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Near-infrared photoactivatable nitric oxide donors with photoacoustic readout

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Abstract

In this chapter, we motivate the need for photoactivatable NO donor molecules and give a brief survey of the existing chemical tools in the field. We then provide detailed protocols for the synthesis and validation of a near-infrared light-activated NO donor molecule, photoNOD-1, developed in our research group. With this tool, NO can be released in vivo in a radiation-dependent manner that can be monitored using photoacoustic imaging.

1. Introduction

Nitric oxide (NO) is an endogenously produced free radical gasotransmitter that regulates a staggering collection of physiological functions. In mammals, NO is biosynthesized by various nitric oxide synthase (NOS) enzymes as a product of arginine metabolism or generated by nitrite reduction under hypoxic conditions (Gladwin, Grubina, & Doyle, 2009). NO exerts diverse downstream effects through soluble guanylate cyclase (sGC)-dependent and sGC-independent pathways. At nanomolar concentrations, NO can bind to the heme cofactor of sGC, which elicits a conformational change and an increase catalytic activity. This in turn enhances the conversion of GTP to cGMP. cGMP then modulates the activity of phosphodiesterase, cGMP-dependent protein kinases and ion-gated channels. Together this affects platelet aggregation, smooth muscle relaxation, leukocyte infiltration, neurotransmission, and a variety of other functions in a tissue-dependent manner (Derbyshire & Marletta, 2012). NO can also react with other reactive oxygen species (such as hydrogen peroxide and superoxide) or exert other sGC-independent functions via the S-nitrosylation of cysteine residues and nitration of tyrosine residues in certain target proteins. These processes are still active areas of research (Bruckdorfer, 2005; Denninger & Marletta, 1999; Smith & Marletta, 2012). In light of its diverse physiological roles, NO must be tightly regulated to maintain homeostasis. Dysregulation of NO has been associated with pathologies including cardiovascular disease (Naseem, 2005), neurodegeneration (Duncan & Heales, 2005), inflammation (Bogdan, 2001), reperfusion injury (Schulz, Kelm, & Heusch, 2004), and cancer (Xu, Liu, Loizidou, Ahmed, & Charles, 2002).

The role of NO in cancer biology is especially interesting. While some studies show NOS or NO levels correlate with tumor suppression, other research shows NO is correlated with

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metastatic potential and tumor growth. These seemingly contradictory results, which have been reviewed in detail, are in part due to the dependence of NO biology on the concentration, duration, tissue type and local microenvironment (Vanini, Kashfi, & Nath, 2015). As a result, NO has earned a reputation as a double-edged sword, and researchers are highly motivated to understand and precisely manipulate NO levels in models of disease.

Several common strategies exist for the modulation of NO levels in vivo. Many of these methods, namely knocking out or silencing NOS enzymes, treating subjects with NOS inhibitors, or applying NO gas or NO-releasing molecules, alter systemic NO levels. While these methods can be informative in certain contexts, they cannot easily distinguish specific effects within a given pathology from global changes in physiological NO signaling. Therefore, there is a rising interest in the use of activatable donors that can release NO in a controlled manner, without eliciting global side effects.

To date, activatable NO releasing molecules (NORMs) have been developed to respond to a variety of stimuli (Wang et al., 2002). In principle, these molecules can be administered systemically, circulate through the subject, and release NO only upon engagement of the target stimulus. The stimulus (for instance, a dramatic difference in pH or enzymatic activity) must be unique to the pathological condition to avoid global side effects. This presents researchers with the challenge of identifying a unique and sufficiently abundant stimulus for each pathology of interest, then developing NO releasing moieties that can selectively respond to that stimulus. This challenge motivated our group's interest in photoactivatable NO donors. Because light can be applied to a subject in a controlled, user-defined manner, photoactivatable NO releasing molecules (photoNORMs) have great potential to provide both temporal and spatial control over NO release.

In this chapter, we highlight the criteria for selecting a photoNORM for a given study. We then provide a brief survey of photoactivatable NO donor classes with a focus on their activation wavelengths, their utility for biological experiments, and applications in fluorescence imaging. Finally, we provide a brief primer on photoacoustic (PA) tomography and outline a detailed guide to our group's development of photoNOD-1, a molecule that can release NO in the presence of near-IR light in a process that can be monitored using PA imaging (Zhou et al., 2018).

2. Selection of photoactivatable nitric oxide releasing molecules

When designing or selecting a photoNORM for a given biological application, several criteria should be considered. First, a photoNORM must be activated by an appropriate irradiation wavelength, depending upon the endpoint application of the photoNORM. For instance, if the NO is to be delivered to two-dimensional cell cultures, a NORM that is labile to UV or visible wavelengths may be sufficient. However, if an experiment requires that a sample be irradiated for extended periods of time, or if NO needs to be delivered to deep tissues, a NORM that is labile to longer-wavelength light may be required to minimize phototoxicity or maximize tissue penetrance, respectively. Second, the photoNORM should be stable to physiological conditions such that NO is selectively released upon irradiation, avoiding undesirable off-target effects. Third, a mechanism for validating NO release and/or

monitoring the progress of NO delivery should be incorporated. This could be accomplished by amperometry, an NO-selective probe, or by incorporating a release readout directly into the photoNORM.

2.1 Photolabile NO releasing moieties

A variety of photoNORMs have been developed that feature a metal-nitrosyl moiety as the NO source. The majority of these are transition-metal based, especially iron, manganese, and ruthenium, which tightly bind to NO. These metal nitrosyls are moderately labile to UV light, but tuning of ligands or coordination of chromophores to the metal center can enable NO release with wavelengths in the visible to near-IR. However, many demonstrate poor stability under physiological conditions and require nanodelivery systems to avoid off-target effects (Sortino, 2010; Xiang, Guo, & Liu, 2017). Moreover, the examples that have successfully delivered NO directly without encapsulation still present questions regarding the toxicity of the metal complexes, and the complexes do not always provide a mechanism by which NO release can be observed.

Organic photoNORMs utilize a variety of functional groups to release NO under UV irradiation, including caged diazeniumdiolates, aryl N-nitrosamines, and nitrobenzenes (Wang et al., 2002). Recently, these strategies have been revamped to incorporate a fluorescent readout of NO delivery upon activation with single photon visible light (He, Xia, et al., 2018; He, Ye, et al., 2018) or two-photon near-IR light (Xie et al., 2017; Zhang et al., 2016). The incorporation of a fluorescence readout makes these molecules excellent tools for studying NO biology in cell culture, without the need of an NO electrode or NO-sensitive fluorescent dye to confirm NO release. These tool compounds are therefore excellent for the delivery of NO to cell cultures and shallow tissues. On the other hand, the poor tissue penetrance of visible light and even two-photon fluorescence methods (which can image tissues at a maximum depth of 1.5mm) (Wang & Yao, 2016) limit their utility in complex, three-dimensional in vivo settings. This motivated our group's interest in developing a photoNORM that can effectively deliver NO to deeper tissues while still maintaining a readout of release.

2.2 Principles of photoacoustic tomography

Photoacoustic (PA) tomography is an imaging technology that interrogates a specimen with innocuous near-IR excitation light and elucidates the features of the specimen via ultrasonic detection. Unlike fluorescence methods, which rely upon emitted photons and are therefore depth-limited by both the excitation and emission wavelengths, PA imaging relies on heat from non-radiative relaxation pathways to afford thermoelastic expansions which propagate through the specimen as ultrasound. Since ultrasound is significantly more tissue penetrant than light, this means that the depth of PA imaging is primarily limited by the tissue penetrance of the near-IR excitation light. Practically, this means that PA imaging can yield high resolution results at depths up to 8–10cm in biological tissues (Reinhardt & Chan, 2018; Wang & Yao, 2016).

