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Development of a ratiometric fluorescent sensor for ratiometric imaging of endogenously produced nitric oxide in macrophage cells†

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We described the development of Cou-Rho-NO as the first small-molecule suitable for ratiometric fluorescent imaging of endogenously produced nitric oxide in macrophage cells.

Nitric oxide (NO) is an endogenous signalling molecule produced by inducible and constitutive nitric oxide synthases.[1] Although the half-life of nitric oxide is only several seconds at low concentrations in biological systems,[2] it is involved in many critical biological processes including signal transduction, smooth muscle relaxation, peristalsis, immune system control, neurotransmission, blood pressure modulation, learning, and memory.[3,4] However, mis-regulation of NO production is implicated with various diseases ranging from stroke, heart disease, hypertension, neurodegeneration, erectile dysfunction, to gastrointestinal distress.[4]

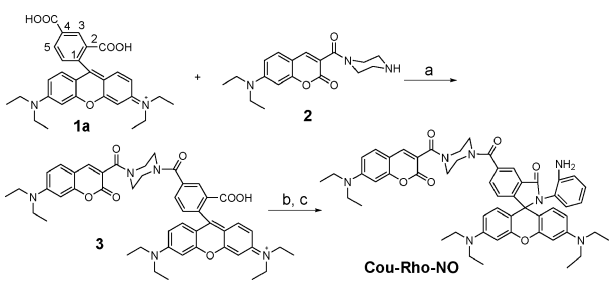
Although a wide variety of techniques have been developed to detect NO, fluorescence sensing has become the gold standard due to its high sensitivity and simplicity.[5] Thus, the construction of small-molecule fluorescent sensors suitable for specific NO detection in living systems has received great attention.[6,7] Metal-based and reaction-based sensors are the two main classes of small-molecule sensing agents specific for NO detection. The recently developed small-molecule fluorescent NO sensors typically exhibit low fluorescence in the absence of NO, but they display a considerable enhancement in fluorescence intensity when incubated with NO. Thus, they respond to NO with a fluorescence turn-on signal.

However, fluorescence intensity-based measurements are influenced by sensor concentration, environment, and excitation intensity, *etc*. By contrast, ratiometric measurements based on the intensity ratios at two wavelengths may alleviate most of these problems.[8] Although ratiometric fluorescent NO sensors suitable for cellular ratiometric imaging are highly sought,[6] to our best knowledge, no such small-molecule ratiometric fluorescent NO sensors have been reported to date.

In this communication, we present compound **Cou-Rho-NO** (Scheme 1) as the first small-molecule fluorescent sensor which can be employed for ratiometric imaging of endogenously produced NO in macrophage cells.

The novel ratiometric sensor **Cou-Rho-NO** was rationally designed based on a coumarin-rhodamine scaffold as the Förster resonance energy transfer (FRET) dyad. When the rhodamine dye is in the ring-closed form, it has no significant absorption in the visible region and is non-fluorescent. However, when the rhodamine dye is in the ring-opened form, it has significant absorption in the visible region and is highly fluorescent. The ring-opening process of the rhodamine dye accompanied by optical property variations has been extensively exploited to design turn-on type fluorescent sensors. Furthermore, a rigid piperazine moiety was selected as the linker to avoid the likely static fluorescence quenching due to the close contact of the coumarin donor and rhodamine acceptor dyes in aqueous environment. In addition, *o*-phenylenediamine was chosen as the potential reaction site for NO. In the absence of NO, the excitation energy of the coumarin donor could not be transferred to the rhodamine acceptor which is in the closed form. Thus, the FRET should be off in the free **Cou-Rho-NO**. In other words, upon excitation at the coumarin donor, only the emission of the coumarin dye is observed. However, we envisioned that addition of NO may induce the rhodamine acceptor to be in the ring-opened form *via* cascade reactions of oxidation and hydrolysis as reported previously.[7a] Thus, the FRET is switched on when the new ratiometric sensor **Cou-Rho-NO** is incubated with NO. Obviously, we should note the emission of the rhodamine acceptor upon excitation at the coumarin donor.

