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Journal Name

COMMUNICATION

Resorufin Based Fluorescent 'turn-on' Chemodosimeter Probe for Nitrosyl (HNO)†

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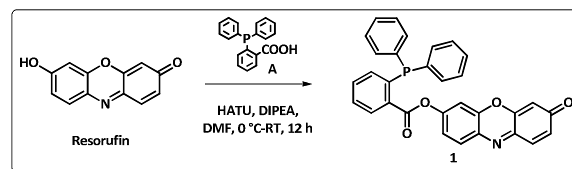
A cellular responsive, highly selective fluorogenic and chromogenic chemodosimeter probe for HNO is developed. This new probe showed ~30 fold fluorescence enhancement in presence of HNO and is sensitive to HNO at concentration as low as 0.02 μM. Further, it is capable of detecting HNO level in cellular milieus as well as in live specimen e.g. *C. Elegan*.

Fluorescent molecular probes have drawn great attention due to their ability to visualize the molecular chemical transformation in normal/pathogen cells in living system. Recently, a great deal of research effort is being focused on development of probe for nitroxyl (HNO). HNO is an one electron reduced or protonated form of nitric oxide (NO) and both nitroxyl (HNO)/ nitric oxide (NO) can be inter-converted in presence of superoxide dismutase(SOD).[1-2] Additionally, in a cellular system under aerobic condition, GSNO reacts with GSH at a rate of $0.01M^{-1}s^{-1}$ at 37 °C to produce HNO.[3] Several studies have suggested a heme enzyme catalyzed two-electron oxidation of hydroxylamine to generate the nitroxyl or nitric oxide in living cells.[4] Moreover, cellular hydrogen sulfide (H_2S) modulate nitroxyl or nitric oxide biosynthesis *via* activation of protein S-nitrosylation.[5] Recent studies have indicated that the nitroxyl (HNO) plays pivotal role in various biochemical processes in living system including resistance to superoxideinfraction in mammalian vascular systems, inhibition of function of aldehyde dehydrogenase and can create redox-potential deference in K^+ channels in mammalian vascular systems.[6] An observation in breast cancer cell indicated that cell proliferation is dramatically enhanced in presence of higher concentration of nitroxyl (HNO).[7] Thus, to detect, and elicit the action mechanism in biological system of such short-lived important species e.g. HNO in living systems is very important.

A number of fluorescent probes have been developed based on HNO induced reduction of Cu^{2+} to Cu^{+},[8] or nitroxide to hydroxylamine, by HNO.[9] Unfortunately these are interfered by highly abundant several biological reductants such as GSH and ascorbate in living system.

To overcome such poor selectivity issues in previous systems, reaction chemistry between HNO and phosphine, have been explored (similar to Staudinger reaction chemistry) to detect HNO without any interference. So far, a few probes have been reported mostly based on naphthalimide, coumarin and fluorescein dye;[10] but these are significantly incapable to provide any bright images in *vivo* or in *vitro* of a large tissue section. Thus we developed a new probe that can provide bright images against dark-back ground without interference by other analytes.



Scheme 1 Synthesis of Probe **1**.

The new probe **1** (scheme 1) was synthesized in a single step, the details of synthetic protocol and characterization procedures are provided in supplementary information. In this probe (**1**), we chose resorufin as 'turn on' fluorescent signalling unit as it has high quantum yield (0.75) and comparative UV absorption at higher wavelength (λ_{abs}= 565-585 nm).[11] Certainly it will allow us to show both absorption changes within visible range along with a 'turn *on'* fluorescent signal.

