoring of Zn^{2+} distribution, uptake, and trafficking in living system is highly demanded [26]. Fluorescent techniques, which have been widely used in imaging various kinds of analytes in living cells and tissues, have some

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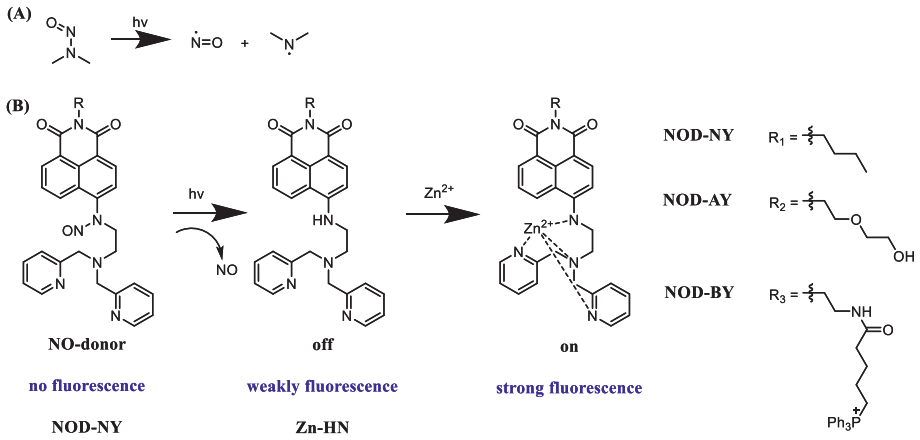

Fig. 1. (A) The structure of nitrosamine and its photolysis. (B) The structure of NO donor and its mechanism of recognizing zinc ion after light activation.

unique advantages, such as non-invasive, higher sensitivity, and excellent temporal-spatial resolution [27]. Zhang *et al.* devised a novel mechanism to allow the release of NO to be triggered by light and accompanied by a drastic fluorescence turn-on to facilitate convenient *in vitro* quantitation of NO dose [8]. Inspired by this work, herein, we designed a series of highly sensitive zinc ion fluorescent probes with photocalibrated NO release from *N*–nitrosated and used for tracking intracellular zinc ions in living cells by using this novel naphthalimide-based fluorophore and *N*,*N*-bis(2-pyridylmethyl)amine (DPA) as the receptor for Zn^{2+} [28-33]. NO donors (NOD-NY, NOD-AY and NOD-BY), whose NO release is triggered by ultraviolet–visible (UV–vis) light at 365 nm on the *N*–nitrosated naphthalimides, can be conveniently synthesized in a few steps (Schemes S1–S3 and Figs. S1–S14 in Supporting information). Electron-withdrawing substituted *N*-nitrosamines are known to homolyze upon photolysis to yield NO (Fig. 1A) [34-37]. Based on the fluorescent recognition mechanism of photoinduced electron transfer (PET), DPA was introduced into the imide moiety of the naphthalimide fluorophore as the receptor of Zn^{2+}. As shown in Fig. 1B, upon phototriggered decomposition, NO donor quantitatively releases NO and converts it into a fluorescent probe with DPA structure, which are weakly fluorescent and can be used to monitor the dose of NO release. After DPA binds Zn^{2+}, the PET process of the probe is inhibited to significantly increase the fluorescence. Donors differ from each other in the substituents on the amide nitrogen of naphthalimide.

We first conducted the UV–vis absorption and fluorescence emission spectra of the nitric oxide donor NOD-NY during photolysis. As shown in Figs. 2A and B, with the continuation of the ultraviolet irradiation time, the maximum absorption in the ultraviolet spectrum curve gradually red-shifted from 350 nm to 450 nm. The results indicated that the nitroso group attached to the imine in the donor compound structure was continuously detached and released NO, and the departure of the strong electron-withdrawing group also caused the NOD-NY absorption to gradually red-shift. The photolysis kinetics curve (Fig. 2C) showed that NOD-NY released NO from fast to slow, and the whole process took about 30 s. The fluorescence, with the generation of the zinc probe after the release, gradually increased with the binding of different equivalents of Zn^{2+}. The selectivity and competitiveness of Zn-HN, which was photolysis product of donor NOD-NY, toward various metal ions (Zn^{2+}, Li^{+}, Mn^{2+}, Fe^{3+}, K^{+}, Ca^{2+}, Na^{+}, Ag^{+}, Cr^{3+}, Pb^{2+}, Hg^{2+}, Ni^{+}, Fe^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+} with perchlorate anions) were further investigated in phosphate buffer solution (PBS, 10^{-2} mol/L, pH 7.4). Upon addition of 3 equiv. different metal ions, negligible changes were observed in the fluorescence emission spectra (Fig. 2D). However, the fluorescence intensity was significantly

