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A specific fluorescent probe for NO based on a new NO-binding group†

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By incorporation of a specific NO-binding group, 2-amino-3′-dimethylaminobiphenyl, into a Bodipy dye, fluorescent probe 1 was constructed, which exhibited high selectivity for NO over other ROS/RNS as well as DHA, AA and MGO.

Nitric oxide (NO), produced by nitric oxide synthases, has been regarded as a ubiquitous signaling molecule, and plays key roles in various physiological as well as pathological processes. Endogenous NO mediates multiple processes in the various physiological systems, such as the cardiovascular system, immune system, and the central and peripheral nervous systems.[1] Also, studies have shown that its deregulation correlates with the symptoms of cancer, endothelial dysfunction, and neurodegenerative diseases.[2] However, the mechanisms by which it performs its diverse biological roles still remain elusive. Therefore, an efficient method for sensitively and selectively probing NO in biological systems is highly required for better understanding of its origin, activities, and biological functions.

Due to their simplicity, sensitivity, real-time imaging, and especially nondestructive detection, fluorescence techniques combined with microscopy have been regarded as a powerful tool for sensing and imaging targeted biomolecules in live cells or tissues. Given this, a number of fluorescent NO probes have been reported to date[3] by exploiting the specific reactions of NO with the *o*-phenylenediamine (OPD) moiety,[4,5] metal–ligand complexes,[6] and others.[7] Among these methods, OPD-based fluorescent probes, pioneered by Nagano's group, have shown the immense potential to visualize NO *in vitro* and *in vivo*.[4,5] Moreover, some of them are commercially available,[8] and have greatly promoted NO-related biological investigation. The corresponding sensing mechanism is based on the irreversible reaction of the OPD group with NO^+ or N_2O_3 to give benzotriazole derivatives, by which the off–on fluorescence response is achieved through suppression of the photoinduced electron transfer (PET) process. Although in some cases they can provide useful information, the basic chemical problems still remain. First, dehydroascorbic acid (DHA) and ascorbic acid (AA) (micromolar to millimolar levels in many cells[9]) were reported to be able to condense with the OPD group to turn on the fluorescence of such probes.[10] Second, it was recently reported that a OPD-based Bodipy fluorescent probe could also detect methylglyoxal (MGO),[11] a dicarbonyl metabolite produced by all living cells, under physiological conditions.[12] Third, benzotriazoles are pH-sensitive, and their deprotonation at physiological pH results in an electron-rich triazolate, which may induce the undesirable fluorescence quenching through the PET mechanism to lower the NO detection sensitivity.[4d,f] Therefore, further efforts to overcome these limitations are highly required.

In 2010, Anslyn *et al.* exploited a 2-dimethlyaminophenyl-5-cyano-α-naphthylamine fluorescent probe for NO (Fig. 1A).[13] The probe itself was nonfluorescent, but displayed the obvious fluorescence off–on response upon NO treatment due to the NO-triggered formation of a new fluorophore. Notably, the probe displayed excellent selectivity for NO over other reactive oxygen/nitrogen species (ROS/RNS) as well as DHA, and thus appears to be more advantageous than the conventional OPD-based ones from the viewpoint of selectivity. To extend the method to a versatile design platform, in this work we present its modified

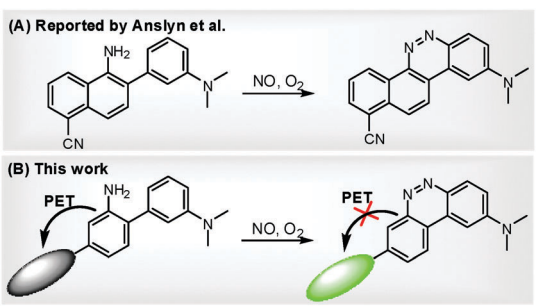

Fig. 1 The design strategies for fluorescent NO probes.