To justify the sensing ability of **1** (5 μM) toward HNO, the probe was incubated at physiological conditions for 30 min with Angeli's salt (AS)[12] (25 eq.) and subjected to UV-Vis and fluorescence analyses. The UV-absorption centered at 575 nm enhanced 18-fold in the presence of HNO (25 eq.) with a visible change from pale yellow to pink, as presented in Fig. 1a. An emission band centered at 590 nm (Fig. 1b) increased 30 fold while excited at 565 nm. These findings indicated that probe **1** is capable to respond both in chromogenic and fluorogenic ways to detect HNO. We measured concentration dependent fluorescence changes of probe **1** in PBS (20% DMSO) and presented in Fig 2. The data indicated that fluorescent intensity at 590 nm gradually increases with increasing concentrations of AS (0- 25 eq.) and then reached to a saturation point. Based on regression equation, we calculated the lower detection limit of probe **1** (Fig. S1) toward HNO to be 0.02 μM. We adopted previous reported[13] protocol to use mixed solvent (PBS with 20% DMSO) for this study; although recently reported few probes showed promising result only in PBS solution due to high polarity of the molecular systems.[14]

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Further, the reaction mechanism as we presumed in Scheme 2 was confirmed by mass spectroscopic analysis (Fig S2). HNO treated sample was subjected to MS study. A molecular ion peak of free resorufin (M^{-} = 212.0353) indicated the cause of fluorescent enhancement.

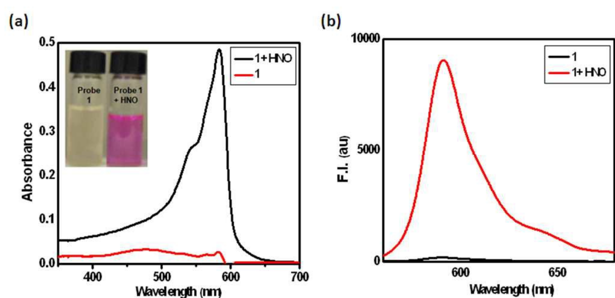


Fig. 1 (a) Fig. 1 (a) UV-visible spectra of probe **1** (5 μM) with or without HNO (AS); (b) Fluorescence changes of probe **1** in presence of HNO (125 μM) and alone in buffer (5 μM). Excitation was effected at 565 nm with the excitation and emission slit widths both set at 3 nm.[Inset: photograph of probe **1** and probe **1** upon addition of HNO (125 μM), which was taken under (a) visible].

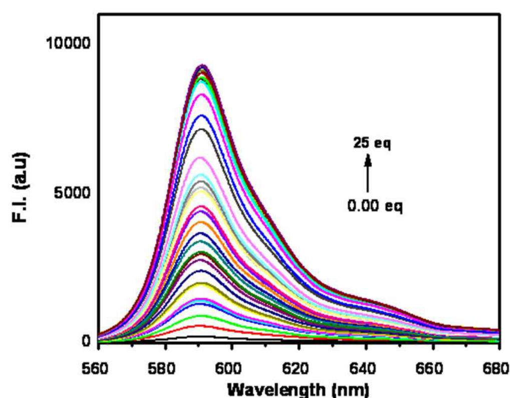
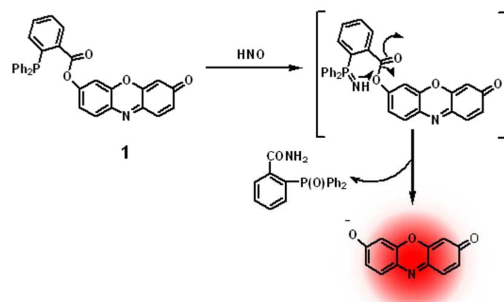


Fig.2 Fluorescence change of probe **1** (5 μM) recorded in the presence of increasing concentrations of HNO (0–25 eq.) in PBS (20.0 % DMSO). Probe **1** was incubated with HNO for 30 min at 37 ºC. Excitation was effected at 565 nm with the excitation and emission slit widths both set at 3 nm.



Scheme 2 Reaction between probe **1** and HNO.