3. Design of photoNOD-1

photoNOD-1 consists of an NO-releasing moiety (an aryl *N*-nitroso) appended to an aza-BODIPY core. The aza-BODIPY dye, which exhibits a large extinction coefficient ($>10^4 \text{ M}^{-1} \text{ cm}^{-1}$) at near-IR wavelengths, absorbs energy upon irradiation to facilitate homolytic cleavage of the N-nitroso bond. This yields an aniline-containing dye product (rNOD-1) and the desired NO radical as well as a significant shift in the absorbance wavelength of the aza-BODIPY dye. This wavelength shift enables simultaneous PA detection of photoNOD-1 and rNOD-1 and enables monitoring of NO release (Scheme 1).

4. Synthesis of photoNOD-1

rNOD-1 can be synthesized in six steps from 4-aminoacetophenone and 4-methoxyacetophenone. One additional nitrosation step is required to generate photoNOD-1 from rNOD-1 (Scheme 2). The following synthetic procedures require understanding of standard organic chemistry techniques and the availability of basic synthetic laboratory equipment. All running solvents are defined as percent volume to volume.

***tert*-Butyl (4-acetylphenyl)carbamate (1)**

1. Preheat an oil bath to 100 °C.
2. Add 4'-aminoacetophenone (6.7 g, 50 mmol, 1.0 equiv.) and di-*tert*-butyl dicarbonate (13 g, 60 mmol, 1.2 equiv.) to a 250 mL round bottom flask equipped with a magnetic stir bar and a rubber septum.
3. Flush the reaction vessel with nitrogen.
4. Measure 1,4-dioxane (60 mL) using a graduated cylinder. Briefly remove the rubber septum from the reaction vessel, pour the solvent directly onto the combined solids with continuous magnetic stirring, and quickly cap the reaction mixture with the septum.
5. Heat the reaction vessel in an oil bath for 8.5h, or until the reaction is complete by thin layer chromatography (TLC) (25% ethyl acetate in hexanes).
6. Concentrate the reaction mixture by rotary evaporation to isolate the crude product as a solid. If the crude product does not readily solidify, allow the oil to stand on the benchtop until it crystallizes to a solid. This process can be accelerated by gently scratching the sides of the flask with a spatula or glass rod.
7. NOTE: Steps 6–9 are optional. Alternatively, the crude product can be used directly in the next reaction.
8. Suspend the solids in a small volume of 20–25% ethyl acetate in hexanes and isolate the first batch of solid product via vacuum filtration. Confirm the purity of the product by proton NMR (CDCl_3).
9. Isolate the filtrate from Step 8. Concentrate the filtrate by rotary evaporation.
10. Repeat steps 8–9 until no more pure batches of product (>95% purity by NMR) can be obtained. NOTE: We obtained 86% yield in two batches.

***tert*-butyl (4-acetylphenyl)(methyl)carbamate (2)**

1. Add *tert*-butyl (4-acetylphenyl)carbamate (**1**, 3.0 g, 12.8 mmol, 1.0 equiv.) to a 250 mL round bottom flask equipped with a magnetic stir bar and a rubber septum.
2. Flush the reaction vessel with nitrogen. Maintain a gentle flow of nitrogen through the reaction vessel using a nitrogen line and a vent needle.
3. Add anhydrous THF (40mL) and stir continuously at room temperature until the solids are completely dissolved.
4. Cool the reaction mixture to 0 °C on an ice bath.
5. Add sodium hydride (60 wt% dispersion in mineral oil) (0.61 g, 15.3 mmol, 1.2 equiv.) in five equal portions over the course of 10min, briefly removing the rubber septum for each addition.
6. Continue stirring the suspension for an additional 20 min at this temperature.
7. Add methyl iodide (1.57 mL, 31.9 mmol, 2.5 equiv.) in one portion via syringe.
8. Warm the reaction mixture to room temperature. Continue stirring for an additional 40 min, or until reaction is complete by TLC (15% ethyl acetate in hexanes).
9. Completely remove the solvent by rotary evaporation to isolate the crude alkylated product.
10. Transfer the crude residue to a separatory funnel with ethyl acetate and water. Allow the layers to separate. Isolate the organic layer and wash vigorously with an equal volume of brine (3 ×).
11. Dry the organic layer over sodium sulfate and concentrate by rotary evaporation to isolate the alkylated product as a yellow oil in quantitative yield. Use directly without purification.

***tert*-Butyl methyl (4-(4-Nitro-3-phenylbutanoyl)phenyl) carbamate (3)**

1. Add *tert*-butyl (4-acetylphenyl)(methyl)carbamate (**2**, 0.59 g, 2.38 mmol, 1.0 equiv.) and ethanol (10 mL) to a 50 mL round bottom flask equipped with a magnetic stir bar.
2. Add benzaldehyde (0.27 mL, 2.62 mmol, 1.1 equiv.) and potassium hydroxide (10 M aqueous solution, 0.72 mL, 7.15 mmol, 3.0 equiv.) via syringe. Stir at room temperature for 2.5 h.
3. Add nitromethane via syringe (1.90 mL, 35.7 mmol, 15 equiv.) and stir the reaction mixture at room temperature for 10.5 h. NOTE: Over the course of the reaction, the solution should turn dark brown in color.
4. Concentrate the reaction mixture by rotary evaporation to remove most of the ethanol. Dilute the crude mixture in brine and extract with dichloromethane (3 ×).

5. Dry the combined organic extracts over sodium sulfate and concentrate to isolate the crude product.
6. Dry load the crude product onto celite and purify the product by flash column chromatography (25% ethyl acetate in hexanes). This will afford the product as a yellow oil (0.47 g, 1.4 mmol, 59% yield). NOTE: Compounds **3** and **4** can be stored under ambient conditions.

However, the products may slowly decompose over time, generating a dark brown color. While older batches may be used, repurification may be necessary if low yields are observed in later synthetic steps.

1-(4-Methoxyphenyl)-4-nitro-3-phenylbutan-1-one (4)

1. Add 4-methoxyacetophenone (10 g, 67 mmol, 1.0 equiv.) and ethanol (130 mL) to a 500 mL round bottom flask equipped with a magnetic stir bar. Stir until the solids are fully dissolved.
2. Add benzaldehyde (6.8 mL, 62 mmol, 0.9 equiv.) via syringe.
3. Cool the reaction mixture to 0 °C and add potassium hydroxide (10 M aqueous solution, 21 mL, 207 mmol, 3 equiv.).
4. Allow the reaction mixture to warm to room temperature and stir for 1.5 h, yielding a white precipitate, until the reaction is complete by TLC.
5. Isolate the white solids by vacuum filtration to obtain the intermediate chalcone in 87% yield.
6. Resuspend the intermediate (4.8 g, 20 mmol, 1 equiv.) in ethanol (35 mL) in a 100 mL round bottom flask equipped with a stir bar.
7. Add nitromethane (13.6 mL, 254 mmol, 13 equiv.) and potassium hydroxide (10 M aqueous solution, 0.34 mL, 3.4 mmol, 0.2 equiv.) and stir the reaction at room temperature for 3 h, or until the reaction is complete by TLC.
8. Concentrate the reaction mixture by rotary evaporation to remove most of the ethanol.
9. Dilute the crude mixture in saturated aqueous ammonium chloride and extract with ethyl acetate (3 ×).
10. Dry the organic layer over sodium sulfate and concentrate to isolate the crude product as a white solid in 91% yield (79% from 4-methoxyacetophenone). Use without purification.

