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# A Water-Soluble, Green-Light Triggered, and Photo-Calibrated Nitric Oxide Donor for Biological Applications

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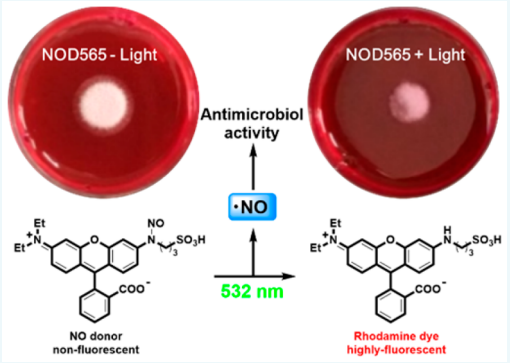
Supporting Information

**ABSTRACT:** Nitric oxide (NO) is a versatile endogenous molecule, involved in various physiological processes and implicated in the progression of many pathological conditions. Therefore, NO donors are valuable tools in NO related basic and applied applications. The traditional spontaneous NO donors are limited in scenarios where flux, localization, and dose of NO could be monitored. This has promoted the development of novel NO donors, whose NO release is not only under control, but also self-calibrated. Herein, we reported a phototriggered and photocalibrated NO donor (**NOD565**) with an N-nitroso group on a rhodamine dye. **NOD565** is nonfluorescent and could release NO efficiently upon irradiation by green light. A bright rhodamine dye is generated as a side-product and its fluorescence can be used to monitor the NO release. The potentials of **NOD565** in practical applications are showcased in in vitro studies, e.g., platelet aggregation inhibition and fungi growth suppression.




## INTRODUCTION

The biological implications of nitric oxide (NO) were not recognized until the 1970s. Murad et al. proved that nitro-based vasodilators function by production of nitric oxide in vivo, which induces the formation of cGMP and leads to vasodilation.[1] Furchgott et al. discovered that endothelial cells could release an unknown substance (EDRF) to induce vasodilation.[2] He further hypothesized that EDRF could be NO and this was confirmed independently by Moncada et al.[3] and Ignarro et al.[4] To date, NO has been found to be involved in numerous physiological and pathological processes in mammalian cells,[5,6] plants cells,[7,8] and prokaryotic organisms.[9,10] Recently, Bossy-Wetzel reported that mitochondrial dynamics is also regulated by nitric oxide.[11] NO donors, a group of chemicals capable of decomposing to release NO, are sought-after for both basic biological studies and therapeutics applications.[12−23]

Existing NO donors are diverse in structure and mechanism. Trinitroglycerin,[24] isoamyl nitrite,[25] and sodium nitroprusside[26] are among the oldest nitric oxide donors, which have been used in treating hypertensive emergencies in the 19th century. Nitrosothiols, diazeniumdiolates, and (benzo-/)furoxans are recent additions to the field.[18−23] They spontaneously release NO in a biological milieu in the presence of various biological analytes or enzymes without the necessity of external stimuli. The biological response of NO is profoundly influenced by where, when, and how much NO is released.[27−30] This has greatly limited the scope of these spontaneous NO donors in basic mechanistic studies. The development of the first phototriggered NO donor (**CNOs**), i.e., an *o*-nitrobenzyl

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protected NONOate constructed by protection of a spontaneous NO donor with a photolabile group, has been enlightening.[31] Another notable group of phototriggered NO donors involves photoinduced sensitization of an electron-rich N-nitrosamine (**NO-Rosa**).[32] We have recently proposed to develop phototriggered NO donors with a concomitant fluorescent turn-on. Such phototriggered and photocalibrated NO donors are advantageous because they not only render a spatiotemporal control over the release of NO, but also offer a convenient and sensitive method for monitoring. The first examples of such novel NO donors are a group of N-nitrosated naphthalimide dyes (**NOD545**) (Figure 1).[33]

