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NitroxylFluor: A Thiol-Based Fluorescent Probe for Live-Cell Imaging of Nitroxyl

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Supporting Information

ABSTRACT: Detection of nitroxyl (HNO), the transient one-electron reduced form of nitric oxide, is a significant challenge owing to its high reactivity with biological thiols (with rate constants as high as $10^9\ M^{-1}\ s^{-1}$). To address this, we report a new thiol-based HNO-responsive trigger that can compete against reactive thiols for HNO. This process forms a common *N*-hydroxysulfenamide intermediate that cyclizes to release a masked fluorophore leading to fluorescence enhancement. To ensure that the cyclization step is rapid, our design capitalizes on two established physical organic phenomena; the alpha-effect and the Thorpe-Ingold effect. Using this new trigger, we developed NitroxylFluor, a selective HNO-responsive fluorescent probe. Treatment of NitroxylFluor with an HNO donor results in a 16-fold turn-on. This probe also exhibits excellent selectivity over various reactive nitrogen, oxygen, and sulfur species and efficacy in the presence of thiols (e.g., glutathione in mM concentrations). Lastly, we successfully performed live cell imaging of HNO using NitroxylFluor.

Nitroxyl (HNO), the one-electron reduced derivative of nitric oxide (NO), has emerged as a potential therapeutic agent for the treatment of cardiovascular disorders due to its unique ability to increase cardiac output by decreasing venous resistance.[1] HNO is also recognized to protect against myocardial ischemia-reperfusion injury[2] and has gained attention as a possible anticancer agent.[3] The cellular production of HNO is proposed to involve either non-enzymatic or enzymatic mechanisms. For example, HNO has been generated via reduction of NO by thiols,[4] or enzymatic reduction of L-arginine by nitric oxide synthase (NOS) under tetrahydrobiopterin-free conditions.[4] It is also speculated that hydroxylamine and *N*-hydroxy-L-arginine may be oxidized to produce HNO in reactions mediated by heme proteins, such as peroxidases.[5,6]

The detection of HNO in a biological context is an immense challenge due to its highly reactive chemical nature. For instance, HNO can spontaneously dimerize and dehydrate in a sequence that affords N_2O ($k = 8 \times 10^6\ M^{-1}\ s^{-1}$).[7] HNO can also act as a potent electrophile in a reaction with nucleophilic biological thiols to form *N*-hydroxysulfenamide intermediates (Figure 1a). The rate constants for this reaction range from $2 \times 10^6\ M^{-1}\ s^{-1}$, for low molecular weight thiols (e.g., glutathione (GSH))[8] to greater than $10^9\ M^{-1}\ s^{-1}$ for proteins exhibiting a low thiol pK_a (e.g., glyceraldehyde 3-phosphate dehydrogenase).[9] Once formed, the *N*-hydroxysulfenamide intermediate can rearrange to a sulfinamide product or react with another thiol to yield a disulfide (Figure 1a). Thus, any detection strategy must proceed with comparable kinetics to intercept HNO before it is metabolized.

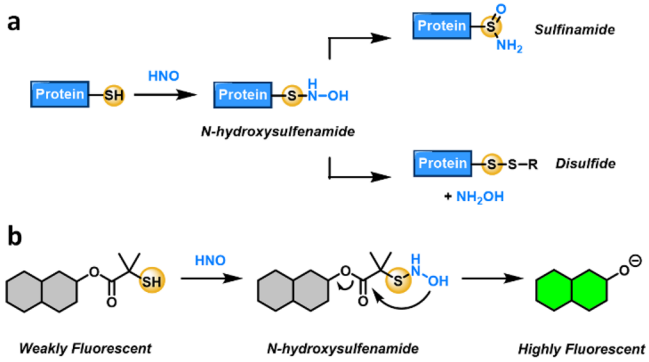

Figure 1. (a) HNO reacts with protein thiols to form an *N*-hydroxysulfenamide intermediate, which rearranges to a sulfinamide or is attacked by another thiol to form a disulfide. (b) HNO reacts with our new thiol-based trigger in the same fashion to afford a common *N*-hydroxysulfenamide intermediate, which cyclizes to release the masked dye.

