1 Visible light-triggered NO generation from Naphthalimide-based probe 2 for photoreceptor-mediated plant root growth regulation

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9 **ABSTRACT:** An efficient visible light-triggered nitric oxide (NO) releasing fluorescent molecule is designed and
10 synthesized by coupling 2,6-dimethyl nitrobenzene moiety at the *peri*-position of 1, 8-naphthalimide through
11 an alkene bond. The NO-releasing ability is investigated in details using various spectroscopic techniques, and
12 the photoproduct was also characterized. Further, the photo-generated NO has been employed to examine
13 the effect of photoreceptor-mediated NO uptake on plant root growth regulation.

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15 Nitrogen monoxide is considered one of the most important signaling agents, exists in three interrelated
16 chemical forms including nitrosonium cation (NO⁺), nitric oxide (NO[•]), and nitroxyl anion (NO⁻).¹⁻² Nitric oxide
17 (NO) exists as a highly reactive free radical or reactive oxygen species (ROS), sometimes also referred to as
18 reactive nitrogen species (RNS), performs as an important multitasked signaling agent in various biological
19 processes.³⁻⁵ NO has also been identified as an endothelium-derived relaxing factor (EDRF) in blood vessels
20 and the nitric oxide synthase is mainly responsible for NO production in living systems.⁶⁻¹⁰ Besides, several
21 diseases such as schizophrenia, Alzheimer's disease, and cancer are related to malfunction of NO signaling and
22 its dynamics.¹¹⁻¹³ For the past few decades, researchers also found that NO performs a crucial role in plant
23 growth.¹⁴⁻¹⁵ In the metabolism of inorganic nitrogenous compounds in higher plants and nitrogen-fixing
24 organism, NO is generated as a pivotal intermediate.¹⁶ Recent literature on plant biology reveals that NO
25 influences primary root development through the initiation of cell-cycle genes and patterns of cellulose
26 synthesis.¹⁷⁻¹⁸ Additionally, being an essential signaling agent, deficiency of NO affects auxin biosynthesis,
27 transport, and signaling.¹⁹ The highly reactive nature of NO and its tremendous biological importance
28 fascinated the researchers for *in-situ* generation of NO in biological systems.²⁰⁻²¹ For potential therapeutic
29 applications in living systems, the NO-donors must release NO in a time-controlled and site-specific manner.²²⁻
30 ²³ To date, numerous NO-donors such as 1-hydroxy-2-oxo-3-(aminoalkyl)-1-triazenes, 4-alkyl-2 hydroxyimino-
31 5-nitro-3-hexenes, *etc.* are reported in the literature.²⁴⁻²⁵ Among them, photo-triggered NO-donors secure
32 extraordinary attention to the researchers due to the release of NO with high spatiotemporal control. Miyata
33 group reported 6-Nitrobenzo[*a*] pyrene derivative that generates NO in the presence of visible light.²⁶ Later,
34 they reported a series of NO-releasing molecules, comprising of a hindered nitrobenzene derivative, but all
35 those molecules are poorly water-soluble.²⁷ However, the recently reported light-induced NO-donors suffer

from ultraviolet and two-photon excitation.²⁸⁻²⁹⁻³¹ Therefore, there is an urgent need of a visible-light triggered efficient NO-donor with considerable water-solubility.

In this contribution, we have designed and synthesized a naphthalene monoimide-based fluorescent molecule (Ni-NO₂) for a visible light-induced NO generation. Thus, we have attached a sterically hindered nitrobenzene moiety, i.e., 2, 6-dimethyl nitrobenzene, at the *peri*-position of 1, 8-naphthalimide ring through an alkene spacer. Subsequently, elaborative spectroscopic investigations were performed to understand the visible light-triggered NO release and further employed to comprehend the effect of NO in photoreceptor-mediated plant root growth regulation. The design strategy of Ni-NO₂ is based on photo-isomerization of nitro group of the

Scheme 1. Synthesis of Ni-NO₂ for visible-light triggered NO generation

sterically-hindered nitrobenzene moiety, connected to the naphthalimide ring. Considering the photo-isomerization possibility, we have synthesized Ni-NO₂ according to the above synthetic scheme (Scheme 1), the detailed synthetic scheme is shown in Scheme S1. Density functional study reveals that the nitro group is in non co-planar conformation with a twist angle of *ca.* 42°, with respect to the benzene ring due to the steric hindrance of two *ortho*-methyl groups (Scheme S2a-b and Fig. S1). Upon photo-irradiation, the twisted nitro group rearranges and isomerizes to nitrite ester,²⁸ owing to the generation of NO (as shown in Scheme S2c).