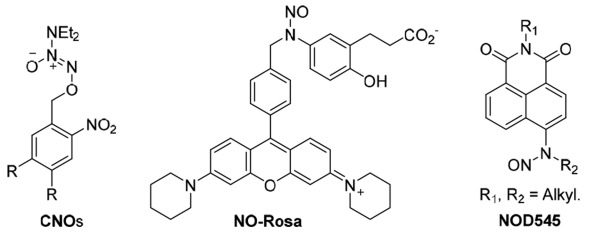


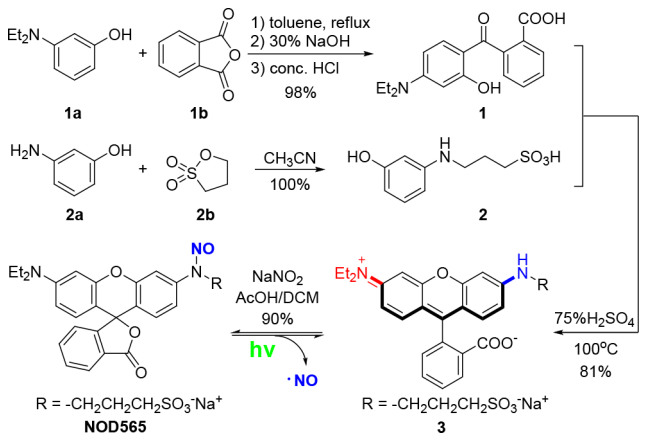

**Figure 1.** Examples of notable photoactivated NO donors.

**NOD545** have a few limitations yet to be addressed. First, it requires UV light to trigger NO release. Also, the fluorescence of naphthalimide dyes is moderate in brightness. Third, their scaffolds are rather hydrophobic and exhibit limited water-solubility. Therefore, it is the ambition of this work to develop highly water-soluble NO donors, which can be triggered to release NO by a longer-wavelength green light and can release a brighter fluorophore, i.e., rhodamine.

## RESULTS AND DISCUSSION

We have previously showcased that N-nitrosated push−pull dyes are viable general strategies for the development of novel phototriggered and photocalibrated NO donors. We designed the structure of **NOD565** which employed a highly fluorescent rhodamine scaffold. Also, a sulfonate group is installed to promote aqueous solubility of the NO donor. It is conveniently synthesized in 4 steps (Scheme 1). The intermediate **1** was obtained by condensation of *m-N,N*-diethylaminophenol (**1a**) and stoichiometric phthalide anhydride (**1b**) upon refluxing in toluene in a 98% yield. The coupling partner (**2**) was obtained by alkylation of *m*-aminophenol (**2a**) with 1,3-propane sultone (**2a**) in acetonitrile at room temperature in quantitative yield. Subsequent condensation of **1** and **2** in 75% $H_2SO_4$ at 100 °C furnished the rhodamine **3** in a 81% yield. Treatment of **3** with $NaNO_2$ in a mixed solvent of dichloromethane and acetic acid at 0 °C yielded the desired NO donor (**NOD565**) in a 90% yield.

**Scheme 1. Design of Photo-Triggered and Photo-Calibrated NO Donors**




The UV−vis absorption and fluorescence spectra of **NOD565** and **3** were acquired in phosphate buffer (50 mM, pH = 7.4). **NOD565** and **3** exhibited a favorable water-solubility, which was tested to be at least 11 mg/mL, equivalent to 20 mM. The solution **NOD565** has a unique absorption band from ∼420 to 570 nm. Three peaks were identified to be at 475 nm ($\varepsilon$ = 19,900 $cm^{-1}$ $M^{-1}$), 505 nm ($\varepsilon$ = 28,800 $cm^{-1}$ $M^{-1}$), and 537 nm ($\varepsilon$ = 23,700 $cm^{-1}$ $M^{-1}$). The fluorescence emission spectrum of this solution was obtained with an excitation at 505 nm. Overall, a very low but finite emission band with a maximum at 565 nm could be observed. The UV−vis absorption spectrum of **3** is very typical of a rhodamine, an intense band with a maximum at 540 nm ($\varepsilon$ = 70,300 $cm^{-1}$ $M^{-1}$) and minor shoulder at 510 nm ($\varepsilon$ = 32,300 $cm^{-1}$ $M^{-1}$). Upon excitation at 540 nm, an emission band at 565 nm was observed with a fluorescence quantum yield of 0.14.

