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Ratiometric direct detection of nitric oxide based on a novel signal-switching mechanism†

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A novel fluorescent probe, which could be the first example of a ratiometric molecular probe for direct monitoring of NO production, has been developed using a 'spin-exchange' mechanism.

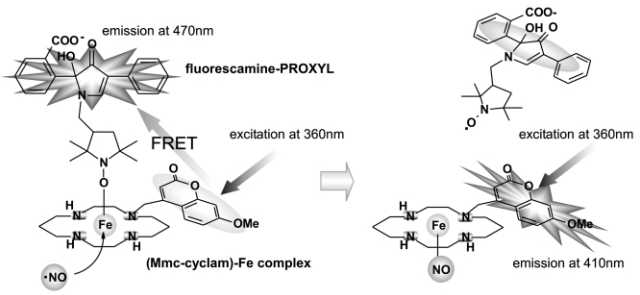
Nitric oxide (NO) has been the focus of keen interest since it was recognized as the endothelium-derived relaxing factor (EDRF) in the 1980s.[1] NO is now thought to play many important physiological roles, acting as a vasodilator, a neuromodulator and an effector molecule in the immune system.[2] NO is a diffusible gaseous free radical, and the molecule thus tends to form various derivatives rapidly in physiological conditions. Since some NO derivatives are thought to display biological actions that are either similar to or different from those of NO,[3] the radical has to be monitored directly near the sites of production and action.

To uncover the biological mechanisms of biomolecules, one of the most promising methods is detection with a fluorescent probe, as demonstrated by the availability of Ca^{2+} probes.[4] The temporal and spatial information given by a NO fluorescent probe would make it possible to clarify unknown roles of endogenous NO in the same way. Thus, many efforts have been made to develop a fluorescent probe for monitoring NO production. Diaminofluorescein (DAF),[5] which is probably most widely used as a useful NO fluorescent probe, increases its fluorescence under NO-generating conditions. However, the probe reacts with NO oxidants such as N_2O_3, but not with NO directly. Fluorescent nitric oxide cheletropic trap (FNOCT) is another unique and intriguing fluorescent probe, which can capture NO directly.[6] However, to increase its fluorescence, the probe requires the following physiological reduction by some reducing agent, such as ascorbic acid. Co(DATI-4), which has aminotroponiminate rings modified with dansyl fluorophores, was also reported as a fluorescent probe for the direct detection of NO.[7] Although this probe is also quite attractive, its use is limited, at this stage, to organic solvents such as dichloromethane.

Recently, we proposed a new type of NO fluorescent probe, which is composed of a dithiocarbamate–Fe complex and 2,2,6,6-tetramethylpiperidine-*N*-oxide (TEMPO) labeled with acridine, and confirmed that the probe could detect NO directly in aqueous conditions.[8,9] Unfortunately, however, the probe quenches its fluorescence with the capture of NO. Herein, we report a novel NO fluorescent probe that reacts with NO directly and exhibits a shift in its fluorescent spectrum with the capture of NO. A detection system, composed of dye-labeled cytochrome *c* attached to an optical fiber along with fluorescent microspheres, was reported only for the ratiometric detection of NO.[10] Therefore, our fluorescent probe would be the first example of a ratiometric molecular probe that can detect NO directly.

The design of this novel fluorescent probe was based on the spin-exchange theory, which was reported in our previous papers.[9,11] The theory was inspired by the activation mechanism of guanylyl cyclase, which has both a heme moiety and an imidazole moiety in the regulatory domain. In guanylyl cyclase, NO binds strongly to the heme iron to make the nitrosyl–Fe bond and releases the imidazole moiety coordinated to the central iron.[12] We previously prepared the complex (dithiocarbamate–Fe–TEMPO) as a novel spin probe for NO by replacing the heme moiety and the imidazole moiety in the enzyme with dithiocarbamate–Fe complex and TEMPO, respectively, and then confirmed the TEMPO release from the dithiocarbamate–Fe–TEMPO complex with the NO binding to the central iron.[9,11] In the present study, to create a ratiometric NO fluorescent probe, we replaced the heme moiety and the imidazole moiety in guanylyl cyclase with an iron complex of a fluorescent methoxycoumarin-pendant cyclam (Mmc-cyclam) and 2,2,5,5-tetramethylpyrrolidine-*N*-oxide (PROXYL) labeled with fluorescamine (fluorescamine-PROXYL), respectively (Scheme 1).

