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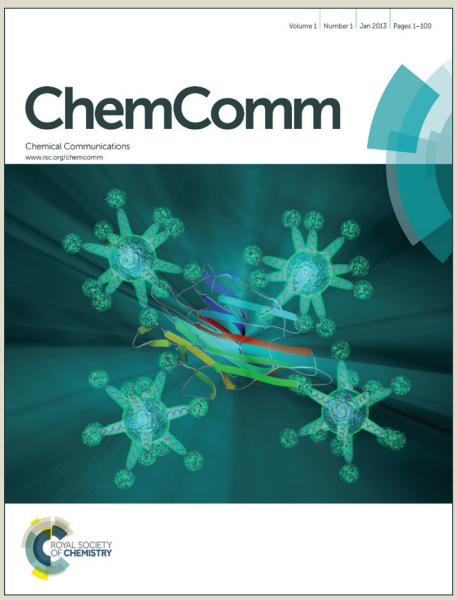

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ARTICLE TYPE

Rational Design of a Highly Sensitive and Selective Fluorogenic Probe for Detecting Nitric Oxide

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A rationally designed small-molecule fluorogenic probe for nitric oxide (NO) based on a new switching mechanism has been developed. Attaching a NO-responsive dihydropyridine pendant group to a fluorophore led to a probe that displays a very high sensitivity to NO concentrations down to the low nM range and a very high specificity to NO while being insensitive to other oxidative oxygen/nitrogen species that often interfere with the sensing of NO.

Since its discovery as the endothelium-derived relaxing factor (EDRF),[1-3] nitric oxide (NO) has been found to be involved in numerous physiological processes including neurotransmission, wound healing, blood pressure regulation, platelet adhesion, and immune responses.[4-8] Although increasing evidence suggests that NO serves as a crucial intermediate in diverse biological signaling pathways, in many cases the mechanisms on how NO plays its roles are still poorly understood. The availability of effective methods for detecting NO under various settings, especially those involving biological systems, should greatly advance the understanding of this important molecule. To this end, a number of strategies for NO detection, such as those based on chemiluminescence,[9] colorimetry,[10,11] electron paramagnetic resonance[12,13] and electrochemistry,[14,15] have been reported. In comparison to these approaches, fluorescence-based techniques have shown their unique advantages such as high sensitivity, high spatiotemporal resolution and easy usage for NO imaging and sensing.

A number of small-molecule fluorescence probes for NO detection have been reported.[16-33] A widely adopted approach involves a two-component "fluorophore-modulator" strategy for designing reaction-based fluorogenic NO probes. The reaction between *ortho*-diaminophenyl and NO is the most well known and widely used modulating mechanism in known designs of fluorogenic NO probes. It is believed that an aromatic vicinal diamine can efficiently quench a fluorophore subunit through photoinduced electron transfer (PET) mechanism. The reaction of an aromatic vincinal diamine with NO in the presence of oxygen leads to the formation of triazole that blocks nonradiative PET relaxation pathway and restores fluorescence emission of the fluorophore.[33] Although this strategy has been successfully employed in the development of many NO fluorescent probes,[25-31] it still has a few limitations. For example, electron-rich aromatic diamines are intrinsically susceptible to self-oxidation. The reactions of such diamines with other reactive oxygen/nitrogen species such as dehydroascorbic acid (DHA) and ascorbic acid (AA) also lead to the generation of fluorescence species.[35] Besides, rather than directly responding to NO itself, vicinal aromatic diamines need to react with oxidized NO species.[33,34] These limitations inevitably complicate NO sensing. Other alternative strategies such as probes based on metal complexes have been exploited.[14] Very recently, a method based on diazo ring formation through N-nitrosation of aromatic primary monoamines under aerobic conditions was reported.[20-24] This probe provides an elegant example of avoiding complications caused by electron-rich vicinal diamine in NO chemosensing.[32] In spite of the progress made so far, discovering new fluorescence modulating mechanism that is highly sensitive and specific for NO is essential for the development of the next-generation fluorogenic NO probes, which still presents a major challenge in this field.