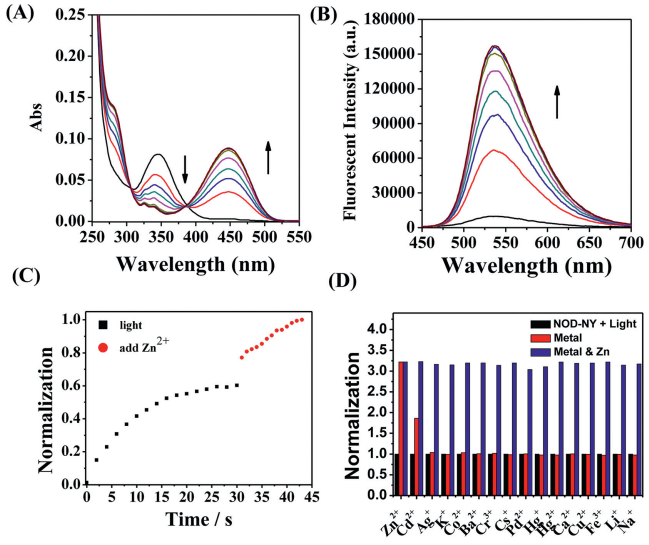

Fig. 2. UV–vis absorption spectrum (A) and fluorescence emission spectrum (B) of NOD-NY in PBS buffer (10^{-2} mol/L, pH 7.4) under UV light at 365 nm. (C) NOD-NY photolysis kinetic spectrum (blank) and relative fluorescence intensity changes (red) after adding Zn^{2+}. (D) Fluorescence intensity of NOD-NY (10^{-5} mol/L) determined after the addition of various cations (3×10^{-5} mol/L, black) followed by Zn^{2+} (10^{-5} mol/L, red) in PBS buffer (10^{-2} mol/L, pH 7.4). $\lambda_{ex} = 450$ nm, $\lambda_{em} = 545$ nm.

enhanced after Zn^{2+} was added. Even in the presence of other metal ions, the fluorescence intensity exhibited similar enhancement when Zn^{2+} was added. A slight enhancement of fluorescence was observed upon the addition of Cd^{2+}, since the DPA receptor also can bind cadmium ions [31-33]. However, considering its low content in the organism, the interference of Cd^{2+} can be ignored in the further cell experiments. Therefore, probe Zn-HN should have the potential in imaging intracellular Zn^{2+}. We also validated two other donors, NOD-AY and NOD-BY, in quantitatively releasing NO and the selectivity and competitiveness of their photolysis products, Zn-HA and Zn-HB, towards Zn^{2+}. After light triggered decomposition, similar results were obtained in the UV absorption curve, fluorescence emission curve, and photolysis kinetics spectrum of donor NOD-AY and NOD-BY (Fig. S15 in Supporting information). The donor photolysis products Zn-HA and Zn-HB also exhibited excellent selectivity and competitiveness towards Zn^{2+} (Fig. S16 in Supporting information).

Additionally, we demonstrated by high-performance liquid chromatography (HPLC) that the product of NOD-AY photolysis was the corresponding Zn^{2+} fluorescent probe Zn-HA. The reten-

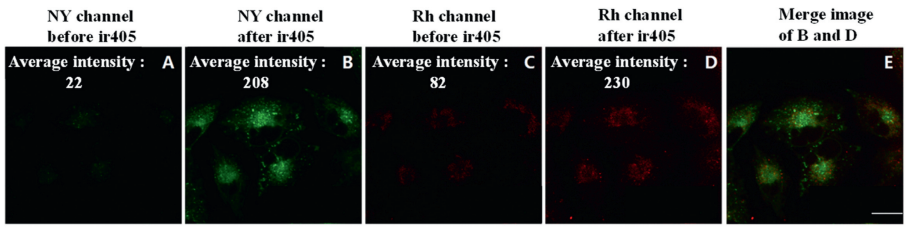

Fig. 3. Confocal fluorescence imaging of donor NOD-NY (5×10^{-6} mol/L) before (A) and after (B) illumination in A549 cells. Green channel ($\lambda_{ex} = 488$ nm, $\lambda_{em} = 500–560$ nm). NO-recognizable fluorescent probe Mito-NO (5×10^{-6} mol/L) located in mitochondria before (C) and after (D) illumination under the same vision. Red channel ($\lambda_{ex} = 559$ nm, $\lambda_{em} = 570–670$ nm). (E) Overlay of (B) and (D). Scale bar: 20 μm.