A promising approach to detect analytes in general is to utilize reaction-based probes that couple fluorescent enhancement with analyte-associated reactivity.[10] In the context of HNO sensing, a variety of reaction-based HNO probes for fluorescence imaging have been developed.[11,12] Such probes are based on either redox coordination[11,13−18] or phosphine-mediated Staudinger chemistry.[19−21] However, metal-based HNO fluorescent probes are limited by slow reaction kinetics relative to thiols and exhibit small dynamic range (typically less than 5-fold fluorescent turn-on).[11,14,16,18] Phosphine-based probes suffer from reduced sensitivity because for every equivalent of HNO, two probes are required for sensing because one equivalent is consumed to generate an unproductive phosphine oxide byproduct.[20] Additionally, some phosphine-based probes also exhibit cross-reactivity with NO donors.[12] Herein, we report the rational design of a new bioinspired thiol-based trigger for the development of a series of HNO-responsive fluorescent probes (Figure 1b). We

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demonstrate excellent selectivity against a panel of reactive oxygen species, reactive sulfur species and reactive nitrogen species, as well as efficacy for HNO detection in competition experiments in the presence of biological thiols. In this study, we employ our best probe, NitroxylFluor, to successfully image HNO in living MDA-MB-231 human breast adenocarcinoma cells.

We propose that for any HNO-responsive trigger to be competent in a biological setting, it must exhibit sufficient reactivity with HNO to outcompete abundant biological thiols present in the cellular milieu. Our initial design featured a 2-mercaptoacetate trigger that can be used to cap the hydroxyl group on various dye platforms to quench its fluorescence (Figure 2a). In the absence of HNO, we anticipated that the

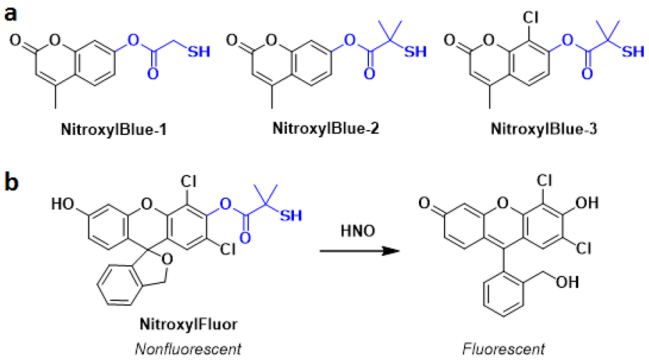


Figure 2. Chemical structures of (a) the NitroxylBlue series and (b) NitroxylFluor and the fluorescent turned over product.

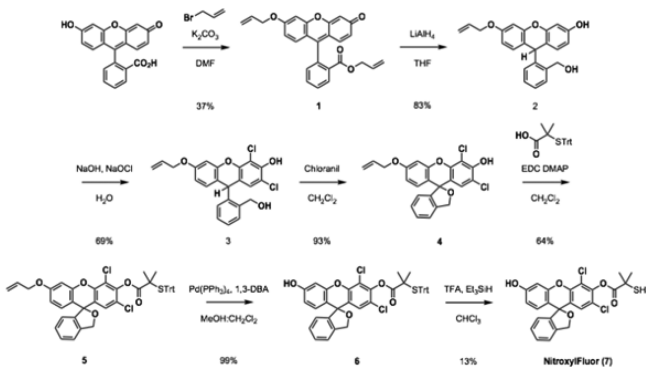
cyclization to release the dye would be unlikely since the product would be a strained α-thiolactone. However, we hypothesized that reaction with HNO would yield a highly reactive nucleophile (*N*-hydroxysulfenamide intermediate) that could readily cyclize due to the lone-pair electrons on the nitrogen atom, which enhances the nucleophilicity of the adjacent −OH group (alpha-effect).[22,23]