Photoinduced decomposition of **NOD565** and triggered release of NO was studied. An $Ar^+$ laser line (532 nm, 200 mW) was used as the light source. The UV−vis absorption and fluorescence emission of a solution of **NOD565** (10 $\mu$M) in aqueous phosphate buffer (50 mM, pH = 7.4) were monitored before and intermittently after photoirradiation (Figure 2). The

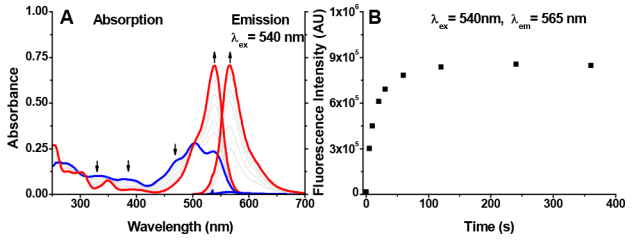



**Figure 2.** (A) UV−vis absorption spectral and fluorescence emission spectral changes of **NOD565** solution upon photoirradiation by 532 nm. Blue lines: the absorption and emission of **NOD565**. Red lines: the absorption and emission of **3**. (B) The enhancement of emission intensity at 565 nm of the solution with respect to the duration of photoirradiation.

absorption spectrum has changed dramatically. The absorbance at 540 nm was enhanced by ∼3-fold before and after complete photodecomposition. An isosbestic point was found at 503 nm and the absorbance in the range of 310−503 nm underwent a drop. An abrupt fluorescence enhancement of 53-fold was seen at 565 nm. The majority of decomposition and fluorescence recovery occurred within the first 100 s. Continuous photo-irridation for 532 nm did not induce further fluorescence fluctuation.

The phototriggered NO release was verified by a commercial NO probe (**DAN**), which reacts with NO in the presence of oxygen to produce **NAT** (Figure 3A).[34] All three compounds (**DAN**, **NAT** and rhodamine **3**) are excitable by 300 nm. Therefore, it is possible to simultaneously monitor the three species with a three channel ratiometric emission scan ($\lambda_{ex}$ = 300 nm). A solution containing both **NOD565** (10 $\mu$M) and **DAN** (10 $\mu$M) in aqueous phosphate buffer (50 mM, pH = 7.4) with 5% DMSO was prepared. The use of higher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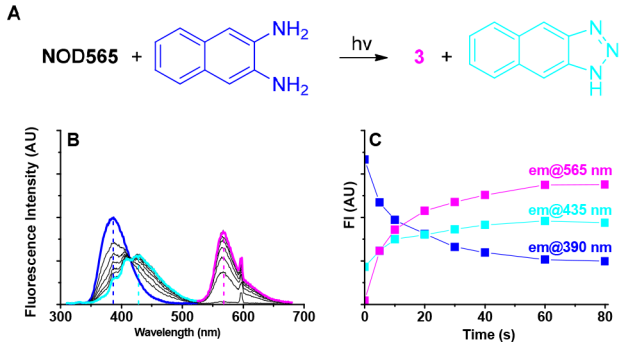


**Figure 3.** (A) Capture of NO released from **NOD565** upon photoirradiation. (B) Fluorescence changes of a solution containing **NOD565** (10 $\mu$M) and **DAN** (10 $\mu$M) in aqueous phosphate buffer (50 mM, pH = 7.4) upon photoirradiation at 532 nm for 80 s, $\lambda_{ex}$ = 300 nm. (C) Gradual change of emission intensity at 390 nm (**DAN**), 435 nm (**NAT**), and 565 nm (**3**) of the solution is plotted with respect to the duration of photoirradiation.

percentage of DMSO is owing to the poor solubility of both **DAN** and **NAT** in an aqueous medium. The solution was then irradiated with 532 nm light and the fluorescence emission spectrum of the solution was collected intermittently. The fluorescence of **3** gradually increased with respect to the exposure time (Figure 3B), consistent with Figure 2. At the same time, the emission band of **DAN** at 390 nm gradually decreased and the emission band of **NAT** at 435 nm increased (Figure 3A), essentially at the same kinetic profile (Figure 3C). The release of NO was also independently verified by EPR study (Figure S1).