After the synthesis and characterization, we validated the optical purity by comparing UV-Vis. and excitation spectra in phosphate buffer (PB, pH 6, Fig. 1a). Ni-NO₂ has an absorption maximum in PB at around 400 nm, whereas it emits around 517 nm (Fig. 1a) with a large Stokes shift of 117 nm. To understand the swiftness

Fig. 1 (a) The absorption, excitation, and emission spectra of Ni-NO₂ in PB at pH 6.0, (b) kinetic monitoring of fluorescence intensity of 1 μ M Ni-NO₂ at 540 nm in PB upon excitation of 410 nm light; inset shows fluorescence spectra of the same at different time interval

of NO-releasing properties of Ni-NO₂, we have kinetically monitored its fluorescence properties under visible-light excitation (113 Lux) in 1 mM PB at pH 6. The fluorescence intensity of Ni-NO₂ gets attenuated upon visible-light mediated excited-state -isomerization of the nitro group. Interestingly, within 20 minutes of irradiation, we have obtained a plateau in the emission intensity indicating completion of NO release (Fig. 1b). The fluorescence quenching is possibly due to the formation of the phenolic group and its interaction with the solvent.³²⁻³³ Further, to verify the effect of light intensity on the NO release rate, we have performed kinetic monitoring of NO release experiment using a neutral density filter and recorded the emission spectra of Ni-NO₂. As expected, we obtained a plateau after 30 min (Fig. S2-3) confirming the role of light intensity for NO release. Furthermore, we have performed a modified Griess assay to validate NO generation through the photolysis of Ni-NO₂.²⁷ The photo-induced production of NO was verified by the detection of NO₂⁻, arising from the auto-oxidation of NO in PB medium.³⁴ The appearance of red color solution having absorption at 542 nm supports the diazo-coupling reaction of NO₂⁻ with the Griess reagents (GR, Scheme S3). To perform this assay, we have taken 50-150 μ M of Ni-NO₂ in CHCl₃ and excited by 400 nm light for ~50 min, and then the mixture was allowed to react with GR. Finally, the absorption spectrum of the red color solution was measured. The increase optical density at 542 nm upon increasing concentration of Ni-NO₂, signifies the successful generation of NO (Fig. 2a). Further, to testify the photo-triggered generation of NO, an EPR experiment was also performed using 2-phenyl-4, 4, 5, 5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO) as a spin trap for NO.^{29, 35} After the photo-irradiation of a mixture of PTIO and Ni-NO₂ for 30 min the EPR spectrum was recorded.

Fig. 2 (a) Absorption spectra of the reaction between Griess solution with a photo-irradiated Ni-NO₂ solution, (b) chemical structure of the photo-generated product of Ni-NO₂ and (c) the mass-spectrum of the product after photodecomposition, (d) HPLC profile of photo-triggered NO generation experiments and (e) time-dependent absorption spectra of Ni-NO₂ (2 μM) in DMSO upon photo-irradiation using white light.

The characteristic EPR spectrum further confirms the generation of NO from Ni-NO₂ (Fig. S4). Additionally, we have performed a HPLC experiment to monitor the progress of the reaction. For HPLC measurement, we have irradiated 10 μM of Ni-NO₂ in acetonitrile using 390 nm light at 0, 15, 30, 60, and 80 min interval (see Fig. 2b and SI for details). A linear decrease in the absorption intensity corresponding to the reactant having an elution time 12.8 minute and subsequent increase in the absorption intensity for the product at an elution time of 4.6 minutes were observed (Fig. 2b). The photodecomposition quantum yield of NO release was measured as *ca.* 0.036 in acetonitrile (for details see SI, Fig. S5-7). The photo-degraded product was also identified by mass spectrometry (Fig. 2c-d). Further, the photo-reaction was also monitored by absorption spectroscopy in different time interval in DMSO (Fig. 2e) and in other solvents (see Fig. S8). The absorption spectra in DMSO reveal a decrease in the absorption maxima at 400 nm with the generation of a new absorption peak at around 427 nm which also confirms the formation of photo-generated product.³³