We also evaluated the selectivity of the probe **1** toward HNO over other competitive, biologically relevant analytes such as S-nitrosoglutathione (GSNO), NO_2^-, NO, NO_3^-, H_2O_2, OCl^- $O_2^{\cdot}$, GSH and ascorbic acid (AA). The analytical data presented in Fig. 3 and Fig. S3 indicated that the probe **1** is highly selective towards HNO over others. This finding suggested that probe **1** may able to note and selectively detect HNO formation or level of HNO without any interference by other biological species inside the cellular milieus.

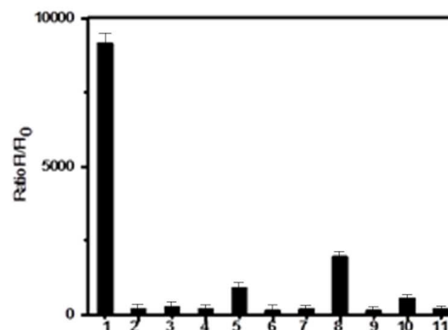


Fig.3 Fluorescence response of probe **1** (5 μM) toward biological relevant analytes (1: HNO, 2: H_2O_2, 3: $HO^{\cdot}$, 4: GSH, 5: NO, 6: NO_2^-, 7: NO_3^-, 8: GSNO, 9: AA, 10: NaOCl, 11: $FeCl_3$ (100 μM). Bars represent comparative fluorescence change at λ_{em} =590 nm; λ_{ex} =565 nm in presence of analytes. Each spectrum was acquired 30 min after addition of analytes at 37 ºC.

Fast response of any probe against target analytes is very crucial; specifically to detect reactive species in cellular milieus. Thus, we carried out a study to monitor the time dependent fluorescence change of probe **1** in presence of HNO (AS). From Fig. S4, it is observed that within 16 min, the fluorescent intensity of probe **1** reached maxima and then plateaued in presence of HNO (AS). Based on this result, we presumed that the probe **1** may be capable of detecting HNO in cellular milieus in presence of other reactive species also.

Further, the role of pH on stability of probe **1** and it's reactivity toward HNO were evaluated in absence and presence of HNO respectively. The results in Fig. S5 is depicted that probe **1** is stable in wide pH range (1-13) as emission intensity (λ_{em} = 590 nm) remains unaltered with low quantum yield (Φ_F = 0.012); whereas in presence of HNO (50.0 μM), the emission intensity enhancement at λ_{em} 590 nm expectedly high within the biological relevant pH range (~4 – 8). This result inferred that probe **1** is capable to detect HNO level unambiguously within wide pH range (~ 4 – 8) and it provoked to be useful for live cell imaging.

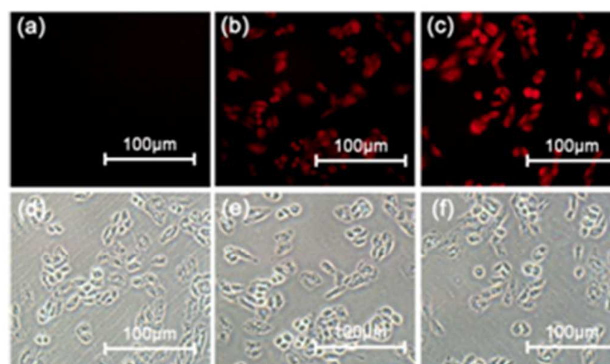


Fig. 4 Fluorescent imaging (top) and phase contrast (bottom) for HNO in CHO cells. (a) Probe **1** (5.0 μM in PBS) only. (b) Probe **1** (5.0 μM in PBS), HNO (75 μM) (c) Probe **1** (5.0 μM), HNO (AS, 125 μM).Cells were incubated with Probe **1** in PBS for 1h and then with or without HNO (AS) for 30 min at 37 ºC. Cell images were obtained using an Olympus BX51 inverted fluorescence microscopy. All images share the same scale bar (100 μm). Cells were using excitation wave length of 540 nm and emission length of 570-630 nm.