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† Electronic supplementary information (ESI) available: Experimental procedures, supplemental spectra, and the ^{1}H-, ^{13}C-NMR, and MS spectra. See DOI: 10.1039/c4cc03540b

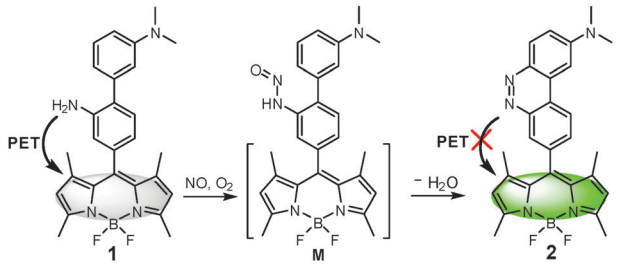


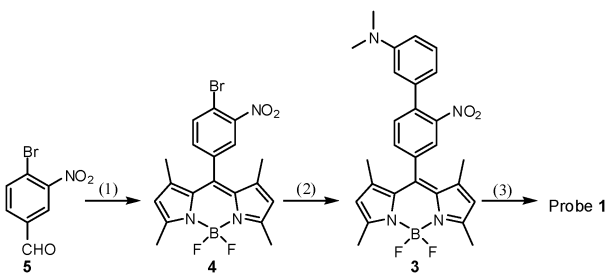
Fig. 2 Proposed sensing mechanism of probe **1** with NO.

version for constructing the fluorescent NO probe through integration of a NO-binding group, 2-amino-3′-dimethylaminobiphenyl (**AD**), and a selected fluorophore (Fig. 1B). We think that the strategy may be more useful for the future design of fluorescent NO probes.

As a proof of concept, we designed probe **1** by attaching the NO-binding group, **AD** into the widely used Bodipy dye[14] (Fig. 2). In fact, **AD** functions not only as a NO-binding group, but also as a fluorescence quencher *via* PET to ensure the low background fluorescence. We reasoned that probe **1** is weakly fluorescent due to the PET process from the electron-rich **AD** group to the excited Bodipy fluorophore. Upon treatment with NO, diazo product **2** was expected to be produced based on the reaction mechanism proposed by Anslyn.[13] Due to the absence of the electron-donating amino group in **2**, the above-mentioned PET process would be inhibited, thereby leading to the fluorescence turn-on of the Bodipy fluorophore.

Probe **1** was synthesized according the route outlined in Scheme 1. To anchor the **AD** group to the Bodipy core, the commercially available 4-bromo-3-nitro-benzaldehyde **5** was used as the starting material. By use of the standard Bodipy synthetic procedures, intermediate **4** could be easily obtained. Suzuki coupling of **4** with 3-dimethylaminophenylboronic acid gave intermediate **3**. The subsequent reduction of **3** with $SnCl_2$ gave probe **1**. The detailed experimental procedure and characterization data are provided in the ESI.†

With probe **1** in hand, we first tested the reaction of **1** with NO in EtOH-PBS buffer (20 mM, pH 7.4, 1 : 1, v/v) at 37 °C by HRMS. DEA·NONOate was used as the source of NO, which is commercially available with a half time of 16 min. The free probe **1** exhibited a main peak at *m/z* 459.2565 (Fig. 3A), corresponding to $[\mathbf{1} + H^+]^+$ (*m/z*, calcd: 459.2532). However, after incubation of the probe with excess of DEA·NONOate, a new peak was observed at *m/z* 470.2338 (Fig. 3B), which could be assigned to $[\mathbf{2} + H^+]^+$ (*m/z*, calcd: 470.2328). Moreover, the reaction also elicited an obvious



Scheme 1 Synthesis of **1**. (1) (i) 2,4-Dimethyl-pyrrole, TFA; (ii) DDQ and (iii) Et_3N, BF_3-Et_2O. (2) 3-Dimethylaminophenylboronic acid, $Pd(PPh_3)_4$, K_2CO_3. (3) $SnCl_2$, conc. HCl.