tion time (Rt) of NOD-AY in the chromatographic column was 8.290 min, and the retention time of NOD-AY photolysis products (Rt = 11.564 min) was consistent with the retention time of Zn^{2+} probe Zn-HA (Rt = 11.510 min) (Fig. S17 in Supporting information). 2-Phenyl-4,4,5,5-tetramethylimidazoline-3-oxide-1-oxyl (PTIO) is a stable nitric oxide scavenger with distinct electron paramagnetic resonance (EPR) characteristic signals, which can react with nitric oxide molecules to generate nitrogen dioxide and 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl (PTI) [38]. Using an EPR instrument to detect photo-controlled generation of nitric oxide free radicals, we found that with the extension of light illumination time, the donor compound had completed photolysis and all nitric oxide free radicals had been released (Fig. S18A in Supporting information). Quantitative release of NO was detected by using commercial NO activity detection agent 2,3-diaminonaphthalene (DAN) [39]. We tested the donor compound NOD-AY at diverse concentrations and found the excellent linear relationship ($R > 0.99$) between the change in fluorescence intensity and the concentration. The results indicated that the designed donor compounds can quantitatively release NO (Fig. S18B and C in Supporting information). As shown in Fig. S19 (Supporting information), NOD-NY, NOD-AY, NOD-BY and their photolysis products exhibited good stability in the physiological range of pH 6.0–8.0.

The biocompatibility and subcellular localization ability of NO donors and their photolysis products were evaluated before living cell imaging. Cell counting kit-8 (CCK8) assay was used to determine the cytotoxicity of three kinds of donors before and after photolysis in Raw264.7 cells and A549 cells. The cells viability was above 80% with the concentration of them ranging from 0 to 2×10^{-5} mol/L after 24 h incubation (Fig. S20 in Supporting information), which indicated that NO donors had low toxicity and could be used for imaging in living cells. Next, we used flow cytometry to analyze the uptake of the three dyes Zn-HN, Zn-HA and Zn-HB by living A549 cells. As shown in Fig. S21 (Supporting information), probe Zn-HA, which contains diethylene glycol amine structure, had the best uptake in living A549 cells. This showed that the hydroxyl group and imine in the diethylene glycol amine structure could increase the water solubility of the molecule and improved the cellular uptake. Then, we investigated the subcellular location of Zn-HN, Zn-HA and Zn-HB in A549 cells with commercial lysosome marker and commercial mitochondrion marker. The experimental results indicated that Zn-HN and Zn-HB were mainly located in the cytoplasm and had no significant organelle localization effect (Figs. S22 and S23 in Supporting information). As shown in Fig. S24 (Supporting information), the fluorescence imaging of Zn-HB was overlapped well with commercial mitochondrial red fluorescent probe, with the Pearson's correlation coefficient at 0.83, which indicated that Zn-HB was mainly localized in mitochondrion.

Subsequently, we verified the NO release process at the cellular level. We incubated NOD-NY and Mito-NO (5 μmol/L, 10 min) with A549 cells in the dark. Mito-NO is a nitric oxide probe that can target mitochondrion [40]. After capturing NO, it can illuminate the fluorescence of the rhodamine channel and make the cells show red fluorescence. As shown in Fig. 3, the imaging experiment was the fluorescence changes of the cells before and after the mercury lamp illumination in the same field of view. After continuous light, the green fluorescence signal gradually increased (Figs. 3A and B), indicating that the green fluorescence was originated from the activation of mercury lamps to generate zinc ion probe Zn-HN to identify free zinc in cells. At the same time, the red fluorescent signal also gradually increased like the green fluorescent signal (Figs. 3C and D), indicating that the Mito-NO captured NO released by NOD-NY. However, the fluorescence imaging of NOD-NY illumination (Fig. 3B) was not overlapped well with Mito-NO (Fig. 3D), with the Pearson's correlation coefficient only at 0.52 (Fig. 3E), which indicated that the NO released by NOD-NY could diffuse into the mitochondrion and be captured by Mito-NO in the mitochondrion, then light up the red light channel.

We also explored the imaging of Zn^{2+} in living cells using confocal microscopy. When the experimental operation was protected from light, A549 cells stained with 5×10^{-6} mol/L of NOD-AY displayed no green fluorescence (Fig. S25A in Supporting information). After illumination, the green fluorescence significantly enhanced for the generated fluorescent probe could bind the free zinc ion in the cells (Fig. S25B in Supporting information). After the subsequent addition of 10^{-6} mol/L or 5×10^{-5} mol/L of membrane-permeable Zn^{2+} chelator, *N*,*N*,*N*',*N*'-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN), the green fluorescent signal had weakened to varying degrees (Fig. S25C and D in Supporting information), indicating that the green fluorescence was originated from the binding of the donor's photolysis product Zn-HA with free Zn^{2+}. It also indicated that fluorescent probe Zn-HA could fluorescently recognize different concentrations of Zn^{2+} in living cells (Fig. S26 in Supporting information).