***tert*-Butyl (Z)-(4-(5-((4-Methoxyphenyl)-3-phenyl-2*H*-pyrrol-2-ylidene)amino)-4-phenyl-1*H*-pyrrol-2-yl)phenyl(methyl)-carbamate (5)**

1. Preheat an oil bath to 60 °C.
2. Add **3** (475 mg, 1.2 mmol, 1.0 equiv.) and **4** (718 mg, 2.4 mmol, 2.0 equiv.) to a 50 mL round bottom flask equipped with a magnetic stir bar.

3. Add ethanol (36 mL) and heat the reaction mixture at 60 °C until the solids are completely dissolved. NOTE: This reaction generates a statistical mixture of the desired heterodimeric product with the two undesired homodimeric products. Therefore, it is critical to dissolve **3** and **4** in the solvent prior to adding ammonium acetate to maximize the potential for heterodimer formation.
4. Add ammonium acetate (1.38 g, 18 mmol, 15 equiv.) to the reaction mixture and attach a reflux condenser to the reaction vessel.
5. Increase the temperature of the oil bath to 90 °C. Reflux the reaction mixture for 15 h.
6. Cool the reaction mixture to room temperature and isolate the precipitated solids by gravity filtration.
7. Dry load the crude product onto celite and purify the crude product by flash column chromatography, gradually increasing the polarity of the running solvent from 100% dichloromethane to 0.5% methanol in dichloromethane to isolate a blue-green solid in 25% yield.

rNOD-1

1. Add **5** (100 mg, 0.166 mmol, 1.0 equiv.) to a 100 mL round bottom flask equipped with a magnetic stir bar.
2. Add dichloromethane (10 mL) and triethylamine (0.333 mL, 2.48 mmol, 15 equiv.) to the reaction vessel to dissolve **5**. Cap the reaction vessel with a rubber septum and flush the reaction vessel with nitrogen.
3. Add boron trifluoride diethyl etherate (0.30 mL, 2.48 mmol, 15 equiv.) dropwise to the rapidly stirring reaction mixture using a syringe. Stir for an additional 15 h. NOTE: All materials that encounter this reagent should be quenched with bicarbonate solution before disposal.
4. Dilute the reaction mixture with dichloromethane and carefully add a saturated aqueous solution of sodium bicarbonate to quench the residual boron trifluoride. NOTE: Great care should be taken to avoid splashing upon quenching the excess boron trifluoride.
5. Pour the quenched reaction into a separatory funnel and allow the phases to separate. Isolate the organic layer, then wash with additional sodium bicarbonate solution (2 ×).
6. Isolate the organic layer, dry over sodium sulfate, and concentrate to a dark residue.
7. Dry load the crude product onto celite and purify by flash column chromatography, gradually increasing the polarity of the running solvent from 30% to 50% ethyl acetate in hexanes to afford the product as a red-brown solid (79% yield).

photoNOD-1

1. Add **rNOD-1** (72.7 mg, 0.130 mmol, 1.0 equiv.) to a 25 mL round bottom flask equipped with a magnetic stir bar.
2. Add tetrahydrofuran (8.7 mL) and dichloromethane (4.35 mL) to dissolve the solid residue.
3. Add acetic acid (4.35 mL) and sodium nitrite (44.8 mg, 0.653 mmol, 5.0 equiv.) and stir the reaction mixture at room temperature for 2 h, or until the reaction appears complete by TLC (80% dichloromethane in hexanes).
4. Dilute the reaction mixture with additional dichloromethane, then quench the acetic acid by slowly adding a saturated solution of sodium bicarbonate until gas evolution ceases. NOTE: Quenching the reaction too quickly may result in rapid bubbling, causing loss of product if the reaction vessel overflows. To avoid this, first transfer the reaction mixture to a larger flask, or place the reaction vessel in a secondary container before quenching.
5. Transfer the quenched reaction mixture to a separatory funnel to isolate the organic layer. Dry the organic layer over sodium sulfate and concentrate to isolate the crude product.
6. Purify the crude product by flash column chromatography using 80% dichloromethane in hexanes as the running solvent. This will yield the product as a green solid (48% yield). NOTE: Low yields are primarily due to the decomposition of photoNOD-1 to rNOD-1 on silica. However, rNOD-1 can be isolated from the column and resubjected to the reaction conditions if needed. photoNOD-1 can be stored as a dry solid at or below -20°C in the dark for several months without noticeable decomposition.

5. In vitro characterization of photoNOD-1 and rNOD-1

Upon completion of photoNORM synthesis, it is necessary to (i) assess the photophysical properties of both the photoNORM and its proposed turnover product, (ii) demonstrate that the compound generates the proposed turnover product upon irradiation with near-IR light with concomitant release of NO, and (iii) confirm that the photoNORM is stable in the absence of irradiation. To expedite our experiments and minimize the error involved in weighing small amounts of compound for each experiment, we recommend aliquoting compounds in advance, as described below.

Equipment

- 1—Microbalance
- 3—Dram vials per compound (1 mL capacity)
- 36—PCR tubes per compound (0.2 mL capacity)
- 1—Rack for storing PCR tubes

3—Micropipettes and tips (P1000, P200, and P10)

Reagents

Compound of interest (3 mg)

CHCl₃, or other organic solvent in which the compound is soluble

Procedure (makes three batches of 12 aliquots)

1. Label the dram vials (A, B, and C).
2. Place three dozen PCR tubes in the tube rack, labeling each set of a dozen tubes with (A, B, or C)
3. Zero the microbalance and acquire the tare weight of each dram vial.
4. Add a small amount of solid compound to each dram vial (1–2 mg each). Note the exact mass of solid added to vials A, B, and C.
5. Based on the molecular weight of the compound, calculate the volume of solvent required to generate a 5 mM solution.
6. Transfer the dram vials to an ice bath (to prevent solvent evaporation) and add the appropriate amount of CHCl₃ to each.
7. Cap each dram vial and shake vigorously or sonicate until the solids are fully dissolved. Return to the ice bath to prevent solvent evaporation.
8. Quickly pipet 20 µL of solution into each PCR tube. Be careful to match the dram vial label with the PCR tube labels, to obtain a dozen aliquots from each vial (A, B, and C).
9. Remove the solvent completely by storing the racks of PCR tubes in a dark vacuum dessicator. NOTE: If the organic solvent is chloroform, the aliquots should dry within 2–3 h under these conditions. However, removal of higher boiling solvents such as DMF and DMSO may take several days.
10. Reserve an aliquot each from batches A, B, and C, and determine the extinction coefficient of the compound. This process is described in Section 5.1.
11. Store the remaining dried aliquots in a foil-covered vessel at or below –20 °C.

When preparing for any experiments that require compound of interest, warm the required number of compound aliquots to room temperature. Redissolve in DMF or DMSO (20 µL of solvent should yield approximately 5 mM compound), then check the true concentration of the aliquot(s) using the following procedure:

1. Add 999 µL of chloroform to a quartz cuvette and baseline the spectrophotometer.
2. Add 1 µL of the compound stock solution. Pipet up and down to mix thoroughly.
3. Scan the sample in the spectrophotometer to identify the absorbance value at 681 nm (the λ_{max} of the dye).