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The results of solution tests encouraged us to apply probe **1** in *vitro* cell imaging depending upon HNO level. Thus we incubated Chinese hamster ovary cell (CHO) cells with HNO, followed by addition of probe **1** (5 μM) and imaged by Olympus BX51 inverted fluorescence microscopy. Fig. S6 depicted that the probe **1** started to provide images within 5 min after treatment with HNO and the fluorescence intensity of labelled cells gradually increases with time. The results inferred that probe **1** has the capacity to detect HNO level in living cells within a short time scale. Further, concentration dependent study in Fig. 4 indicated that the extent of cell labelling increases with increasing conc. of HNO.

Finally, we attempted to apply the probe **1** for turned *on* fluorescence imaging in *vivo* in presence of HNO. For the study, in C. *Elegans*- a nematode was treated with probe **1** followed by incubation with 0-300 μM HNO analogue for 1 h. Fig. 5 indicates that the nematode is fluorescent labelled in presence of HNO and extent of labelling gradually increases with increasing concentrations of HNO. Further, time dependent in *vivo* imaging (Fig. S7) revealed that probe is capable of providing in *vivo* images within 30 min.

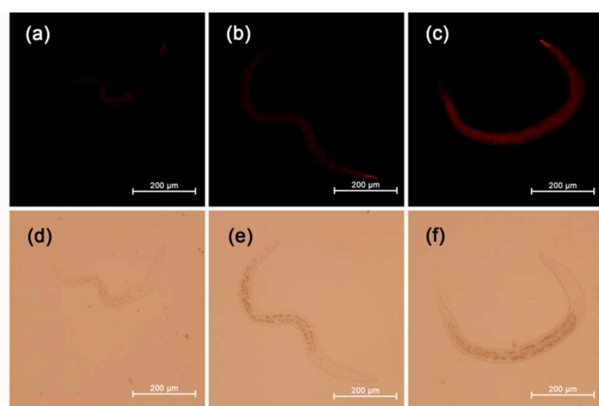

Fig. 5 Fluorescent imaging (top) and phase contrast (bottom) for HNO in *C. elegans*. (a) Probe **1** (5 μM in PBS) only. (b) Probe **1** (5 μM), HNO (AS, 50 μM). (c) Probe **1** (5 μM), HNO (100 μM). *C. elegans* were incubated with Probe **1** in PBS for 1h; then with or without HNO (AS) for 30 min at 20 °C. *C. elegans* images were obtained using an Olympus BX51 inverted fluorescence microscopy. All images share the same scale bar (200 μm). Cells were using excitation wavelength of 540 nm and emission wavelength of 570-630 nm.

Conclusions

We have developed a smart chemodosimeter probe for HNO which has the ability to change '*turn on'* fluorescence signal as well as visual changes from yellow to pink. The probe showed a remarkable *uv-vis*. and fluorescence signal enhancement *e.g.* 18 to 30 fold increment respectively in presence of HNO. A time dependent study indicated that the probe is highly reactive toward HNO. The probe displayed sensitivity toward HNO as low as 0.02 μM in physiological alike buffered saline milieus. In *vitro* cellular imaging and in *vivo* nematode imaging implied that this probe is highly capable of monitoring the HNO level in living systems.

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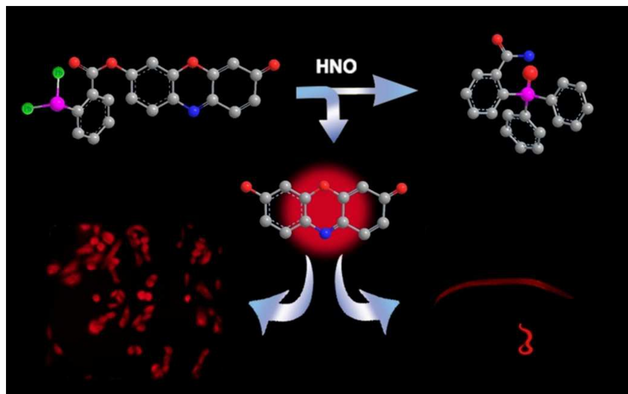

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