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A highly sensitive and reductant-resistant fluorescent probe for nitroxyl in aqueous solution and serum†

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A novel coumarin-based fluorescent probe, P-CM, for quantitative detection of nitroxyl (HNO) was developed. P-CM exhibits a selective response to HNO over other biological reductants and was also applied for quantitative detection of HNO in bovine serum with satisfactory results.

Nitric oxide (NO) has been discovered to play a critical role in several physiological and pathological processes, including anticancer activity,[1] vasodilation,[2] neurotransmission,[3] and regulation of the immune response by macrophages.[4] Nitroxyl (HNO), the one-electron reduced form of NO with a pK_a of 11.4, which exists primarily in the protonated form under physiological conditions[11a] and exhibits biological effects distinct from NO,[5] has gained increasing interest in the past decade. For example, HNO reacts with protein thiols to cause inhibition of aldehyde dehydrogenase,[6] can activate voltage-dependent K^+ channels in mammalian vascular systems,[7] and is resistant to scavenging by superoxide.[8] Moreover, biochemical studies suggest that HNO can be formed directly from nitric oxide synthase under appropriate conditions,[9] and that NO and HNO may be able to interconvert in the presence of superoxide dismutase (SOD).[10] HNO is a reactive molecule that could spontaneously dimerize and subsequently dehydrates to form nitrous oxide (N_2O),[11] making its direct detection in solution or biologically relevant samples difficult. As a consequence, the studies on HNO chemistry and biology have been hampered by the lack of efficient detection methods. Thus, the development of highly sensitive and selective methods for the detection of biological nitroxyl is of great importance.

Several methods, including mass spectrometry,[12] high-performance liquid chromatography,[13] colorimetry,[14] electrochemical analysis,[15] NMR,[16] and fluorescent probes,[17–20] have been developed for the detection of HNO in various samples. Among them, fluorescent probes have attracted particular interest due to their advantages such as high sensitivity, fast analysis with high spatio-temporal resolution and non-destructive sample preparation.[21–23] However, most of the previously reported fluorescent probes for HNO were based on the reduction of Cu(II) to Cu(I),[17] or nitroxide to hydroxylamine[19] by HNO. Therefore, these probes suffered from interference from other biological reductants, such as glutathione and ascorbate, which are abundant in biological systems and limited their applications in detection of HNO in practical biological samples. The development of fluorescent probes that could discriminate HNO from other biological reductants is desired.

HNO was reported to be able to react with phosphines with a high reaction rate constant (estimated to be 9×10^5 M^{-1} s^{-1}) to give the corresponding aza-ylide, intramolecular nucleophilic attack of which on the carbonyl carbon of the ester is expected to occur in the presence of a electrophilic ester, leading to the formation of alcohol and amide.[14e,16a,20] On the other hand, 7-hydroxycoumarin was often chosen as a fluorophore of a probe since it possesses excellent photophysical properties, including good solubility, high fluorescence quantum yield and large Stokes shift.[24] Moreover, the fluorescence properties of 7-hydroxycoumarin derivatives depend on their molecular substitution, being influenced by the degree of intramolecular charge transfer from 7-substituents to the coumarin ring. Based on these facts, we designed a new probe **P-CM** for HNO with 7-hydroxycoumarin as the fluorophore and a triphenylphosphine group as the HNO recognition unit (Scheme 1).

Probe **P-CM** was synthesized following the synthetic route shown in Scheme S1 (see ESI†). The structure of **P-CM** was confirmed by ^{1}HNMR and MS (see Fig. S6 and S7, ESI†).

The fluorescent properties of the probe **P-CM** and its HNO-induced product 7-hydroxycoumarin were first assessed (see Fig. S1, ESI†). A 76-fold fluorescence enhancement at 450 nm was observed between **P-CM** and 7-hydroxycoumarin, indicating that **P-CM** is suitable for constructing a "turn-on" fluorescence probe. Next, we evaluated the capability of **P-CM** to detect HNO in aqueous buffer. The time-dependent fluorescence response of **P-CM** to different concentrations of AS was recorded (see Fig. S1, ESI†). The results revealed that the response time of the probe toward AS increased

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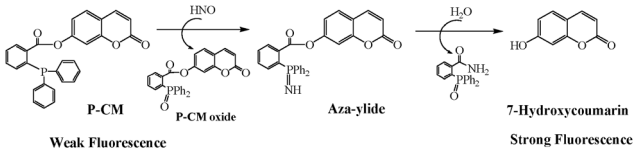

Scheme 1 Structure and response mechanism of the probe **P-CM** for HNO.