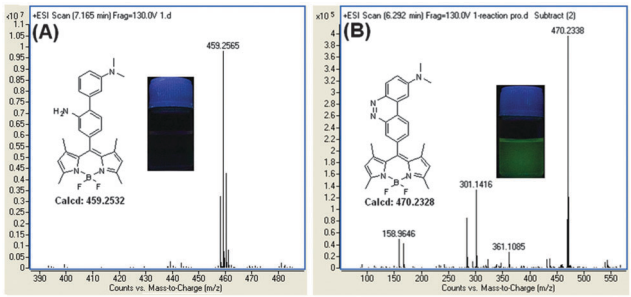


Fig. 3 HRMS charts of probe **1** in the absence (A) and presence (B) of the excess of DEA·NONOate in EtOH-PBS buffer (20 mM, pH 7.4, 1 : 1, v/v). Inset: fluorescent images of probe **1** in the absence (A) and presence (B) of the excess of DEA·NONOate in the same buffer.

fluorescence turn-on response from a dark background (Fig. 3, inset). The preliminary results are in accordance with our proposed sensing mechanism.

Next, we performed the time-dependent fluorescence spectral studies of probe **1** treated with different concentrations of DEA·NONOate in EtOH-PBS buffer (20 mM, pH 7.4, 1 : 1, v/v). The obtained results indicated that the reaction could be completed within 30 min (Fig. 4A). With a time point of 30 min after addition of DEA·NONOate, we performed the fluorescence titration studies of **1** for NO. As shown in Fig. 4B, probe **1** exhibited a weak emission at 518 nm due to PET from the aniline unit to the excited Bodipy fluorophore. Treatment with DEA·NONOate elicited an obvious enhancement. The changes in the emission intensities became less obvious when the amount of DEA·NONOate was more than 75 μM. In this case, an approximately 5-fold fluorescence enhancement was observed. Moreover, the fluorescence intensities of **1** at 518 nm showed a good linear relationship with DEA·NONOate concentrations from 0 to 15 μM (Fig. 4B, inset), and the detection limit was estimated to be 30 nM based on $S/N = 3$. Thus, probe **1** is potentially useful for monitoring NO in living cells.

Further, we evaluated the specificity of probe **1** for NO. We screened a wide array of possible competitive species, including H_2O_2, ClO^-, $^{\bullet}OH$, O_2^-, $^1O_2$, $ONOO^-$, NO_2^-, NO_3^-, DHA, AA, and MGO, all of which are biologically related (Fig. 5A). In fact, probe **1** was inert to most of these ROS and RNS, and only $^1O_2$ and $ONOO^-$ elicited a minor fluorescence enhancement of 1.4- and 1.5-folds, respectively. Notably, probe **1** did not show

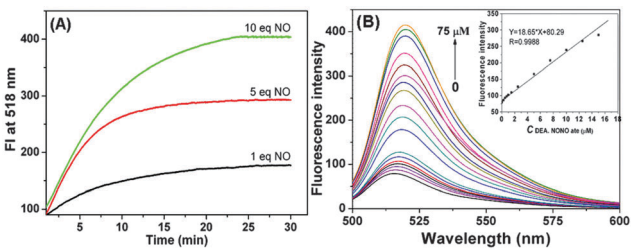


Fig. 4 (A) Time-dependent fluorescence intensity changes of **1** (5 μM) upon addition of varied concentrations of DEA·NONOate in EtOH-PBS buffer. (B) Fluorescence spectra of probe **1** (5 μM) upon addition of DEA·NONOate (0–75 μM) recorded after 30 min. Condition: EtOH-PBS buffer (20 mM, pH 7.4, 1 : 1, v/v); 37 °C; λ_{ex} = 480 nm; λ_{em} = 518 nm; slits: 5/5 nm.