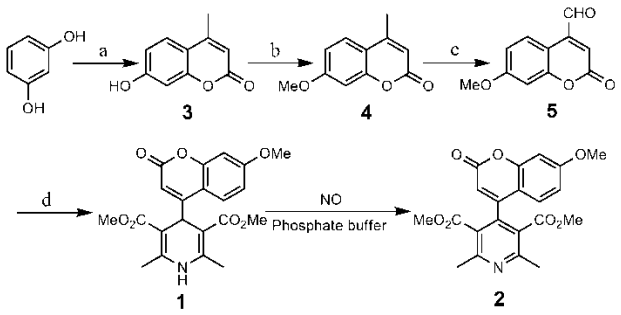
Recently, Nagano and co-workers demonstrated that fluorescence emission of a fluorophore can be modulated by varying the electron structures of a pendant group attached to the fluorophore via the PET mechanism. Furthermore, these authors reported that there was a direct relationship between the quantum yield (ϕ) of the fluorophore and the calculated energy level of the highest occupied molecular orbital (E_{HOMO}) of its pendant group.[38-42] This discovery has been shown to serve as a practical guide in design of PET type fluorogenic probes.[43]

We report herein the rational design and experimental validation of a highly selective fluorogenic probe for NO sensing based on a fluorescence switching-on mechanism. Hantzsch ester, a dihydropyridine derivative, has been known to able to quantitatively react with NO to give the corresponding pyridine, a reaction that is independent of oxygen.[36,37] A dramatic electron structure change (from non-aromatic to aromatic) accompanies this chemical transformation, which makes Hantzsch ester a likely candidate as a pendant group for developing PET based fluorescence NO probes. Hence, we first calculated the E_{HOMO} value of a Hantzsch ester and its corresponding pyridine product with density functional theory (DFT) at the B3LYP/6-31+G(d) level (ESI†). Results from our calculation revealed that the E_{HOMO} level of the Hantzsch ester was about -5.89 eV and that of the corresponding pyridine product was much lower (-7.19 eV). Thus, if such a Hantzsch ester, as a pendant modulating group, is covalently attached to a fluorophore with an E_{HOMO} level lying in between -5.89 to -7.19 eV, the fluorophore should become almost non-fluorescent due to the PET process between the Hantzsch

ester and the excited fluorophore. Upon NO-induced aromatization of the Hantzsch ester, the E_{HOMO} level of the pendant group will be below that of the fluorophore, which should abolish the PET process and therefore turn on the fluorescence of the probe.

As a proof of concept, a number of fluorophores commonly used in biological research were screened for their E_{HOMO} values. 7-Methoxycoumarin, which has an E_{HOMO} value of -6.38 eV, was chosen as the model fluorophore due to its desirable photophysical properties including a large Stokes' shift, excitation and emission wavelengths in the visible region,[44,45] and its biocompatibility and facile synthesis. Fluorogenic probe **1**, which consists of such a coumarin moiety and a substructure derived from the Hantzsch ester, was designed (Scheme 1).

Scheme 1. Synthesis of probe **1** and NO detection mechanism[a]



[a]Reagent and conditions: (a) Ethyl acetoacetate, H_3PO_4, rt; (b) Acetone, K_2CO_3, CH_3I, reflux; (c) SeO_2, xylene, reflux; (d) Methy acetoacetate, $NH_3{\bullet}H_2O$, EtOH, reflux.

In the design of **1**, the Hantzsch ester moiety is directly attached to 7-methoxycoumarin at its 4-position since it is known that the photo-physical properties of a coumarin derivative is strongly influenced by substituents attached to its 4-position.[24] As shown in Scheme 1, 7-methoxy-coumarin **5** was prepared from resorcinol in three steps via Knoevenagel condensation. The condensation of **5** with methyl acetoacetate in the presence of aqueous ammonia led to the efficient formation of **1**. The reaction of probe **1** with NO was then evaluated in both organic solvent (dichloromethane and methanol) and in phosphate buffer (50 mM, pH 7.4). It was found that the reactions proceeded quantitatively. Compound **2**, resulted from the aromatization of **1**, was the only product observed in each one of the reactions. The identities of **1** and **2** were confirmed by satisfactory analytical data based on NMR spectroscopy and mass spectrometry. The structures of probe **1** and its reaction product with NO, compound **2**, were further confirmed by X-ray crystallographic analysis (Fig. S1, ESI†).