Excellent chemostability of NO donors is desired in biological studies to avoid unintended NO release and fluorescence turn-on. Theoretically, N-nitroso compounds may cause denitrosation in the presence of nucleophiles, e.g., thiols,[35] and reducing agents.[36] Hence, the stability of **NOD565** was tested in phosphate buffer (50 mM, pH = 7.4) with cysteine, glutathione, and ascorbic acid in the dark. The absorbance spectra of these solutions were collected and plotted in Figure S3. All three agents at very high concentrations (up to 10 mM) did not induce any spectra changes at room temperature in 30 min, suggesting competent chemostability of **NOD565**. The absorbance values for 24 h also comfirmed the result except for conditions at 37 °C or with ascorbic acid, which exhibited an absorbance enhancement by ca. 5% decomposition of **NOD565** (Figure S4). Overall, **NOD565** displayed high chemostability and feasibility for practical applications.

In vitro NO release of **NOD565** was investigated in HeLa cells. An aqueous solution of **NOD565** (20 $\mu$M) was added into the HeLa cell culture and incubated for 30 min. The fluorescence images were collected before and after irradiation by 532 nm (Figure 4). As shown in Figure 3B, the cells exhibited no background as expected. However, upon photoirradiation by 532 nm for 60 s, punctate fluorescence was captured. This highlighted its potentials as an exogenous NO donor for in vitro NO studies. The feasibility of **NOD565** in zebrafish embryos was also preliminarily tested. An aliquot of **NOD565** solution (1 mM, 3 nL) in DMSO was injected into the yolk sac region of a zebrafish egg, which we have indicated in SI. As shown in Figure S7, the fluorescence was turned on upon green light irradiation, which suggested that **NOD565** could release NO and dye **3** in this case.

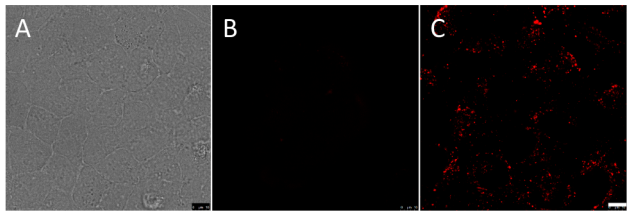


**Figure 4.** Confocal fluorescence imaging of HeLa cells invubated with **NOD565** (20 $\mu$M) for 30 min. (A) Bright-field image. (B) Fluorescence image before irradiation. (C) Fluorescence image after irradiation by 532 nm for 60 s. Scale bar: 10 $\mu$M.

**Inhibition of Platelet Aggregation.** NO plays a role in platelet aggregation inhibition.[37,38] Hence, we evaluated the potentials of our donors in such an application. The inhibition percentage of platelet aggregation was measured at different conditions (Figure 5). First, at the time point indicated by

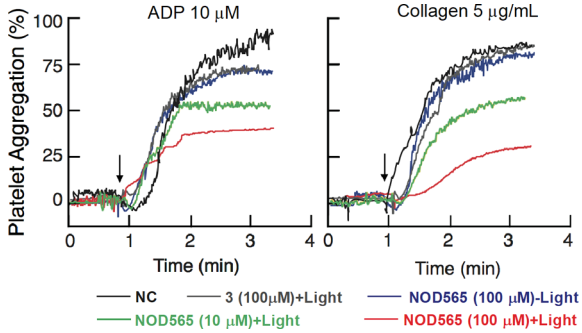


**Figure 5.** Inhibition effect of **NOD565** (in aqueous solvent) toward platelet aggregation in the presence of ADP (10 $\mu$M) and collagen (5 $\mu$g/mL). NC: negative control group without **3** or **NOD565** or light irradiation. Light: 3 W green LED light. Light duration: 10 min.

arrows, adenosine diphosphate (ADP, 10 $\mu$M) and collagen (5 $\mu$g/mL) were added into the platelet-rich plasma to induce aggregation. By the end of 3.5 min, platelet aggregation was nearly 90% (NC). The addition of **3** (100 $\mu$M) or **NOD565** (100 $\mu$M) without photoirradiation did not significantly inhibit platelet aggregation to any appreciable extent. In the presence of **NOD565** (10 $\mu$M) and green light irradiation, aggregation percentage dropped to ca. 50%, suggesting that NO produced from photolysis of **NOD565** indeed inhibited platelet aggregation. Platelet aggregation inhibition by **NOD565** under photoirradiation was dose dependent. Raising the concentration of **NOD565** (to 100 $\mu$M) helped to further inhibit the platelet aggregation percentage (to below 50%).