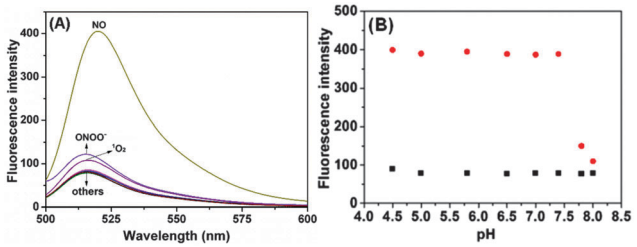


Fig. 5 (A) Fluorescence spectra of **1** (5 μM) treated with various species (10 equiv. of NO, $ONOO^-$ and $^1O_2$; 100 equiv. of H_2O_2, ClO^-, •OH, O_2^-, NO_2^-, NO_3^-, DHA, AA, and MGO) in EtOH-PBS buffer (20 mM, pH 7.4, 1 : 1, v/v). (B) Effects of pH on the fluorescence intensity of **1** (5 μM) in the absence (■) or presence (●) of DEA·NONOate (10 equiv.). λ_{ex} = 480 nm, λ_{em} = 518 nm, slits: 5/5 nm.

any response to DHA, AA, and MGO. By comparison, only NO could elicit a big increase of 5-fold in the fluorescence intensity at 518 nm, suggesting a high selectivity of probe **1** toward NO. We attributed the high selectivity of **1** toward NO over DHA, AA, and MGO to the **AD** group in **1**, which fails to condense with the 1,2-dicarbonyl group of DHA/AA/MGO to form quinoxaline heterocycles.

Also, we studied the emission behaviour of **1** treated with DEA·NONOate in different pH environments. As shown in Fig. 5B, probe **1** showed a stable and weak emission in the pH region of 4.5–8.0, and displayed the best response for NO in the pH region of 4.5–7.5. Thus, probe **1** can function well under physiological conditions. However, under alkaline conditions, the fluorescence enhancement was inhibited, likely due to the fact that DEA·NONOate generates NO in a pH-dependent manner, and is more stable in a high pH environment.

Encouraged by the above results, we evaluated the capability of **1** to selectively sense NO in the cellular environment. HL-7702 cells (human liver cells) showed no fluorescence in the green channel (Fig. 6A). After incubation with **1** (5 μM) in culture medium for 1 h at 37 °C, HL-7702 cells showed a very weak fluorescence (Fig. 6B), indicating that **1** is cell-permeable. However, strong fluorescence in the cells was observed after the cells were pretreated with **1** for 1 h and further incubated with DEA·NONOate (50 μM) for 1 h (Fig. 6C). These preliminary results confirmed that probe **1** has the potential to visualize NO levels in living cells.

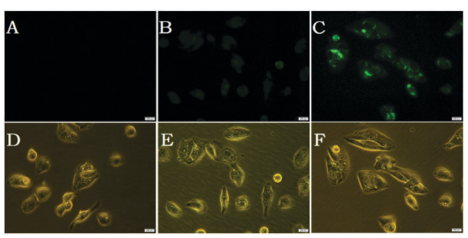


Fig. 6 Fluorescent images of NO in HL-7702 cells using probe **1** (5 μM) at 37 °C. (A) HL-7702 cells. (B) HL-7702 cells incubated with **1** for 1 h. (C) HL-7702 cells pretreated with **1** for 1 h and then incubated with DEA·NONOate (50 μM) for 1 h. (D–F) The corresponding bright-field images. Scale bar: 200 μm.

In summary, we have presented a novel fluorescent probe for NO by incorporation of a new NO-binding group, 2-amino-3′-dimethylaminobiphenyl, into a Bodipy dye. The probe can selectively sense NO over ROS/RNS as well as AA/DHA/MGO with a detection limit as low as 30 nM. The preliminary cell imaging experiments indicate its potential to probe NO chemistry in biological systems.

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