As Fig. 1 shows, the UV-vis spectra of probe **1** displays an absorption band centered at 324 nm (ε = 23,630 $M^{-1}cm^{-1}$). For compound **2**, an absorption band centered at 334 nm (ε = 21,258 $M^{-1}cm^{-1}$) is observed. Despite a small red shift (10 nm) in their maximum absorption wavelengths, the absorption profiles of these two compounds share similar features. As expected, probe **1** is nearly non-fluorescent (ϕ = 0.009). Upon reaction with NO, an intense emission band centered at 450 nm and a significant increase in quantum yield (ϕ = 0.39) were observed (Fig. 1). The dramatic enhancement of quantum yield observed for **2** proved the validity of NO-induced aromatization of Hantzsch ester as a reliable fluorescence modulating mechanism.

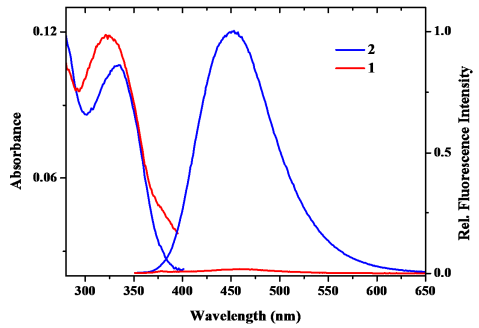


Fig. 1 UV-vis absorption and fluorescence emission spectra (λ_{ex} = 334 nm) of probe **1** (red) and compound **2** (blue) (5 μM in phosphate buffer, 50 mM, pH 7.4 for both probe **1** and compound **2**).

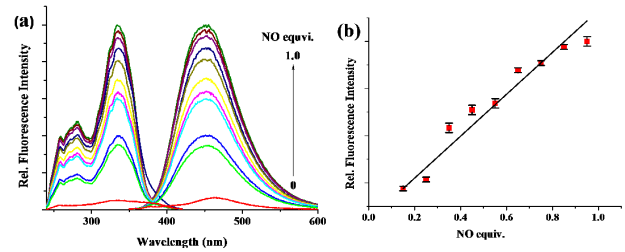


Fig. 2 (a) Enhancement of the fluorescence intensity upon addition of NO from 0 – 1.0 equiv. to a solution of probe **1** (10 μM) in 50 mM sodium phosphate buffer at pH 7.4 (λ_{ex} = 334 nm, λ_{em} = 450 nm); (b) Plot of fluorescence intensities of probe **1** (10 μM) at 450 nm as a function of NO concentration from 0 – 1.0 equiv., λ_{ex} = 334 nm.

The detection of NO with probe **1** was then systematically evaluated. As shown in Fig. 2, with increasing proportion of NO (added as a aliquots of NO-saturated water, 1.9 mM),[46,47] significant fluorescence enhancement in the presence of probe **1** was observed (Fig. 2a). A linear correlation (R^2 = 0.94) was found between the observed fluorescence enhancement and NO concentrations (Fig. 2b). The lower limit of detection, 3s/k, in which s is the standard deviation of blank measurements, k is the slope of the linear regression plot, was estimated to be 13 nM.

Since variation of pH values exists among different types of cells, or in different compartments within the same type of cells, the consistent performance of a fluorescence probe for NO against pH fluctuations is a highly desirable property. Tests showed that the emissive response of probe **1** remained almost unchanged as pH values varied in a wide range, from pH 4 to 9 (Fig. S2, ESI†).

Furthermore, the responses of probe **1** to NO and other reactive nitrogen or oxygen species that exist in living organisms were compared (Fig. S4, ESI†). Except for NO, no fluorescence enhancement significantly above the background (blank) was detected for other tested reactive species. These results demonstrate that **1** can not only tolerate a wide range of pH variations, but also exhibits a superb selectivity for NO in the presence of other potentially interfering oxidative species.

To gain insights into the nature of this novel fluorescence switching-on mechanism based on NO-induced aromatization of Hantzsch ester, computational studies based on density functional theory (DFT) and time-dependence DFT (TDDFT) were performed (ESI†). The obtained results indicate that the transition

between the electronic ground state (S_0) and the first excited state (S_1) of probe **1** is inefficient or "forbidden" due to an insufficient electronic wave function overlap.[48-50] The radiative locally excited (^{1}LE) state of probe **1** is rapidly converted into a nonradiative electron transfer state (^{1}ET) that subsequently decays back to the ground state. In contrast, compound **2**, formed from the aromatization of **1**, has well overlaped HOMO and LUMO located on the fluorophore (i.e., the coumarin moiety). The electronic transition between the S_0 and S_1 states of **2** now becomes highly efficient or "allowed". Moreover, the aromatized Hantzsch ester moiety of **2** has a significantly decreased HOMO potential (from -5.89 eV to -7.19 eV). This decrease of the donor's reduction potential disfavors the crossing from the emissive locally excited state to the dark electron transfer state. Results from our theoretical calculations thus predict a fluorescence enhancement of **2** due to the suppression of nonradiative pathways. In addition, the experimentally observed high quantum yield of **2** was also confirmed computationally.