The synthesis of compound **Cou-Rho-NO** started with the preparation of the intermediate rhodamine acid **1a** (Scheme S1†)



Scheme 1 Synthesis of the ratiometric sensor **Cou-Rho-NO**. Reagents and conditions: (a) DCC, DMAP, CH_2Cl_2; (b) phosphorus oxychloride, 1,2-dichloroethane; (c) *o*-diaminobenzene, triethylamine.

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and piperazyl-coumarin **2** (Scheme S2†). Condensation of 3-(diethylamino)phenol **4** with commercially available 1,2,4-benzenetricarboxylic anhydride **5** led to the formation of the mixture of 4′- and 5′-carboxyrhodamines **1a**/**b** (Scheme S1†). However, the separation of the resulting mixture to afford pure regioisomers **1a** and **1b** is very challenging due to their quite similar structures and high polarity. Extensive optimization was performed to identify suitable conditions for the separation, and we eventually found that the mixture could be resolved by preparative thin layer chromatography (CH_2Cl_2 : CH_3OH, 6 : 1). The 4- and 5-position regioisomers **1a** and **1b** were identified by ^1H NMR as they have distinct ^1H NMR profiles, especially for the three phenyl protons (Fig. S1†). The other key intermediate, piperazyl-coumarin **2**, was obtained in two steps using coumarin acid **6** as the starting material (Scheme S2†). Coupling of coumarin acid **6** with *t*-Boc-piperazine **7** followed by deprotection under acidic conditions gave piperazyl-coumarin **2**.

With the key intermediates **1a** and **2** in hand, compound **Cou-Rho-NO** was synthesized in two steps *via* the coumarin-rhodamine intermediate **3** (Scheme 1). Condensation of piperazyl-coumarin **2** with rhodamine acid **1a** by the standard coupling chemistry yielded compound **3**. Interestingly, the coupling almost exclusively occurred at the 4-position carboxylic acid, in accordance with the results of our preliminary studies (Scheme S3†). The high region-selectivity can be ascribed to the less steric hindrance of the 4-position carboxylic acid when compared to the 2-position one. Then, acid **3** was converted into an acid chloride, which was further reacted with *o*-diaminobenzene to afford the desired product, **Cou-Rho-NO**.

Then, we examined the spectral properties of the ratiometric sensor **Cou-Rho-NO** (1 μM) in the absence or presence of NO. As designed, in aqueous PBS buffer, the free sensor only displayed the characteristic absorption peak of the coumarin donor at around 416 nm but no featured absorption band of the rhodamine dye at around 560 nm (Fig. S2†), as the rhodamine acceptor is in the ring-closed form. However, addition of DEA/NONOate (a commercially available NO donor) elicited the formation of a significant absorption peak at around 560 nm, suggesting that the rhodamine acceptor is in the ring-opened form in the presence of NO.

In good agreement with the results of the absorption spectral studies, upon excitation at 410 nm, the free sensor only exhibited the emission peak of the coumarin dye at 473 nm but no typical emission of the rhodamine dye at around 583 nm, as the rhodamine acceptor is in the ring-closed form and there is no FRET in the free **Cou-Rho-NO**. By sharp contrast, as shown in Fig. 1a, addition of NO renders the fluorescence intensity at around 473 nm significantly diminished and simultaneous formation of a new red-shifted emission band at around 583 nm, attributed to the emission of the rhodamine acceptor. A well-defined isoemission point at 548 nm in the emission spectra was observed. Remarkably, there is a *ca.* 420-fold variation in the fluorescence ratio (I_{583}/I_{473}) from 0.061 in the absence of NO to 26.6 in the presence of 100 equiv. NO (Fig. 1b). It should be noted that such a big change of signal ratios at two wavelengths is very desirable for ratiometric fluorescent sensors, as the sensitivity as well as the dynamic range of ratiometric sensors are controlled by the ratios.[8a] The large ratiometric fluorescence response is further