**Antimicrobial Activity.** The immune system harnesses NO as a defense against infectious microbes and intracellular parasites.[39] To further test the feasibility of our NO donors in biological applications, we carried on qualitative and quantitative studies on cytotoxicity of released NO to microbes. *Aspergillus nidulans* is one of the model fungi, exhibiting sensitivity to NO, generated from NO donors, e.g., NOCs (10 mM) or acidified nitrite (1−40 mM).[40,41] First, we examined the sensitivity of wild type fungus *A. nidulans* to NO released from **NOD565** on the agar plate. As shown in Figure 6, after cultivation for 30 h, compared to strains grown under the control conditions without **NOD565**, the cell growth was clearly suppressed by the NO released from **NOD565** (3 mM) triggered by an LED light (532 nm, 16 W). No obvious growth impairment was observed on the media containing **NOD565** (3


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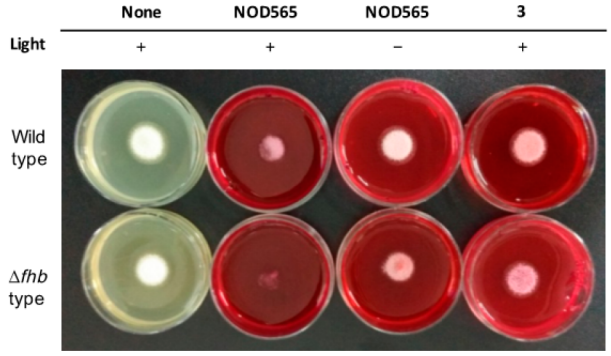


**Figure 6.** Sensitivities of fungi to NO, derived from **NOD565**. Conidia ($2 \times 10^4$) of wild type and Δ*fhb A. nidulans* were grown on DPY agar plates. The concentrations of **NOD565** and **3** were all 3 mM. The plates were incubated at 37 °C for 30 h with or without photoirradiation by 532 nm (16 W LED light) as indicated.

mM) in the dark or the photolysis product **3** (3 mM). This experiment excludes the possibility that **NOD565** itself or its fluorophore product (**3**) inhibited the cell growth, and verifies that the cytotoxicity was induced by NO. Furthermore, we performed the experiment with *A. nidulans* Δ*fhb*, which is deficient of flavohemoglobin, a NO detoxifying enzyme. Similarly to wild type cells, only NO releasing conditions inhibited the cell growth. Moreover, the NO cytotoxicity deriving from **NOD565** to the mutant was indeed enhanced compared to the wild type.

It is noteworthy that the dose of NO release is readily monitored by the fluorescence from the agar media. Also, the spectroscopic analysis (Figure S8) indicated **NOD565** in the agar media did not decompose without light irradiation, in agreement with the chemostability study in Figure S3. These results indicated that 3 mM NO could suppress and abolish the growth of wild type and Δ*fhb* of this fungus, respectively. As an important immunological molecule, antimicrobial properties of NO have been intensively studied. However, most studies focused on the NO detoxification pathways and resistant abilities of the microbial enzymes in qualitative levels rather than quantitative investigations due to the difficulties to quantify the dose of NO release from the existing NO donors. Our present NO donor overcame the shortcomings of the common NO donors. Additionally, being triggered by green-light rather than the cytotoxic UV-light makes **NOD565** potentially suitable for investigation of the physiological or pathological roles of NO in microorganisms.

## CONCLUSIONS

A green-light triggered NO donor was developed by mono N-nitrosation of a bright water-soluble rhodamine fluorophore. Upon NO release, a dynamic fluorescence turn-on was observed and could be harnessed to quantify the kinetics, localization, and dose of NO release. NO release was unambiguously verified by a fluorescent probe for NO and by EPR. The potentials of **NOD565** in platelet aggregation inhibition and microbial growth inhibition were exemplified. Our work reinforced the viability of the design rational, i.e., N-nitrosated push−pull dye, for novel phototriggered and photocalibrated NO donors. We also believe that **NOD565** has broad potentials in biological and translational studies.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.7b00821.

General synthetic methods, experimental, compound characterizations, photophysical results, chemostability study, $^1$H NMR, $^{13}$C NMR and HRMS spectra (PDF)

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### Notes

The authors declare no competing financial interest.

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