Fig. 3 Response of *Arabidopsis* (Col-0) roots to Ni-NO₂ under dark and light. (a) 6 days old *Arabidopsis* seedlings grown on MS media plates supplemented with 0, 10 μM Ni-NO₂ in continuous dark and cycling white light at 110 $\mu\text{M m}^{-2} \text{s}^{-1}$ fluence level. Part of seedling below white arrow shown in (a) indicates root that is inhibited by Ni-NO₂ in light due to the release of NO. (b) Primary root length of Col-0 seedlings treated with 0, 0.2, 1, 5, 10, 30 μM Ni-NO₂ and SNP at two different fluence levels 35 and 150 $\mu\text{M m}^{-2} \text{s}^{-1}$ of cycling white light (c) and in continuous white light (110 $\mu\text{M m}^{-2} \text{s}^{-1}$) and flash of white light (110 $\mu\text{M m}^{-2} \text{s}^{-1}$ for 1 hour). Scale bar, 1 cm in (a).

After the spectroscopic characterization of the NO release in the presence of visible-light, we have investigated the effect of NO on plant roots and root-hair growth. A thorough literature survey suggests that NO inhibits root growth in a dose-dependent manner.³⁶ Hence, to determine the effect of NO released from Ni-NO₂, we used *Arabidopsis thaliana* as a model plant and its root growth inhibition as a functional assay. *Arabidopsis* seeds (Col-0 ecotype) were inoculated on growth medium supplied with 0, 0.2, 1.0, 5.0, 10.0, 30.0 μM of Ni-NO₂. The NO-donor Ni-NO₂ was added inside the upper cover of plates at desired concentrations (Fig. S9). Seedlings were grown on Ni-NO₂ free agar medium in the lower portion of the plate to avoid side effects of NO-donor reagents.³⁷ One set of plates was kept under cycling white light (110 $\mu\text{M m}^{-2} \text{s}^{-1}$) while the other set was kept under darkness. After six days seedlings grown under light and containing Ni-NO₂ in the upper lid showed significantly reduced primary root length as compared to the plate without Ni-NO₂ (Fig. 3a). On the other hand, seedlings grown in the dark did not exhibit any significant difference in root length in the presence or absence of Ni-NO₂ (Fig. 3a). These results suggest that the photo-activation of Ni-NO₂

Fig. 4 Inhibition of root hair development by Ni-NO₂ under white light. (a) Root hair image of 6 days old Col-0 seedlings grown on medium supplemented with 0, 2, 5, 10 μM Ni-NO₂ and SNP in continuous white light at 110 μM m⁻² s⁻¹ fluence level (b) Root hair length measurement of Col-0 seedlings treated as described in (a). (c) Root hair number measurement of Col-0 seedlings treated as described in (a). Mean values and S.E. were calculated from at least 30 seedlings. Scale bar, 2 mm in (a).

promotes the release of NO that causes root growth inhibition in plants. In order to evaluate the efficiency of Ni-NO₂ derived NO on root growth inhibition, we used sodium nitroprusside (SNP), a well-established NO-donor, and compared the effects on primary root growth inhibition (Fig. 3b-c).³⁸ At similar concentrations of these two NO-donors the root elongation defect was either comparable or more severe in Ni-NO₂ compared to SNP (Fig. 3b-c). Further, we examined the effect of Ni-NO₂ and SNP with varying fluence levels of cycling white light, 35, 85 and 150 μM m⁻² sec⁻¹ (Fig. 3b and Fig. S10a-b). At all fluence levels, both the NO-donors showed reduced primary root length in a dose-dependent manner. We also determined the effect of continuous white light and flash of white light (1 h at 150 μM m⁻² sec⁻¹) on NO-donors by using different doses of Ni-NO₂ and SNP (0, 2 and 20 μM) and exposing them to continuous or flash of white light. Both the light conditions exhibited an inhibitory effect on primary root growth (Fig. 3c and Fig. S11a, S12a). Further, we investigated the effect of monochromatic blue light on NO-donors by exposing plants to continuous blue light (150 μM m⁻² sec⁻¹) and a flash of blue light (1 h at 150 μM m⁻² sec⁻¹, see SI). Both Ni-NO₂ and SNP markedly inhibited primary root length under continuous blue light (SI, Fig. S11b, S12b). Under flash of blue light, Ni-NO₂ at 2 μM concentration promoted primary root length while at 20 μM concentration it showed inhibitory effect on root growth (Fig. S12b, S13b). This might be due to cell elongation effect at a low concentration of NO under blue light.³⁹ We also examined the effect of different concentrations (0-10 μM) of Ni-NO₂ and SNP on root-hair growth when exposed to continuous white light (110 μM m⁻² sec⁻¹). Root hair growth is inhibited (reduced root