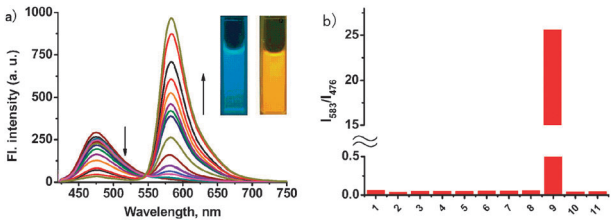

Fig. 1 (a) Emission spectra of **Cou-Rho-NO** (1 μM) in the presence of various amounts of NO (0–100 equiv.). The inset shows the visual fluorescence color of compound **Cou-Rho-NO** (1 μM) before (left) and after (right) addition of 100 equiv. of DEA/NONOate on excitation at 365 nm using a handheld UV lamp. (b) Emission intensity ratio (I_{583}/I_{473}) of **Cou-Rho-NO** (1 μM) in the presence of various reactive oxygen or nitrogen species (100 equiv. for NO and 1000 equiv. for other analytes). 1, blank; 2, H_2O_2; 3, HClO; 4, $O_2^{\bullet-}$; 5, $OH^{\bullet}$; 6, $^1O_2$; 7, NO_3^-; 8, NO_2^-; 9, NO; 10, AA; 11, DHA. The data were collected from the emission spectra excited at 410 nm. The spectra were recorded after incubation of **Cou-Rho-NO** with DEA/NONOate and other analytes for 30 min at 37 °C in PBS (pH 7.4, containing 20% CH_3CN as a cosolvent) with excitation at 410 nm.

supported by a drastic change of emission color from green to orange (the inset in Fig. 1a). The detection limit (S/N = 3) of the ratiometric sensor was determined to be 30 nM, indicating that **Cou-Rho-NO** is potentially useful for monitoring NO production in living cells.

Although the *o*-phenylenediamine-based reaction site has been used in development of fluorescent turn-on type NO sensors, it has not been employed in the context of ratiometric sensor design. Based on the earlier literature report about the turn-on sensing mechanism of *o*-phenylenediamine-based fluorescent turn-on type NO sensors,[7a,f] a likely NO-induced FRET sensing mechanism in **Cou-Rho-NO** is proposed (Scheme S4†). To get insight into the proposed ratiometric sensing mechanism, we decided to monitor the progress of the reaction of **Cou-Rho-NO** with NO by both mass and fluorescence spectroscopy. As shown in Fig. S3†, the free sensor **Cou-Rho-NO** exhibited intense peaks at *m*/*z* 888.5 and 910.5 in the ESI-MS spectrum, corresponding to (**Cou-Rho-NO** + H)$^+$ and (**Cou-Rho-NO** + Na)$^+$, respectively. However, after incubation of NO with the sensor for 0.5 min, a new peak at *m*/*z* 899.4 corresponding to the intermediate triazole was observed (Fig. S4†). Longer incubation time (30 min) led to the complete disappearance of the peak at *m*/*z* 899.4 and the formation of two novel peaks at *m*/*z* 798.5 and 820.3, corresponding to (**3**)$^+$ and (**3** + Na − H)$^+$ (Fig. S5†). In addition, there is a minor time-dependent shift in the emission wavelength from around 600 to 583 nm upon treatment of NO with **Cou-Rho-NO** (Fig. S6†), further supporting the presence of the intermediate triazole in the titration process.[7a] Thus, the results of time-dependent mass and fluorescence spectroscopic studies are consistent with the proposed ratiometric sensing mechanism.

To examine the selectivity, ratiometric sensor **Cou-Rho-NO** was incubated with various reactive oxygen or nitrogen species including H_2O_2, HClO, $O_2^{\bullet-}$, $OH^{\bullet}$, $^1O_2$, NO_3^-, NO_2^-, ascorbic acid (AA), and dehydroascorbic acid (DHA) at up to 1 mM. Importantly, these species do not elicit an observable change in the fluorescence intensity ratio (Fig. 1b), indicating that the ratiometric sensor is highly selective for NO. It is known that