With this design in mind, we developed NitroxylBlue-1 (NB-1), a coumarin-based HNO probe featuring a 2-mercaptoacetate trigger (Figure 2a). The maximum absorbance and emission of NB-1 were centered at 350 and 448 nm, respectively. The probe itself was weakly fluorescent indicating efficient fluorescent quenching. Ten minutes after treatment with Angeli's salt, an HNO donor, we observed insignificant fluorescence enhancement over the background (Figure S1). We speculate that the lack of response was due to cyclization kinetics that were too slow. Additionally, we observed that the ester moiety was prone to hydrolysis (~2-fold turn-on after 10 min at 37 °C), which can lead to false positives (Figure S1). To address these shortcomings, we installed an α-geminal dimethyl group to afford NitroxylBlue-2 (NB-2) (Figure 2a). This modification was made to increase stability by shielding the ester from hydrolysis with greater steric bulk. We also aimed to leverage the Thorpe-Ingold effect, which describes the acceleration of cyclization from the substitution of hydrogen atoms for alkyl groups on the carbons tethering two reaction centers.[24] Although incubating NB-2 with Angeli's salt did result in a more significant 3.5-fold turn-on response, we were surprised to find that NB-2 was not stable in aqueous media as the background enhancement at 37 °C was 1.6-fold after 10 min (Figure S1). To determine whether this was due to hydrolysis or α-thiolactonization, we developed a control reagent by blocking the reactivity of the thiol via methylation of NB-2. No hydrolysis was observed after incubation in aqueous media, providing compelling evidence that the release of the coumarin was indeed due to α-thiolactonization (Figure S2). To address this instability issue we sought to further stabilize the ester moiety by exploiting X···C═O $n\rightarrow\pi^*$ interactions (where X = halogen) by installing an ortho-chloro substituent to give rise to NitroxyBlue-3 (NB-3) (Figure 2a).[25] We reasoned this modification would be sufficient to prevent intramolecular attack by the thiol, yet would allow for cyclization of the more reactive *N*-hydroxysulfenamide α-nucleophile. Indeed, when NB-3 was treated with a vehicle control, we did not observe any fluorescence enhancement, indicating the ortho-chloro substituent was sufficient to stabilize the ester from α-thiolactonization (Figure S1). On the other hand, the addition of Angeli's salt resulted in a 3.6-fold turn-on response (Figure S1).

Having established a new thiol-based HNO-responsive trigger on a commercially available coumarin scaffold, we sought to develop a custom HNO probe with an absorbance and emission profile in the visible spectrum. Visible wavelength probes are more desirable than their UV counterparts for biological studies because visible light is less phototoxic. This led to the development of our final probe in this series, NitroxylFluor, which is based on a chlorinated hydroxymethyl fluorescein platform. Synthesis of NitroxylFluor was achieved by diallylation of fluorescein with allyl bromide to afford **1** in 37% yield. Lithium aluminum hydride reduction of the allyl ester also resulted in reduction of the xanthene moiety to give the hydroxymethyl intermediate **2** in 83% yield. Disruption of the xanthene π-system facilitated regioselective ortho,ortho-dichlorination of the resulting phenol moiety under basic conditions using sodium hypochlorite to yield **3**. Of note, we did not exhaustively try to obtain the monochloro product since dichlorination would likely lead to greater stabilization against α-lactonization. The chromophore was reestablished via chloranil oxidation to afford **4**, which was subsequently capped using a tritylated 2-mercaptoisobutyric acid building block under Steglich coupling conditions to give **5** in 60% yield over 2-steps. The *O*-allyl functionality was removed under Tsuji-Trost deallylation conditions to afford **6** in near quantitative yields. Finally, the trityl protecting group was removed under acidic conditions to give NitroxylFluor in 13% yield (Scheme 1).