By using the commercial Zn^{2+} fluorescent probe Zinquin, we next assessed the change of intracellular free zinc concentration with the release of NO by NOD-NY. As shown in Fig. 4A, The NOD-NY photolysis of the nitric oxide donor continuously generated zinc ion probe Zn-HN and recognized the free zinc in the living A549 cells, which gradually increased the fluorescence of the green light channel. In addition, the NO released by the donor NOD-NY, which could induce free Zn^{2+} release from MTs, increased the blue fluorescence of the Zinquin. The reason for the green fluorescence was originated from the binding of Zn-HN with free Zn^{2+}. Under the living A549 cells in the same field of view, a 5.88-fold and a 2.50-fold enhancement in the fluorescence intensity of NOD-NY and Zinquin were observed (Fig. 4B) after light, respectively. Upon addition of 5×10^{-5} mol/L of membrane-permeable Zn^{2+} chelator, TPEN, the green and blue fluorescence signal both gradually decreased. These results indicated that NOD-NY could be activated by light to generate "off-on" type zinc ion fluorescent probe Zn-HN. The probe Zn-HN was a sensitive fluorescent probe for the real-time monitoring of intracellular endogenous Zn^{2+} affected by NO.

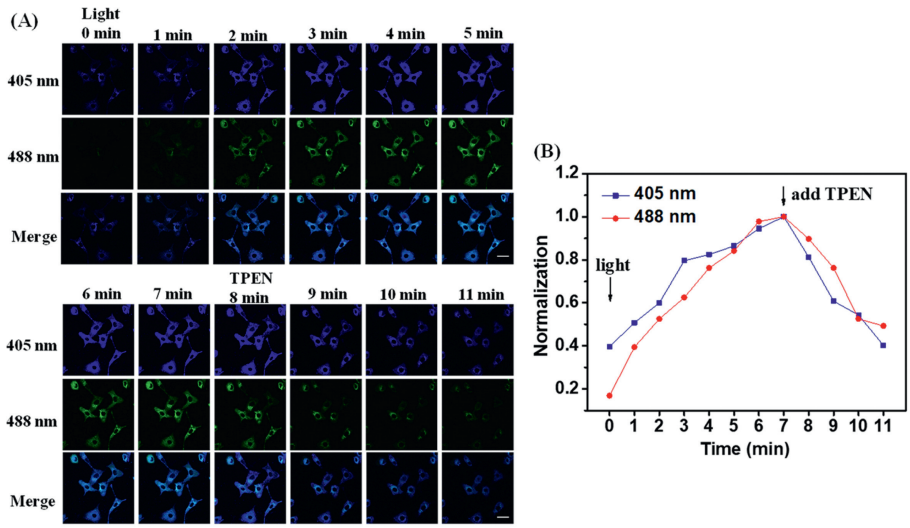

Fig. 4. (A) Light-controlled release of NO induces the production of Zn^{2+} in A549 cells. The green channel (λ_{ex}= 488 nm, λ_{em}= 500–560 nm) was NOD-NY (5×10^{-6} mol/L), and the blue channel (λ_{ex}= 405 nm, λ_{em}= 450–500 nm) was Zinquin (5×10^{-6} mol/L). Scale bar: 25 μm. (B) Normalized of the change in fluorescence intensity of green channel (red) and blue channel (blue).

In summary, we have successfully combined a NO donor with light-controlled release function and a fluorescent probe for Zn^{2+} concentration detection. The target compound possesses the universal applicability of biological research and mitochondrial targeting. In dynamic imaging of A549 cells, nitric oxide released by donor NOD-NY can stimulate metallothionein to release Zn^{2+}, while light-induced *in situ* generated zinc ion probe Zn-HN can detect intracellular free zinc in real time. This strategy provides a useful tool for the study of the physiological and pathological interactions between nitric oxide and zinc ions in vivo.

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.

CRediT authorship contribution statement

Zhixiao Xiong: Writing – original draft, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Shanni Qiu:** Validation, Methodology, Data curation. **Yuyu Wang:** Validation, Data curation. **Houna Duan:** Validation, Methodology, Data curation. **Yi Xiao:** Validation, Data curation. **Yufang Xu:** Writing – review & editing, Supervision. **Weiping Zhu:** Writing – review & editing, Supervision, Project administration, Funding acquisition. **Xuhong Qian:** Supervision, Project administration, Funding acquisition.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cclet.2024.110002.

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