The Mmc-cyclam was synthesized by the reaction of cyclam with 4-bromomethyl-7-methoxycoumarin (Br-Mmc) in dichloromethane at room temperature.‡ When iron sulfate was added to Mmc-cyclam in 100 mM phosphate buffer solution (pH 7.4) at a final concentration of 40 μM each, the fluorescence intensity at 410 nm from the Mmc moiety gradually decreased to 0.71-fold with excitation at 360 nm, probably due to the electronic interaction between the iron and the Mmc-cyclam as the complex formed. The fluorescamine-PROXYL was then added at a final concentration of 40 μM to the resultant solution containing the iron complex of Mmc-cyclam for the preparation of the fluorescent probe. Fig. 1 shows emission spectra of the iron complex of Mmc-cyclam before and after the addition of fluorescamine-PROXYL with excitation at 360 nm. With the addition of fluorescamine-PROXYL, the fluorescence intensity at 410 nm derived from the Mmc fluorophore was decreased. On the other hand, the fluorescence intensity at 470 nm from the fluorescamine fluorophore was simultaneously increased. This result suggested that fluorescamine-PROXYL coordinated to the iron complex of Mmc-cyclam through the redox interaction between the nitroxide radical of fluorescamine-PROXYL and the central iron of the complex; in the previous study[9,11] 2,2,6,6-tetramethyl-4-hydroxylpiperidine-*N*-oxide (TEMPOL) coordinated to the dithiocarbamate–Fe complex and, as a result, fluorescence resonance energy transfer (FRET) was induced from the Mmc donor to the fluorescamine acceptor. FRET, which is a radiationless transmission of an energy quantum, is



Scheme 1 Schematic illustration of detection mechanism of NO using the novel probe.

† Electronic supplementary information (ESI) available: Fig. S1. See http://www.rsc/org.suppdata/cc/b2/b207726d/

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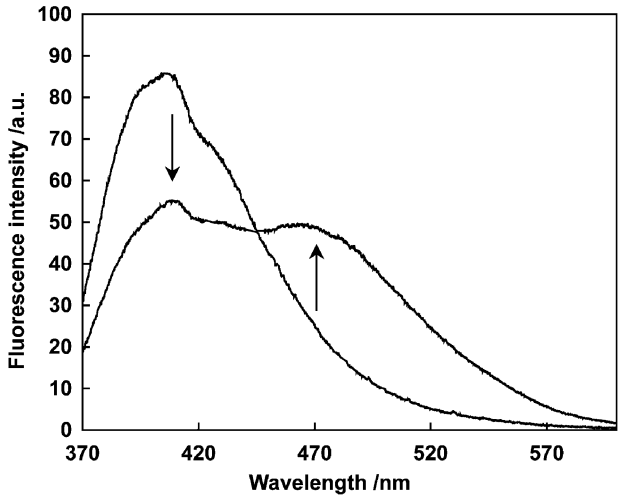

Fig. 1 Emission spectra of (Mmc-cyclam)–Fe complex (40 μM) before and after the addition of fluorescamine-PROXYL (40 μM) with the excitation at 360 nm in 100 mM anaerobic sodium phosphate buffer (pH 7.4) at 37 °C.

known to occur when two fluorophores having an overlap in their emission and excitation spectra are close to each other. The emission spectrum of the Mmc fluorophore (λ_{em} = 410 nm) overlaps well with the excitation spectrum of the fluorescamine fluorophore (λ_{ex} = 385 nm), thus inducing effective FRET between the two fluorophores. Finally, the fluorescence intensities at 410 nm and 470 nm were 0.65-fold and 1.92-fold, respectively.