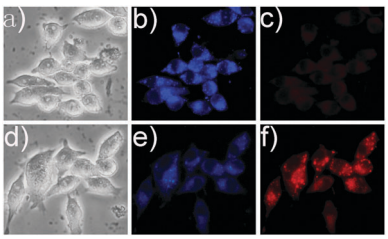

Fig. 2 Images of RAW 264.7 macrophages treated with the ratiometric sensor **Cou-Rho-NO**. (a) DIC image of RAW 264.7 macrophages incubated with only **Cou-Rho-NO** (5 μM); (b) fluorescence image of (a) from blue channel; (c) fluorescence image of (a) from red channel; (d) DIC image of RAW 264.7 macrophages co-incubated with 5 μM ratiometric sensor **Cou-Rho-NO**, 1.25 μg mL^{-1} LPS, and 1000 U mL^{-1} IFN-γ; (e) fluorescence image of (d) from blue channel; (f) fluorescence image of (d) from red channel.

DHA and AA often interfere with the NO detection in *o*-diaminobenzene-based fluorescent turn-on NO sensors.[7b,9] However, it is worthwhile to note that **Cou-Rho-NO** has essentially no ratiometric response to both DHA and AA, further supporting that **Cou-Rho-NO** is a superior sensing agent for specific detection of NO. Furthermore, **Cou-Rho-NO** can respond to NO over a wide pH range from 3 to 10 (Fig. S7†). In addition, the MTT assays (see the ESI†, Fig. S8) suggest that the sensor has low cytotoxicity to the cells.[10]

We then assessed the ability of **Cou-Rho-NO** to operate in live cells. For proof-of-concept, **Cou-Rho-NO** was initially incubated with HeLa cells. Fig. S9† indicates that **Cou-Rho-NO** can penetrate the cell membrane and respond to intracellular NO in a ratiometric fashion. The promising results of the ratiometric imaging of NO in HeLa cells encourage us to further evaluate the feasibility of **Cou-Rho-NO** to detect endogenously produced NO. Macrophage cells can generate NO in micromolar levels when inducible nitric oxide synthase (iNOS) is activated in response to a pathogenic attack.[7g,11] Lipopolysaccharide (LPS) and interferon-γ (IFN-γ) may stimulate expression of the iNOS gene leading to NO production several hours later. The RAW264.7 macrophage cells loaded with **Cou-Rho-NO** (5 μM) displayed a strong blue emission (Fig. 2b) and almost no red emission (Fig. 2c). However, after the RAW264.7 macrophage cells have been co-incubated with LPS (1.25 μg mL^{-1}), IFN-γ (1000 U mL^{-1}), and **Cou-Rho-NO** (5 μM) for 12 h, a decrease in the blue emission (Fig. 2e) and a dramatic enhancement in the red emission (Fig. 2f) was observed. The ratiometric fluorescence images are shown in Fig. S10†. These data indicate that **Cou-Rho-NO** is capable of ratiometric fluorescent imaging of endogenously produced NO. To the best of our knowledge, this represents the first report of ratiometric fluorescent imaging of endogenously produced NO in macrophage cells.

In summary, we have judiciously designed and synthesized **Cou-Rho-NO** as the first small-molecule suitable for ratiometric fluorescent imaging of endogenously produced NO in macrophage cells. In addition, we have preliminarily studied the likely NO-induced ratiometric sensing mechanism of **Cou-Rho-NO**. Remarkably, the novel ratiometric fluorescent sensor exhibited a very large variation (up to 420-fold) in the fluorescence ratio (I_{583}/I_{473}). The other prominent features of **Cou-Rho-NO** include high sensitivity, high specificity, functioning-well at physiological pH, low cytotoxicity, and good cell membrane permeability. Importantly, we have demonstrated for the first time ratiometric imaging of endogenously produced NO in macrophage cells by using the novel ratiometric sensor **Cou-Rho-NO**. We expect that the favorable features of **Cou-Rho-NO** will render it a very useful ratiometric imaging agent for NO detection. Furthermore, the modular nature of the ratiometric sensor design and synthesis holds great promise to access to a wide variety of ratiometric fluorescent sensors by simply replacing the NO reaction site with other reaction sites of interest.

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