with the increase in AS concentration. The **P-CM** itself shows a low background fluorescence emission. However, the treatment of **P-CM** (10 μM) with 10 equiv. of Angeli's salt (AS, a HNO source) resulted in a remarkable fluorescence enhancement, with a 57-fold fluorescence enhancement observed after 30 minutes. The changes in the fluorescence spectra of **P-CM** upon the gradual addition of HNO were further investigated (Fig. 1). The intensity increased linearly with the concentration of AS ranging from 0.05 μM to 5 μM. A detection limit of 20 nM was estimated for HNO (based on 3σ/slope), which is much lower than the limits of previously reported fluorescence probes for nitroxyl.[17–19] The improved sensitivity of the probe might be ascribed to the smaller size of probe **P-CM**, which affords a weaker steric hindrance for HNO attaching.

For a probe with potential application in complex biological samples, a highly selective response to the target species over other potentially competing species is a necessity. Therefore, we assessed the selectivity of **P-CM** for HNO over other biologically-related species (Fig. 2). The responses of **P-CM** were found to be highly selective for HNO over the reactive oxygen species (ROS), the reactive nitrogen species (RNS), biological-related metal ions, and reductants. RNS and ROS, including NO, GSNO, NO_2^-, NO_3^-, $ONOO^-$, H_2O_2 and ClO^-, failed to induce significant fluorescence enhancement of **P-CM**. Additionally, no obvious turn-on signal was induced when 100 equiv. of K^+, Na^+, Ca^{2+}, Mg^{2+}, Zn^{2+}, or Fe^{3+} was added. It is particularly noteworthy that the fluorescence intensity of **P-CM** was hardly affected by biological reductants, including GSH, AA, and Na_2S (a hydrogen sulfide source). The proposed probe **P-CM** seems to be greatly superior to the previously reported Cu(II)-based probes which show high fluorescence in the presence of large amounts of various biological reductants.[17] Moreover, the competitive experiments were conducted with other testing species in the presence of 100 μM of AS (see Fig. S2, ESI†). The fluorescence intensity of the probe did not vary much except for Fe^{3+}, GSH, AA and Na_2S, which caused a decrease in the fluorescence intensity. It may be because HNO was consumed by Fe^{3+}, GSH, AA or H_2S since HNO is both an electrophile that can oxidize thiols and a nucleophile that can reduce metal ions.[25] All these results demonstrated that **P-CM** probe is highly selective for HNO over other biologically related species, and could meet the selective requirements for detection of HNO in complex biological samples.

The effect of pH on the fluorescence intensity of **P-CM** in the absence and presence of HNO was also investigated (see Fig. S3, ESI†). The fluorescence intensity of **P-CM** was independent of pH over the range of pH 5.0–10.0, indicating that the ester group is stable in pH 5.0–10.0. After the probe was incubated with AS for 30 min, the fluorescence intensity of **P-CM** notably increased with the increase in pH between pH 5.0–9.0. It would be due to this that the reaction product 7-hydroxycoumarin exhibits higher fluorescence intensity under alkaline conditions because of deprotonation of the hydroxyl group.[26] At a pH value larger than 9.0, the fluorescence intensity of **P-CM** showed a decreasing tendency, which might be ascribed to the decreased decomposition rate of AS into nitroxyl under strong basic conditions.[27]

To test the feasibility of the practical application of the probe **P-CM**, we further conducted the HNO detection in 20% bovine serum (Fig. 3). Upon addition of AS, the solution of **P-CM** in bovine serum showed moderate fluorescence enhancements. The fluorescence enhancement in bovine serum was not as large as that in aqueous solution, which is because HNO can react with thiols and thiol

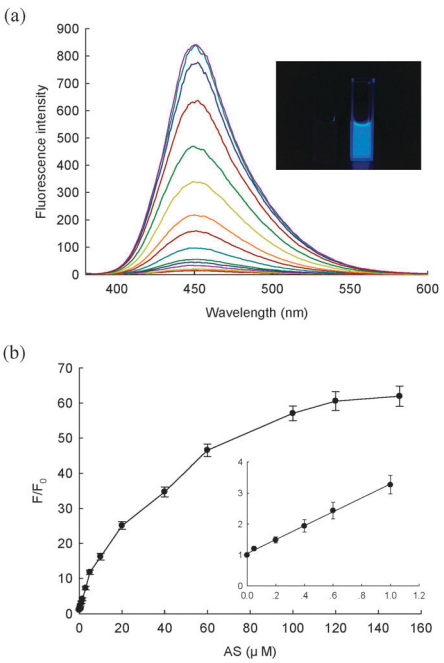

Fig. 1 (a) The fluorescence emission spectra of **P-CM** (10 μM) in the presence of different concentrations of AS (0, 0.05, 0.2, 0.6, 1, 1.5, 3, 5, 10, 20, 40, 60, 100, 120, 150 μM) in buffered (pH 7.4) aqueous DMF solution. Inset show the visual fluorescence of **P-CM** before (left) and after (right) incubation with AS (100 μM) for 30 min (UV lamp, 365 nm). (b) Calibration curve of **P-CM** to AS. The curve was plotted with the fluorescence intensity *vs.* AS concentration after their incubation for 30 min. Inset shows the linear responses at low AS concentrations.