4. Using the Beer-Lambert law, determine the true concentration of the stock solution based upon the extinction coefficient of the dye determined in Section 5.1. NOTE: Using the extinction coefficient to back-calculate the true concentration ensures that slight differences in compound concentration between batches (A, B, and C) will not affect the experimental outcomes. Moreover, the concentration of dye can be precisely reproduced for every experiment.
5. Adjust the solution to the desired concentration by diluting in additional solvent, or by reconstituting additional aliquots, and verifying the concentration by repeating steps 1–4.

5.1 Photophysical characterization of photoNOD-1 and rNOD-1

This experiment identifies each compound's wavelength of maximum absorption and the compound's extinction coefficient at that wavelength. This information is vital, as it allows the user to reliably check the concentration of a compound stock solution prior to all future experiments. Below, we describe our characterization of photoNOD-1 and rNOD-1 as an example. The properties of these two compounds are described in Table 1.

Equipment

- 1—UV–visible range spectrophotometer
- 1—Quartz cuvettes with corresponding cuvette caps
- 3—Micropipettes and tips (P10, P200, P1000)

Reagents

- 3 aliquots—photoNOD-1
- 1 aliquot—rNOD-1
- Organic solvents: CHCl_3 , DMF
- Aqueous buffer: 50% ethanol, 20mM HEPES, adjusted to pH 7.4

Procedure

1. Warm up the spectrophotometer at least 15 min in advance of the experiment.
2. Setup the experiment to collect absorbance spectra from 400 to 900 nm, at 0.5 nm increments.
3. Meanwhile, prepare a stock solution of dye.
4. Add a known volume of CHCl_3 or aqueous buffer to a quartz cuvette (0.5–1mL) and cap the cuvette to minimize solvent evaporation.
5. Blank the spectrophotometer using the baseline function.
6. Briefly remove the cuvette cap and titrate in the compound stock solution, taking note of the volume added so that the dye concentration can be calculated.

7. Mix thoroughly by pipetting up and down 10 times. Re-cap the cuvette.
8. Collect an absorbance spectrum.
9. Continue steps 5–7 until at least five scans have been collected in which the peak absorbance value falls between 0.1 and 1.0 a.u.
10. Repeat steps 2–8 for each batch of compound (A, B, and C) to obtain a total of 15 data points.
11. Identify the wavelength of maximum absorbance for the selected compound. Generate a linear regression for absorbance vs. concentration and use the Beer-Lambert Law to determine the extinction coefficient of the compound of interest.

5.2 Tissue phantom preparation

The purpose of a tissue phantom is to provide a medium through which incident irradiation light will be scattered. This phantom benefits in vitro PA studies in two ways. First, it mimics the light scattering that occurs in tissues. Second, it enables irradiation of a larger region of the sample, rather than irradiating just the portion of the sample that is aligned with the laser.

Equipment

- 1—Microwave (standard food-grade)
- 1—Glass beaker (250mL capacity)
- 1—Spatula
- 1 pair—Heat resistant gloves (optional)
- 1—Tissue phantom mold (custom) equipped with two pieces of hypodermic steel tubing.

NOTE: In the absence of a custom tissue phantom mold, 50 mL falcon tube equipped with the hypodermic steel tubing can serve as an adequate substitute.

Reagents

- Deionized water (78 mL)
- Agarose LE (Molecular Biology Grade, Gold Bio) (4 g)
- 2% fat milk (2 mL)

Procedure

1. Weigh out agarose LE (4 g) into a 250 mL beaker.
2. Add deionized water (78 mL) and 2% milk (2 mL) and stir vigorously with a spatula to suspend the solids.
3. Place the beaker into a standard microwave and heat for 45 s or until a viscous, translucent gel is formed, pausing briefly at 30 s to stir. NOTE: Microwave

strengths vary, so watch carefully and pause the heating as needed to prevent excessive bubbling or overflow.

4. Remove the beaker from the microwave and stir vigorously to ensure an even consistency. NOTE: Beaker will be very hot; use heat resistant gloves if needed.
5. Immediately pour the hot gel into the custom Teflon mold, completely submerging the steel tubing. Tap the mold vigorously on the benchtop to displace any bubbles in the gel.
6. Cool the gel in the Teflon mold for at least 2 h at 4 °C.
7. Carefully remove the steel tubes, then turn the Teflon mold upside-down and gently tap on the benchtop to remove the solidified phantom (see Fig. 1). NOTE: Phantoms can be used immediately, or stored submerged in deionized water for up to 5 days at 4 °C. The phantom may be prepared up to 5 days in advance, but at the minimum the phantom should be poured at least 2 h prior to the start of this experiment.

Alternatively, to make a phantom using a 50 mL falcon tube, follow the following procedure (Scheme 3):

1. Drill two parallel 0.08 in. channels through the diameter of the falcon tube.
2. Thread the channels with hypodermic steel tubing (or FEP tubing).
3. Pour the hot gel into the mold, completely submerging the tubing.
4. Tap the mold vigorously to remove bubbles.
5. Cool the gel for at least 4 h at 4 °C.
6. Carefully remove the tubing, then invert the falcon tube and gently squeeze the sides to remove the tissue phantom from the tube.
7. Cut the phantom into a rectangular prism using a razor blade. The final phantom should be a rectangular prism with 1 cm of phantom material on either side of the channels that will contain the FEP sample tubes.

5.3 Optical and photoacoustic validation of rNOD-1 release upon irradiation

In order to monitor the release of NO from a photoNORM using an imaging modality, it is necessary to detect a spectral change during release.

Here, we monitor the loss of photoNOD-1 and the formation of rNOD-1 by both absorbance and PA imaging.

Equipment

1—Photoacoustic tomographer (Nexus 128, Endra Life Sciences)

1—UV–visible spectrophotometer

1—Tissue-mimicking phantom (prepared as in Section 5.2)

2—FEP tubes: FEP tubing (0.08 in. diameter, 5cm length)

4—FEP caps: FEP tubing (0.12 in. diameter, 0.25cm length)

2—Quartz cuvettes (0.5 mL capacity)

Reagents

Organic solvents: CHCl_3 , DMF

photoNOD-1 (5 mM in DMF)

milli-Q water

Procedure

1. Warm up the photoacoustic tomographer at least 30 min in advance of the experiment.
2. Warm up the spectrophotometer at least 15 min in advance of the experiment.
3. Prepare a 5 mM stock solution of photoNOD-1 in DMF. Verify the concentration.
4. Using the DMF stock, prepare a 10 μM solution of photoNOD-1 in CHCl_3 and acquire an initial absorbance spectrum (400–900 nm).
5. Load the photoNOD-1 solution into the tissue mimicking phantom:
 - a. Using a P200 micropipette, carefully transfer 200 μL of the photoNOD-1 solution into an FEP tube.
 - b. Seal the tube on end by folding the edge of the tube upon itself and securing the crease with an FEP cap.
 - c. Thread the uncapped edge of the FEP tube through the phantom.
 - d. Seal the uncapped edge as described in step 5b.
 - e. Repeat steps 5a–5d with the second FEP tube so that the phantom is loaded with two identical solutions of dye (see Fig. 1).
6. Place the phantom into the bowl of the photoacoustic tomographer. Add enough deionized (Milli-Q) water to the bowl to just cover the phantom. Optional: Place a small weight on top of the phantom to prevent it from floating in the bowl.
7. Acquire PA measurements using the schedule shown in Table 2.
 - a. Recall that in chloroform, photoNOD-1 can be detected at $\lambda_{\text{blue}} = 680$ nm and rNOD-1 can be detected at $\lambda_{\text{red}} = 730$ nm.
 - b. Recall that irradiating photoNOD-1 at λ_{blue} drives the release of NO and the formation of rNOD-1.
8. After the 40 min timepoint is complete, remove the samples from the tissue phantom:

- a. Remove one FEP cap from each FEP tube.
 - b. Gently unfold the exposed crease in the FEP tube and carefully slide the tube from the phantom. Take care not to damage the phantom in the process. NOTE: The creased end of the FEP tube can also be cut off with a pair of sharp scissors or a razor blade, if more convenient.
9. Combine the contents of both FEP tubes in an empty quartz cuvette and acquire a final absorbance spectrum (400–900 nm).
10. Approximate the quantification of NO release upon irradiation by matching the absorbance spectrum from step 10 with a linear combination of the spectra for photoNOD-1 and rNOD-1 (see Fig. 2).
11. Repeat steps 5–10 twice more in order to collect three datapoints for each timepoint.
12. Analyze the PA data:
 - a. Reconstruct the PA images for each timepoint.
 - b. Quantify the average PA signal of both FEP tubes at each wavelength for each timepoint.
 - c. Plot the relative PA signal (PA at λ_{red} divided by PA at λ_{blue}) as a function of irradiation time (see Fig. 2).

5.4 Validation of NO release upon irradiation of photoNOD-1 by EPR

For a given photoNORM, it is critical to confirm that NO is in fact the species released upon irradiation (vs. nitrosonium [NO^+] or nitroxyl [HNO]). One of the most effective techniques for this determination is EPR spectroscopy, which enables the detection of paramagnetic centers. Here we describe the trapping and EPR detection of NO upon photorelease from photoNOD-1.

Equipment

- 1—Photoacoustic tomographer (Nexus 128, Endra Life Sciences)
- 1—X-band Continuous Wave EPR spectrometer
- 1—Schlenk line equipped with nitrogen gas and high vacuum capabilities
- 21—Hematocrit capillary tubes
- 1—Capillary tube sealing tray with wax plate
- 1—Quartz EPR tube
- 12—Septa capped dram vials (4 mL capacity)
- 1—Microsyringe (50 μL)

Reagents

photoNOD-1 (5 mM in DMF)

MAHMA-NONOate (a commercially available NO donor that releases 2 equiv. NO)

Potassium hydroxide (10 mM in water)

FeSO₄·7H₂O

N-dithiocarbamoyl-*N*-methyl-D-glucamine (MGD)

Deionized water

DMF

Procedure

1. Warm up the photoacoustic tomographer for at least 30 min prior to starting the experiment. This ensures adequate heating of the water bath and optical system.
2. Degas septum-capped vials of water, DMF, and 10 mM KOH solution by bubbling nitrogen vigorously through each solution for at least 30 min at room temperature. NOTE: Great care must be taken to eliminate all sources of oxygen from this experiment until the irradiation of the samples has been completed. Samples should also be protected from light throughout this experiment.
3. Prepare a 5 mM stock solution of photoNOD-1 in degassed DMF.
4. Prepare solutions of iron sulfate heptahydrate (8 mM) and MGD (40 mM):
 - a. Transfer solid iron sulfate heptahydrate or solid MGD to a septum-capped dram vial.
 - b. Evacuate the vial under high vacuum.
 - c. Flush the vial with nitrogen gas to remove any remaining oxygen.
 - d. Add degassed water through the septum
5. Evacuate three 4mL septum capped dram vials under high vacuum and flush with nitrogen.
6. In the indicated order, add following volumes of degassed water, degassed DMF, aqueous iron sulfate heptahydrate, aqueous MGD solution, and photoNOD-1 solution to each of three dram vials. This yields a 1:1 solution of water and DMF containing 0.8 mM Fe(II), 4.0 mM MGD, and 0.2 mM photoNOD-1. NOTE: The iron sulfate heptahydrate and MGD generate the NO spin-trapping complex, Fe-(MGD)₂, in situ.
 - a. Water, 120 µL
 - b. DMF, 184 µL
 - c. 8 mM Fe(II), 40 µL

- d. 40 mM MGD, 40 μ L
 - e. 5 mM photoNOD-1, 16 μ L
- 7. Irradiate each vial containing photoNOD-1 and Fe-(MGD)₂ in the photoacoustic tomographer at λ_{blue} (680 nm) for 0, 5, or 40 min using step-and-shoot mode. NOTE: We use the photoacoustic tomographer as the source of excitation light. In principle, alternative sources of continuous 680 nm excitation light may be used as a substitute.
- 8. After the irradiations are complete, open the vial and place a hematocrit capillary tube inside. Allow the tube to fill by capillary action.
- 9. Seal the capillary tube on each side with the wax tube sealant.
- 10. Place the capillary tube into the quartz EPR tube to acquire the EPR spectra.
- 11. Acquire the EPR spectra at room temperature on the EPR spectrometer using the following parameters:
 - a. Modulation frequency: 100 kHz
 - b. Modulation amplitude: 2.5 G
 - c. Gain: 4000
 - d. Sweep width: 100 G
 - e. Scan time: 0.5 min per scan, six scans
 - f. Microwave power: 12 dB
 - g. Microwave frequency: 9.30 GHz
- 12. Generate a calibration curve to quantify NO release using MAHMA-NONOate:
 - a. Prepare a range of MAHMA-NONOate stock solutions (0, 2, 4, and 8mM) in degassed 10 mM KOH.
 - b. Repeat steps 5–12, substituting the various MAHMA-NONOate solutions in the place of photoNOD-1, to generate 0, 50, 100, and 200 μ M solutions of MAHMA-NONOate in the presence of Fe-(MGD)₂.
 - c. Incubate the samples in the dark at room temperature for an hour (>20 half-lives, $t_{1/2} = 3$ min at room temperature) to ensure complete NO release.
 - d. Acquire the EPR spectrum of each sample as described in step 11.
 - e. Plot the EPR intensity (peak height) against the concentration of NO released (see Fig. 3).
- 13. Determine the concentration of NO released by the irradiation of photoNOD-1 using the calibration curve generated in step 12.

5.5 Assessing stability of photoNOD-1

To ensure that NO will not be released from a photoNORM in the absence of irradiation, it is necessary to verify the stability of the photoNORM under conditions that could encourage transnitrosylation (for instance, to glutathione, forming S-nitrosoglutathione), denitrosylation, and denitrosation. In this experiment, we treat 5 μ M photoNOD-1 with 1 mM reduced glutathione, 20 μ M metal ions (including copper (I) and (II), iron (II) and (III)), rat liver microsomes with high CYP450 activity, or 20% human plasma in aqueous buffers at 37 °C and show that photoNOD-1 is stable to these conditions. This experimental procedure can be modified to screen for a variety of other analytes.