hair length and number) in a dose- dependent manner (Fig. 4a-c). This result suggests that the regulatory effect of Ni-NO₂ on root growth of Arabidopsis seedlings is light dependent. Upon exposure to light, the photolysis of Ni-NO₂ releases NO which inhibits root growth. Light-mediated release of NO also activates various photoreceptors in plants. Phytochrome (PHY) and cryptochrome (CRY), red and blue light receptors respectively are reported to be NO-responsive. NO release causes nuclear accumulation of *phyB-9*

Fig. 5 Response of WT, *cry1cry2* and *phyB-9* mutants to NO under continuous white and blue light (a) WT, *cry1cry2* and *phyB-9* seedlings growing on medium supplemented with SNP and Ni-NO₂ at various concentration under continuous WL at 110 μM m⁻² s⁻¹ fluence. (b) Root length of WT, *cry1cry2* and *phyB-9* seedlings treated as in (a). (c) WT, *cry1cry2* and *phyB-9* seedlings growing on medium supplemented with SNP and Ni-NO₂ at 0, 2 and 10 μM concentration under continuous blue light at 35 μM m⁻² s⁻¹ fluence. (d) Root length of WT, *cry1cry2* and *phyB-9* seedlings treated as in (c). Mean value and SE were calculated from at least 20 seedlings. Significant difference was analyzed student's t-test with Welch's corrections compared to WT under same condition are indicated by asterisks: **P*<.05, ** *P*<.01, *** *P*<.001.

which positively regulates primary root growth inhibition.⁴⁰ To verify, if the NO-induced primary root growth inhibition is photoreceptor mediated, we quantified root length in the photoreceptor mutants *phyB-9* and *cry1cry2* (Fig. 5 and S14-18). Our results indicate that *phyB-9* and *cry1cry2* exhibit hyposensitivity to the NO released by 2 μM Ni-NO₂. At 10 μM concentration of Ni-NO₂ the root growth inhibition was also observed in the mutants especially under white light (WL) possibly due to the activation of other photoreceptors in the single mutant (Fig. 5a-b). Under the monochromatic blue light condition, *cry1cry2* double mutant display

almost insensitivity at a higher concentration indicating that NO-induced primary root growth inhibition is photoreceptor mediated (Fig. 5c-d).

In conclusion, we have designed and developed a naphthalene monoimide-based fluorescent NO-donor Ni-NO₂ by attaching 2,6 dimethyl nitrobenzene at the *peri*-position of 1,8-naphthalimide ring through an alkene bond. The synthesized molecule releases NO upon photoexcitation with visible light and the NO generation was confirmed by Griess assay, EPR spin trapping, HRMS and optical spectroscopic investigation in various solvents. Further, the progress of the reaction was monitored using HPLC. The photodecomposition quantum yield of Ni-NO₂ in acetonitrile was also determined. The fluorescence intensity of the probe gets quenched by releasing NO in aqueous solution and its get saturated within 20 min signifying the rapid release of NO. Finally, the released NO was employed for Arabidopsis root growth assay either by continuous or flash of white and blue light irradiation. Our results indicate NO-mediated regulation of primary root and root hair growth by Ni-NO₂ in a photoreceptor mediated manner. These findings can potentially open up prospects for generation of an effective weedicide in the future.

Supporting Information

Materials and methods, synthesis, photodecomposition QY measurement, kinetic study, DFT study, Griess assay, root growth experiments, characterization by UV-Vis., NMR, EPR and mass spectrometry.

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