With NitroxylFluor in hand, we began to evaluate its response to HNO *in vitro*. NitroxylFluor displays an absorbance maximum centered at 505 nm ($\varepsilon = 1.3 \times 10^3$ M^{-1} cm^{-1}) and an

Scheme 1. Synthesis of NitroxylFluor



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emission maximum at 525 nm. Although the fluorescence was initially attenuated, reaction with HNO afforded a 16-fold fluorescent turn-on response in a matter of seconds (Figure 3a,b). Given the rapid reaction of HNO with thiols, we

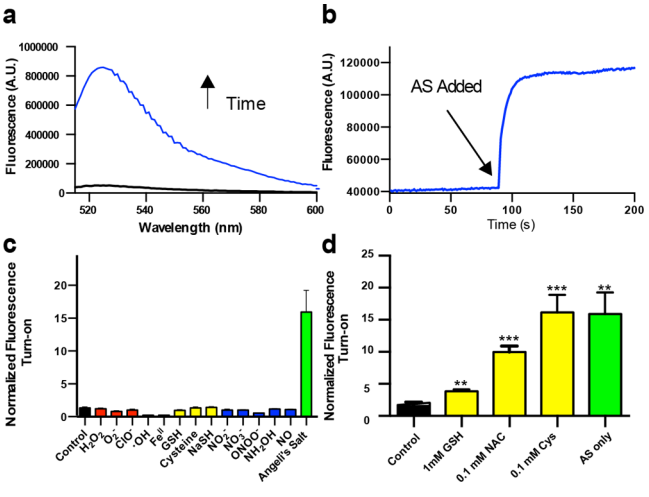

Figure 3. (a) Fluorescence spectra of NitroxylFluor (2 μM) upon addition of AS (100 μM). (b) Fluorescence kinetic trace of NitroxylFluor upon addition of 1 mM Angeli's salt, arrow indicates time of addition. (c) Response of NitroxylFluor to various reactive oxygen, sulfur, and nitrogen species at concentrations of 100 μM (GSH was tested at 1 mM). Measurements were taken 15 min into treatment. (d) Competition assays against various thiols. Control indicates no addition of Angeli's salt. Statistics are compared to the control. *, $p < 0.05$; ***, $p < 0.001$; $p < 0.0001$. ($n = 3$).

speculate the rate-limiting step is not formation of the N-hydroxysulfenamide intermediate, but rather cyclization. Having established excellent responsiveness to HNO, we turned our attention to determining the selectivity profile of the trigger. In particular, NitroxylFluor was treated with various oxidants, including H_2O_2 because it is known that thiols can be oxidized to sulfenic acids (RSOH), sulfinic acids (RSO_2H), and sulfonic acids (RSO_3H), which can potentially cyclize to release the dye.[26] However, we did not observe cross-reactivity with any of the oxidants tested (Figure 3c). Likewise, when NitroxylFluor was incubated with various reactive sulfur species (e.g., H_2S) and reactivate nitrogen species (e.g., NO) there was no change in fluorescence after a 15 min incubation at 37 °C (Figure 3c). Taken together, this demonstrates high selectivity of the trigger for HNO.

We next evaluated whether NitroxylFluor possessed requisite reactivity to compete against biologically relevant thiols for HNO. In these experiments, we treated 10 μM solutions of NitroxylFluor and various thiols such as L-cysteine (100 μM), *N*-acetyl-L-cysteine (100 μM), and GSH (1 mM) with Angeli's salt (200 μM) (Figure 3d). In the case of L-cysteine, the fluorescence response was not attenuated (16.2-fold increase) indicating NitroxlFluor was the more reactive species. Similarly, competition with *N*-acetyl-L-cysteine resulted in a 9.8-fold fluorescence enhancement. GSH, on the other hand, is highly abundant in cells (millimolar concentrations) and reacts rapidly with HNO ($k = 2 \times 10^6\ M^{-1}\ s^{-1}$). Thus, interference from GSH presents a significant challenge for any HNO detection strategy. Even in the presence of 1 mM GSH, treatment of NitroxylFluor with Angeli's salt still gave rise to a 3.9-fold fluorescent turn-on response. To determine the origin of why NitroxylFluor can function in the presence of a large excess GSH, we synthesized a control compound where we replaced the dye with a methoxy nucelofuge (Scheme S5). We did not anticipate that this will significantly alter the pK_a of the thiol. However, the poor leaving group ability of the methoxy suppresses α-thiolactonization, which allowed us to measure a pK_a value of 8.01 by UV/vis spectroscopy (Figure S3). In comparison, the reported pK_a value of GSH is 9.42.[27] The lower pK_a of our probe renders it more reactive toward HNO.