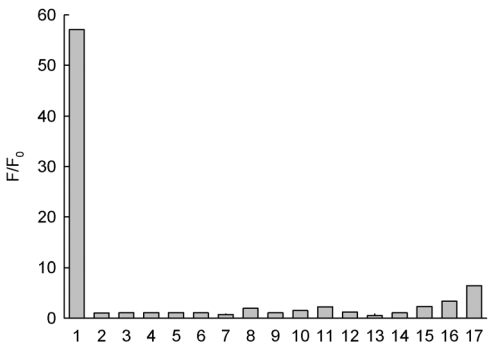

Fig. 2 Fluorescence responses of the **P-CM** (10 μM) to testing species in buffered (pH 7.4) aqueous DMF solution: (1) 100 μM AS; (2) 1 mM K^+; (3) 1 mM Na^+; (4) 1 mM Ca^{2+}; (5) 1 mM Mg^{2+}; (6) 1 mM Zn^{2+}; (7) 1 mM Fe^{3+}; (8) 3 mM GSH; (9) 4 mM AA; (10) 10 mM Na_2S; (11) 500 μM H_2O_2; (12) 4 mM ClO^-; (13) 10 mM NO_2^-; (14) 10 mM NO_3^-; (15) 4 mM $ONOO^-$; (16) 200 μM NO; (17) 200 μM GSNO.

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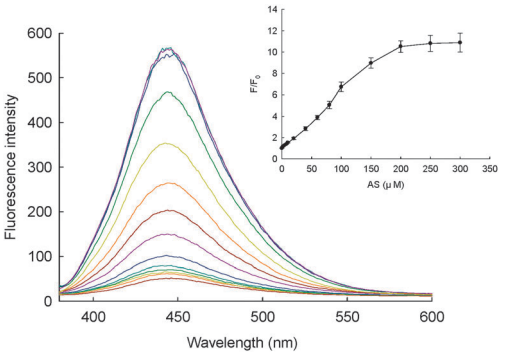

Fig. 3 The fluorescence emission spectra of **P-CM** (10 μM) in the presence of different concentrations of AS (0, 2, 4, 7, 10, 20, 40, 60, 80, 100, 150, 200, 250, 300 μM) in buffered (pH 7.4) aqueous 20% bovine serum solution. Inset shows the calibration curve of **P-CM** to AS.

residues of protein in bovine serum.[28] Moreover, the interactions of 7-hydroxycoumarin with protein may probably cause fluorescence quenching. As shown in Fig. S5 (see ESI†), an excellent linear relationship was obtained. The fluorescence intensity increased linearly with the concentration of AS ranging from 2 μM to 60 μM, which confirmed that the probe **P-CM** was applicable for practical HNO detection in real samples with satisfactory results.

To verify the proposed mechanism (Scheme 1), the sample solutions of **P-CM** before and after treating with AS for 30 min at 37 °C were analyzed by HPLC (see Fig. S4, ESI†). After treatment of **P-CM** with AS for 30 min, the final product 7-hydroxycoumarin was detected concomitantly with the decrease of **P-CM** in the solution. The reaction of **P-CM** with HNO will generate **P-CM** oxide and 7-hydroxycoumarin. **P-CM** oxide could not be observed, probably due to its easy hydrolysis into 7-hydroxycoumarin. All these experimental results indicate that the fluorescence response of the probe is through a HNO-induced cleavage mechanism.

In summary, we have reported a new fluorescent probe **P-CM** for quantitative detection of nitroxyl in aqueous solution and serum. It affords a high sensitivity to HNO in aqueous solutions with a detection limit of 20 nM. Also, **P-CM** exhibits high selectivity for HNO over other biologically relevant species. Especially, **P-CM** is unaffected by various biological reductants in contrast to previously reported fluorescence probes. Moreover, the probe was applied for quantitative detection of HNO in bovine serum with satisfactory results. All these features make **P-CM** favorable for direct quantitative detection of HNO in complex biological samples, demonstrating its value in practical application.

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