The feasibility of employing probe **1** to detect endogenously generated NO was then investigated. Raw 264.7 cells in the presence or absence of bacterial lipopolysaccharide (LPS)[51] that stimulates the endogenous generation of NO were incubated with probe **1** respectively. As shown in Fig. 3, only very weak fluorescence is observed with unstimulatted cells (Fig. 3a), while significantly enhanced emission is clearly visible with simulated cells (Fig. 3b), which indicates that probe **1** is capable of imaging LPS-stimulated endogenous NO production in living cells and the change of NO level in intracellular environments.

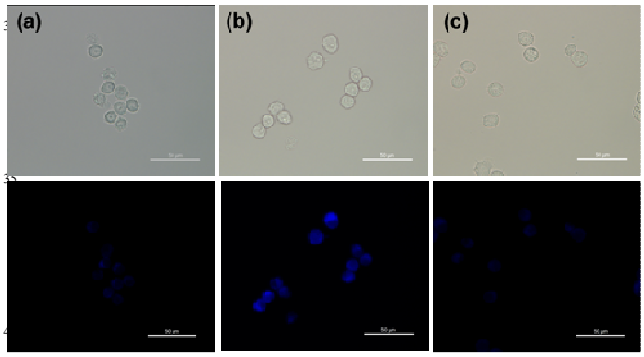


Fig. 3 Probe **1** detection of endogenous NO in Raw 264.7 cells: bright field image (top) and corresponding fluorescence image (bottom) of cells (a) incubated with probe **1** (40 μM) without LPS prestimulation, (b) prestimulated with LPS (0.5 μg/mL) for 4 h and incubated with probe **1** for 8 h, (c) pretreated with NOS inhibitor (L-NMA, 2 mM) 1.5 h and incubated with LPS 4 h then probe **1** (40 μM) 8 h, Scale bars are all 50 μm.

As a control, the fluorescence response for stimulated cells pretreated with NG-methyl-L-arginine (L-NMA), a selective inhibitor of LPS-stimulated NO production, is significantly weaker (Fig. 3c) than untreated cells (Fig. 3b), which confirms that the switched-on fluorescence in the stimulated cells is indeed the result of the direct reaction of probe **1** with NO. Cell viability was examined based on a known assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT).[52] The results showed that neither probe **1** nor the product of its reaction with NO, compound **2**, was toxic to Raw 264.7 cells in the concentration range adopted for our imaging studies (Fig. S5, S6, ESI†). Probe **1** was also tested for visualization of NO exogenously supplied by a donor (sodium nitroprosside, SNP) in HeLa cells. A switch-on fluorescence emission was clearly observed (Fig. S7, ESI†).

Conclusions

In summary, we have demonstrated the rational design, synthesis and experimental verification of a highly efficient fluorogenic probe for NO sensing based on a novel switching mechanism. NO-induced aromatization of Hantzsch ester is proved to be an effective fluorescence switch-on strategy in the design of PET–type NO probes. Photo-physical studies reveal that the model probe **1**, displays a sensitive, linear response to changes of NO level and a high specificity for NO in a wide pH range. This probe has been found to be suitable for both intra- and extracellular NO detection. The fluorogenic probe for NO as described here, with its modular design, should serve as a very useful, readily expandable prototype for the subsequent development of a series of analogous fluorogenic probes. Properties such as further enhanced sensitivity, expanded absorption and emission ranges, as well as compatibility to a variety of conditions, especially those involving biological settings, are expected for such probes.

Notes and references

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† Electronic Supplementary Information (ESI) available: Materials and methods, synthesis of compounds, calculation of the detection limit, calculation methods, Fig. S1-S9. See DOI: 10.1039/b000000x/

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