After demonstrating excellent responsiveness to HNO, exceptional selectivity over a range of biological analytes, and effective competition with thiols, we tested the ability of NitroxylFluor to visualize HNO in living cells. Specifically, we stained MDA-MB-231 cells, a human breast adenocarcinoma cell line, with NitroxylFluor for 15 min before removing the probe solution and replacing with fresh media. Addition of Angeli's salt at various concentrations (250 to 1000 μM) resulted in a dose-dependent fluorescence turn-on response after 15 min (Figure 4a,b). A statistically significant enhancement was observed even for the lowest Angeli's salt concentration. We also performed time-lapse imaging by treating NitroxylFluor stained cells with 500 μM Angeli's salt and recorded images every 1 min after addition for 20 min. We noted a gradual increase in the fluorescence during this time course. Representative images at 0, 10, and 20 min are shown in Figure S5. We also evaluated the cytotoxicity of NitroxylFluor by performing a MTT assay where MDA-MB-231 cells were stained with 10 μM probe for 3, 6, and 24 h. We did not observe cytotoxicity at any time point (Figure S6). The subcellular localization of NitroxylFluor in MDA-MB-231 cells appeared to be lysosomal. We confirmed this by using a commercial lysosome stain (LysoTracker Red) and observed significant overlap in signal with a Pearson's coefficient of 0.932 ± 0.01 (Figure S7).

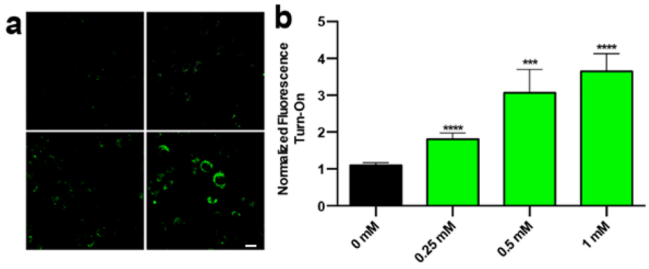

Figure 4. (a) Confocal microscopy images acquired by irradiation of MDA-MB-231 cells treated with 0 μM (vehicle control), 250 μM, 500 μM, 1000 μM Angeli's salt for 15 min at 25 °C with a 488 nm laser. Images taken through a 40× oil immersion objective. Scale bar represents 20 μm. (b) Quantification of imaging data. *, $p < 0.05$; ***, $p < 0.001$; $p < 0.0001$. ($n = 5$).

In conclusion, we have harnessed the known reactivity of thiols with HNO to develop a novel HNO-reactive trigger. We then called upon physical organic phenomena to improve upon this reactivity to develop a probe that competes with the native chemistry, even when known scavengers are in excess. In doing this, we have created a selective fluorescent probe for HNO that avoids inefficient byproducts and the lack of selectivity suffered by previously reported technologies. Our results indicate that the cyclization of the trigger outcompetes foreseeable side reactions. Owing to the culmination of this work, we predict that this technology will allow us and others to

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study HNO with enhanced rate and selectivity to elucidate its biological roles and pharmacological potential.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.7b11471.

Experimental details, including synthetic procedures for NB-1, NB-2, NB-3, and NitroxylFluor (PDF)

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Notes

The authors declare no competing financial interest.

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