Equipment

- 1—UV–visible spectrophotometer
- 21—1.6 mL Eppendorf tubes
- 1—floating foam tube rack
- 1—Nitrogen tank or Schlenk line equipped with nitrogen gas
- 1—Water bath, preheated to 37 °C
- 4—Quartz cuvettes, 1.5 mL capacity
- 4—Small rubber septa to cap the quartz cuvettes
- 3—Micropipettes and tips (P10, P200, and P1000)
- 2—Microsyringes (10, 50 μ L)

Reagents

- Assay buffer (50% ethanol, 20mM HEPES, pH 7.4)
- Phosphate buffered saline (PBS)
- photoNOD-1 (50 μ L, 5 mM in DMF)
- Rat liver microsomes (pooled from male Sprague-Dawley rats, Sigma Aldrich) (50 μ L)
- NADPH (50 μ L, 2 mM in water)
- Human plasma
- Reduced glutathione (50 μ L, 100 mM in water)
- CuCl₂ (50 μ L, 2 mM in water)
- FeCl₃ (50 μ L, 2 mM in water)
- [(CH₃CN)₄Cu]PF₆ (50 μ L, 2 mM in degassed water)

FeSO₄ 7H₂O (50 µL, 2 mM in degassed water)

Procedure

1. Warm up the spectrophotometer at least 15 min in advance of the experiment.
2. Degas a portion of assay buffer by gently bubbling nitrogen through the solution for 30 min.
3. Degas water by gently bubbling nitrogen through the solution for 30 min.
4. Prepare stock solutions of the relevant compounds at the concentrations described above in the reagents section.
5. Assess the stability of photoNOD-1 to CYP450 enzymes:
 - a. To each of three quartz cuvettes, add 986.5 µL of PBS.
 - b. Collect a baseline spectrum on the UV–visible spectrophotometer.
 - c. Add 10 µL of rat liver microsomes followed by 1 µL of 5 mM photoNOD-1 stock solution to each cuvette, carefully pipetting up and down to mix.
 - d. Cap the cuvettes with rubber septa and incubate the cuvettes in the water bath at 37 °C for at least 10 min. NOTE: While photoNOD-1 is generally poorly soluble in aqueous solutions without organic cosolvents, the rat liver microsomes help to solubilize photoNOD-1 in this experiment during this preincubation period.
 - e. Add 2.5 µL of 2 mM NADPH to each cuvette, mix the solution, and immediately acquire initial absorbance spectra (400–900 nm).
 - f. Cap the cuvettes with rubber septa and incubate the cuvettes at 37 °C for 1h in a water bath.
 - g. Mix the solutions and acquire final absorbance spectra.
6. Assess the stability of photoNOD-1 to human plasma:
 - a. To each of four quartz cuvettes, add the following reagents:
 - i. 200 µL of human plasma
 - ii. 799 µL of PBS
 - b. Carefully pipet up and down to mix, then cap the cuvettes with rubber septa and incubate the cuvettes at 37 °C for at least 10 min in a water bath.
 - c. Using one cuvette, collect a baseline spectrum on the UV–visible spectrophotometer.
 - d. Add 1 µL of 5 mM photoNOD-1 stock solution to each of the remaining three cuvettes, carefully pipetting up and down to mix.
 - e. Acquire initial absorbance spectra.

- f. Cap the cuvettes with rubber septa and incubate the cuvettes in the water bath at 37 °C for 1 h.
 - g. Acquire final absorbance spectra (400–900 nm).
7. Assess the stability of photoNOD-1 to glutathione and the reactive metal species. NOTE: For experiments involving Cu(I) or Fe(II), avoid introducing air into the cuvettes by evacuating cuvettes under high vacuum, then flushing the cuvettes with nitrogen. Degassed solutions should be injected through the septum with a micro-syringe. This will minimize oxidation to Cu(II) and Fe(III), respectively.
 - a. To each of three quartz cuvettes, add 989 µL of assay buffer. Use one of the three cuvettes to acquire a baseline spectrum.
 - b. Add 1 µL of 5 mM photoNOD-1 stock solution to each of the three cuvettes.
 - c. Cap the cuvettes with rubber septa and incubate the cuvettes in the water bath at 37 °C for at least 10 min.
 - d. Add 10 µL of stock solution corresponding to the species of interest.
 - e. Acquire initial absorbance spectra.
 - f. Cap the cuvettes with rubber septa and incubate the cuvettes in the water bath at 37 °C for 1 h.
 - g. Acquire final absorbance spectra.
8. To calculate the ratiometric fold turn-on for each experiment (Fig. 4), use the following equation.

$$\text{Ratiometric foldturn - on} = (\text{Abs}_{730\text{nm}}/\text{Abs}_{680\text{nm}})_{\text{final}}/(\text{Abs}_{730\text{nm}}/\text{Abs}_{680\text{nm}})_{\text{initial}}$$

5.6 Validation that photoNOD-1 and rNOD-1 are non-cytotoxic

Because a photoNORM is designed to be used as an activatable NO source in biological experiments, it is important that we confirm that neither the photoNORM nor its product after irradiation is inherently cytotoxic to cellular systems. Confirmation of this fact suggests that any toxicity observed in future experiments can be attributed to the effects of NO, rather than to the donor or dye.

Equipment

96-well plate, clear-bottomed, coated for cell culture

1 cell culture hood

1 incubator suitable for mammalian cell culture, with 5% CO₂ atmosphere

1 epifluorescence microscope equipped with a DAPI filter cube and an RFP filter cube

Reagents

4T1 cells

RPMI 1640 Medium supplemented with 10% FBS and 1% penicillin/streptomycin

photoNOD-1 and rNOD-1 (0, 2.7, 5.4, 13.5, and 27 mM in DMSO)

Propidium iodide (0.5 mg/mL)

Hoechst 33342 stain (0.1 mg/mL)

Procedure

1. Warm the modified DMEM media in a 37 °C water bath.
2. Seed 30 wells with 160 μ L (8.8×10^4 cells/mL of media) of 4T1 cells.
3. Incubate the plate for 12 h at 37 °C, or until the wells approach 50–60% confluence.
4. Meanwhile, prepare the stock solutions of photoNOD-1 and rNOD-1 in DMSO as described in the Reagents list.
5. Dilute each stock solution 1:9 in modified DMEM to generate solutions of 270 μ M, 540 μ M, 1.35 mM, and 2.70 mM in media.
6. Add 20 μ L of the appropriate dye solution to each well such that the cells will be incubated with 0, 30, 60, 150, or 300 μ M of dye (see Fig. 5).
7. Incubate the plate, in the dark, for 24 h.
8. Add 10 μ L of propidium iodide and 10 μ L of Hoechst 33342 to each well.
9. Incubate the plate in the dark for an additional 15 min.
10. Image each of the wells using an epifluorescence microscope:
 - a. Count Hoechst-stained cells using a DAPI filter cube
 - b. Count Propidium iodide-stained cells using the RFP filter cube
11. Calculate percent viability by dividing the number of cells unstained by propidium iodide by the total number of Hoechst-stained cells.

6. Characterization of photoNOD-1 in vivo

Once the release of NO from a given photoNORM has been demonstrated in vitro, validation of the release must also be confirmed in vivo. In this section, we validate that irradiation-mediated release of NO is operative in vivo. We first use authentic photoNOD-1 and rNOD-1 to identify the wavelengths ($\lambda_{\text{blue}} = 700\text{nm}$, $\lambda_{\text{red}} = 830\text{nm}$) that will maximize the PA ratiometric signal change upon release of NO from photoNOD-1 in vivo. We then validate that photoNOD-1 turns over in shallow tissues upon exposure to near-IR light. Finally, we demonstrate that photoNOD-1 can be used as an effective tool to modulate tumor progression in an irradiation-dependent manner, and we show that repression of tumor

growth is likely due to NO-induced apoptosis. These protocols may be adapted for other near-IR activatable photoNORMs to assess their in vivo release efficacy.