To investigate the detection ability of the prepared fluorescent probe, we added NOC-7,[13] a spontaneous NO-releasing agent, to the probe solution and monitored the emission spectrum excited at 360 nm. As shown in Fig. 2, the fluorescence intensity at 410 nm was increased, while that at 470 nm was decreased, both gradually. After 60 min, the fluorescence intensities at 410 nm and 470 nm were 1.17-fold and 0.75-fold, respectively. Fig. S1 (ESI†) shows the time course of the emission ratio at 410 nm to 470 nm with the addition of NOC-7. The ratio increased gradually, tracing a smooth curve, and corresponded with the rate of NO generation from NOC-7, as estimated from the absorption changes at 250 nm. These results demonstrate that NO was bound to the iron moiety of the probe to release the fluorescamine-PROXYL according to the spin-exchange mechanism as shown in Scheme 1. Consequently, the FRET from the Mmc donor to the fluorescamine acceptor was canceled because the two fluorophores could not interact with each other. NO binds to the iron moiety of the fluorescent probe so tight that the spin-exchange reaction is practically irreversible. The reaction rate of NO with the iron complex is so fast that the probe can capture NO efficiently. No significant change in fluorescence was found when NOC-7 was added to a solution containing either Mmc-cyclam or fluorescamine-PROXYL. The results also support the suggestion that the fluorescent change was actually caused by the spin-exchange mechanism. The changes of the probe's emission ratio at 410 nm to 470 nm correspond to the concentrations of NO and good reproducibility was found in the detection system using the fluorescent probe. Bubbling of the probe solution with carbon dioxide or an addition of other oxides of nitrogen did not affect the probe's fluorescence. Excess molecular oxygen changes the probe's fluorescence, however, the interference is so little that it can be ignored for detecting NO under normal experimental conditions. Using the probe system, less than 100 nM of NO could be detected.

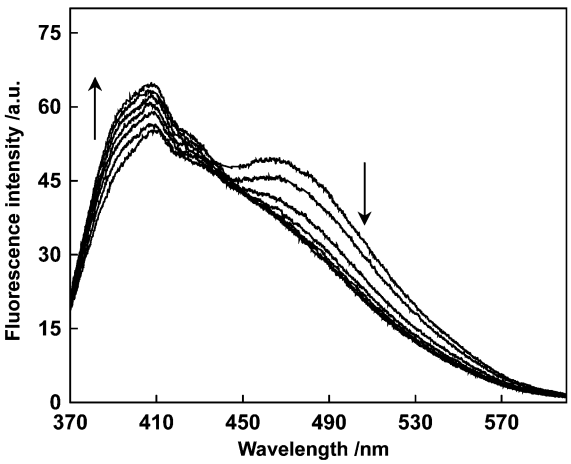

Fig. 2 Emission spectra of the fluorescent probe (40 μM) excited at 360 nm after 0, 10, 20, 30, 40, 50, or 60 min from the addition of NOC-7 (160 μM). The measurements were performed in 100 mM anaerobic sodium phosphate buffer (pH 7.4) at 37 °C.

In conclusion, we designed a novel NO fluorescent probe using the spin-exchange mechanism and demonstrated the probe's detection ability. The probe is quite attractive because it makes possible both the direct detection of NO in an aqueous solution and the ratiometric monitoring of NO production. Normal fluorescent probes, which simply change their fluorescence intensity, tend to be affected by various factors, such as probe concentration and environmental conditions (temperature, pH, *etc.*). In ratiometric measurement, on the other hand, the fluorescent change due to the concentration change of the target can be detected independently of such factors. Thus, our ratiometric probe may be an innovative tool for monitoring endogenous NO more precisely and effectively.

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Notes and references

‡ Synthesis of Mmc-cyclam: 5 mg (18.6 μmol) of Br-Mmc and 7.5 mg (37.2 μmol) of cyclam were mixed in 13 ml of dichloromethane containing 2.5 μl (18.6 μmol) of *N*,*N*-diisopropylethylamine at room temperature in the dark overnight. Dichloromethane was removed *in vacuo* and the residue was purified by reversed-phase HPLC (methanol was used as an eluent). The structure of Mmc-cyclam was confirmed by ^{1}H-NMR and MALDI-TOF/MS.

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