The following experiments require training in the ethics and practice of animal handling and may not be conducted without institutional approval. All in vivo experiments were performed with the approval of the Institutional Animal Care and Use Committee of the University of Illinois at Urbana–Champaign, following the principles outlined by the American Physiological Society on research animal use. Female BALB/c mice (5–7 weeks old) were acquired from The Jackson Laboratory.

6.1 Identification of optimal wavelengths for monitoring photoNOD-1 in vivo

Because photoNOD-1 and rNOD-1 may solubilize differently in tissue than in our in vitro solvent systems, we conduct this experiment to identify the wavelengths at which the dyes can be most easily detected in vivo.

Equipment

- 1—Photoacoustic tomographer (Nexus 128, Endra Life Sciences)
- 1—Vaporizer equipped with isoflurane and oxygen (for anesthesia)
- 2—sterile Eppendorf tubes
- 2—sterile insulin syringes
- 1—electric shaver, for hair removal
- 1—bottle depilatory cream, for hair removal

Animals

- 1—Female BALB/c mouse, 5–7 weeks old

Reagents

- photo NOD-1
- rNOD-1
- Sterile saline containing 2% DMSO by volume

Procedure

1. Warm up the photoacoustic tomographer for at least 30min prior to imaging.
2. Anesthetize the mouse in an isoflurane/oxygen chamber, then maintain an anesthetized state using a stream of isoflurane through a nose cone for the remainder of the experiment.
3. Shave both flanks of the mouse. Remove any residual hair with depilatory cream.

4. After hair removal is complete, gently wash away the residue from the skin with warm damp gauze.
5. Formulate 30 μ M photoNOD-1 in the saline/DMSO solution in a sterile Eppendorf tube.
6. Using an insulin syringe, inject 25 μ L of the 30 μ M photoNOD-1 solution subcutaneously into one flank.
7. Place the mouse in the tomographer bowl.
 - a. Acquire PA spectra for the flank (continuous mode, 6 s rotation time), scanning from 850 to 680 nm at 10 nm intervals. NOTE: It is important to scan from red wavelengths to blue wavelengths, as scanning near λ_{blue} first will cause NO release and rNOD-1 generation that will be detected in the spectrum.
8. Repeat steps 4–7 for rNOD-1, injecting the dye into the opposite flank.
9. Process the data by generating three-dimensional reconstructed images, selecting equal-volume regions of interest (ROIs) from each image, and quantifying the mean PA signal in each ROI, using the Horos DICOM viewer or equivalent software.
10. Plot the PA signal versus excitation wavelength for each compound to determine the optimal wavelengths of interest (see Fig. 6).

6.2 Confirmation of light-dependent conversion of photoNOD-1 to rNOD-1 in vivo

The goal of this experiment is to verify that near-IR irradiation can drive the conversion of photoNOD-1 to rNOD-1 (and thus the release of NO) in live tissues that contain diverse species which may absorb or scatter the incident light. In our hands, irradiation of photoNOD-1 for 5min yields a 1.37-fold ratiometric PA turn-on relative to a theoretical maximum of 2.09-fold, suggesting that NO release can indeed be achieved in live animals.

Equipment

1—Photoacoustic tomographer (Nexus 128, Endra Life Sciences) Vaporizer equipped with isoflurane and oxygen (for anesthesia) Insulin syringes

Animals

3—Female BALB/c mice, 5–7 weeks old

Reagents

photo NOD-1

Sterile saline with 2% DMSO

Ethanol

Procedure

1. Follow steps 1–6 from Section 6.1 for all three animals, injecting photoNOD-1 into both flanks.
2. Acquire initial PA images at λ_{red} (830 nm) and λ_{blue} (700 nm) (continuous mode, 6 s rotation time, 0.2 min irradiation at λ_{blue}).
3. Irradiate one flank at λ_{blue} (step-and-shoot mode, 120 angles, 39 pulses per angle, 4.8 min). Do not irradiate the second flank.
4. Acquire final PA images at λ_{red} and λ_{blue} (continuous mode, 6 s rotation time, 0.2 min irradiation at λ_{blue}).
5. Process the data by generating three-dimensional reconstructed images, selecting equal-volume regions of interest (ROIs) from each image, and quantifying the mean PA signal in each ROI at both wavelengths, at the initial and final timepoints, using the Horos DICOM viewer or equivalent software.
6. Calculate the ratiometric fold turn-on of photoNOD-1 to rNOD-1 with and without irradiation (Fig. 7):

$$\text{Ratiometric foldturn-on} = (PA_{830\text{nm}}/PA_{700\text{nm}})_{\text{final}} / (PA_{830\text{nm}}/PA_{700\text{nm}})_{\text{initial}}$$

6.3 Application of photoNOD-1 to a murine tumor model

The goal of this experiment is to demonstrate that systemically administered photoNOD-1 can be locally activated by irradiation, yielding a significant difference in phenotype between activated and unactivated tissues. Because high concentrations of NO have been cited as toxic to cancer cells, and a variety of NO donors have been demonstrated to effectively inhibit in vivo tumor growth, we selected a tumor model to demonstrate this phenomenon. We implant two tumors of equal size into mice and treat the animals with photoNOD-1. We then irradiate only one tumor with near-IR light, and demonstrate that in the irradiated tumor, growth is severely repressed, whereas the tumor without activation grows unimpeded in comparison to tumors treated with vehicle.

Equipment

- 1—Photoacoustic tomographer (Nexus 128, Endra Life Sciences)
- 1—Vaporizer equipped with isoflurane and oxygen (for anesthesia)
- 24—Insulin syringes

Cells and animals

- 4T1 cells
- 6—Female BALB/c mice, 5–7 weeks old

Reagents

Matrigel

Serum-free RPMI 1640

photo NOD-1

Sterile saline

Trypsin

Procedure

1. Culture 4T1 cells using standard conditions.
2. Three hours before implantation, transfer the Matrigel to a wet ice bath to thaw. Keep cold until just prior to mixing with the cell suspension.
3. Trypsinize and count cells. Prepare a cell suspension in a 1:1 mixture of serum-free medium and Matrigel (5×10^6 cells per mL)
4. Implant 50 μ L of the 4T1 suspension (2.5×10^5 cells) into the subcutaneous space of each flank using an insulin syringe.
5. Five days after implantation, randomly assign half of the mice to the experimental group and half of the mice to the control group.
6. Weigh each mouse and measure its tumor size using calipers. Calculate the tumor volume using the formula $V = (w^2 \times l)/2$, where w is the tumor width and l is the tumor length.
7. Formulate photoNOD-1 in sterile saline containing 20% DMSO such that 150 μ L will deliver 1.2 mg/kg of photoNOD-1 to each mouse. Administer the solution retroorbitally to the experimental group.
8. Administer 150 μ L of sterile saline containing 20% DMSO retroorbitally to the control group.
9. Wait 4 h. Meanwhile, warm up the photoacoustic tomographer for at least 30 min before proceeding to step 9.
10. Treat and monitor the left tumor of each mouse:
 - a. Acquire initial PA images of the left tumor of each mouse at λ_{red} (830 nm) and λ_{blue} (700 nm) (continuous mode, 6 s rotation time, 0.2 min irradiation at λ_{blue}).
 - b. Irradiate the left tumor of each mouse at λ_{blue} (step-and-shoot mode, 120 angles, 39 pulses per angle, 4.8 min).
 - c. Acquire final PA images of the left tumor of each mouse at λ_{red} and λ_{blue} (continuous mode, 6 s rotation time, 0.2 min irradiation at λ_{blue}).
11. Monitor the right tumor of each mouse:

- a. Acquire initial PA images of the right tumor of each mouse at λ_{red} and λ_{blue} (continuous mode, 6 s rotation time, 0.2 min irradiation at λ_{blue}).
 - b. Wait 5 min.
 - c. Acquire final PA images of the right tumor of each mouse at λ_{red} and λ_{blue} (continuous mode, 6 s rotation time, 0.2 min irradiation at λ_{blue}).
12. Repeat steps 5–10 every 2 days, for a total of 7 days (four treatments).
 13. Plot tumor volume over time for each condition (see Fig. 8).

7. Concluding remarks

Herein we describe the synthesis and validation of phtooNOD-1, a near-IR and PA responsive NO donor that can be used for the spatiotemporal control of NO release in live animal systems. We believe this tool compound provides a unique strategy for the delivery of NO in deep tissues. More generally, we present a thorough and systematic approach for the validation of photoactivatable NO donors in living systems, and hope this approach proves useful for researchers interested in probing the many facets of NO biology.

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Scheme 1.

photoNOD-1 exhibits a strong PA signal upon excitation at λ_{blue} , with negligible PA signal upon excitation at λ_{red} . Continuous irradiation at λ_{blue} drives the release of NO, generating rNOD-1 as a byproduct. rNOD-1 exhibits a small PA signal upon excitation at λ_{blue} and a large PA signal at λ_{red} . Therefore, the release of NO can be monitored by the changes in PA signal at λ_{blue} and λ_{red} .

Scheme 2.
Synthetic route to rNOD-1 and photoNOD-1.

Scheme 3.

Process for making a tissue phantom without a custom mold. (A) Drill 0.08 in. diameter parallel holes through a 50 mL falcon tube, approximately 1 cm apart. Insert hypodermic steel tubing or FEP tubing. (B) Pour in prepared tissue mimicking phantom solution and allow to set at 4 °C. (C) Remove the tubing from the mold, then remove the tissue phantoms from the tubes. Cut the phantom into a rectangular prism, such that 1cm of phantom remains above and below the channels in the phantom.

Fig. 1. (Left) Tissue phantom after cooling in the mold. (Center) Phantom after removing hypodermic tubing and separating from the mold. (Right) Representative images phantoms loaded with FEP tubes containing a sample.

Fig. 2.

(Left) Representative quantification of NO release after irradiation of photoNOD-1 in chloroform. A linear combination of the spectra of pure rNOD-1 (red) and pure photoNOD-1 (blue) yield a calculated spectrum (black) that matches the spectrum of the irradiated photoNOD-1 sample (purple). The concentration of rNOD can be approximated using the intensity of the red line with the extinction coefficient of rNOD. (Right) The conversion of photoNOD-1 to rNOD-1 is dependent on the irradiation time at 680 nm.

Fig. 3.

(Left) EPR spectra of spin-trapped NO released from MAHMA-NONOate. (Center) Calibration curve correlating EPR intensity to concentration of NO released, assuming each molecule of MAHMA-NONOate releases 2 equiv. of NO. (Right) EPR spectra of spin-trapped NO released from photoNOD-1 upon irradiation for 0, 5, or 40 min at 680 nm. *Reproduced with permission from ACS.*

Fig. 4. Ratiometric fold turn-on of photoNOD-1 upon incubation with various analytes, as determined from the absorbance spectra before and after stability tests. Data is plotted as the mean $\pm$ SD ($n=3$).

	A	B	C	D	E	F	G... L
1							
2							
3							
4							
5							
6...8							

Legend		
photoNOD-1	rNOD-1	Concentration
		0 μ M
		30 μ M
		60 μ M
		150 μ M
		300 μ M

Fig. 5.

Layout of 96-well plate. Highlighted wells are plated with 4T1 cells. Wells highlighted in blue are incubated with various concentrations of photoNOD-1; wells highlighted in orange are incubated with various concentrations of r NOD-1.

Fig. 6. PA spectra of photoNOD-1 and rNOD-1 in vivo. We select $\lambda_{\text{blue}} = 700$ nm and $\lambda_{\text{red}} = 830$ nm as the ideal wavelengths to track photoNOD-1 and rNOD-1, respectively.

Fig. 7. After 5min irradiation, subcutaneous photoNOD-1 demonstrates a significant PA ratiometric fold turn-on in vivo.

Fig. 8. Monitoring the effect of photoNOD-1 on tumor progression in vivo. (A) Schematic of experimental setup. (B) Representative image of a mouse positioned in the bowl of a photoacoustic tomographer. (C) Representative PA images at λ_{red} (830 nm) showing increase in rNOD-1 signal after 5 min of irradiation. (D) Tumor volumes over time, as measured by calipers on each day of treatment. (E) Tumor volumes on day 7 of treatment. All data presented as mean $\pm$ SD ($n = 3$).

Table 1

Summary of photophysical properties.

Compound	ϵ ($M^{-1}cm^{-1}$) ($CHCl_3$)	λ_{max} ($CHCl_3$) (nm)	λ_{max} (aqueous) (nm)
photoNOD-1	5.59×10^4	681	677
rNOD-1	6.34×10^4	733	746

Table 2

Experimental schedule for in vitro irradiations using the photoacoustic tomographer

Timepoint (min)	Scan mode	Excitation wavelengths (nm)	Rotation time (s) OR [angles/ pulses per angle]	Irradiation time at 680nm (min)	Cumulative irradiation time at 680 nm
0	C	730, 680	6	+0.2	0.2
	C	680	6	+0.3	0.5
0.5	C	730, 680	6	+0.2	0.7
	C	680	6	+0.3	1.0
1	C	730, 680	6	+0.2	1.2
	C	680	9	+1.8	3.0
3	C	730, 680	6	+0.2	3.2
	C	680	9	+1.8	5.0
5	C	730, 680	6	+0.2	5.2
	SS	680	[120/39]	+4.8	10.0
10	C	730, 680	6	+0.2	10.2
	SS	680	[120/39]	+4.8	15
15	C	730, 680	6	+0.2	15.2
	SS	680	[120/39]	+4.8	20
20	C	730, 680	6	+0.2	20.2
	SS	680	[120/39]	+4.8	25
25	C	730, 680	6	+0.2	25.2
	SS	680	[120/39]	+4.8	30
30	C	730, 680	6	+0.2	30.2
	SS	680	[120/39]	+4.8	35
35	C	730, 680	6	+0.2	35.2
	SS	680	[120/39]	+4.8	40
40	C	730, 680	6	+0.2	40.2

C, continuous mode; SS, Step-and-Shoot mode. Gray rows indicate short scans which are used to collect PA images of the samples contained in the tissue phantoms. Collect images at λ_{red} 730 nm first (corresponding to the detection of rNOD-1) followed by 680 nm (corresponding to the detection and irradiation of photoNOD-1). White rows indicate scans used to irradiate the samples for extended periods of time.