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A ratiometric fluorescent probe for imaging hydroxyl radicals in living cells†

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A novel fluorescent probe, the detection mechanism of which is based on the 'on-off' switching of a FRET triggered by the ·OH-induced cleavage of a DNA strand, has been developed for the ratiometric imaging of ·OH.

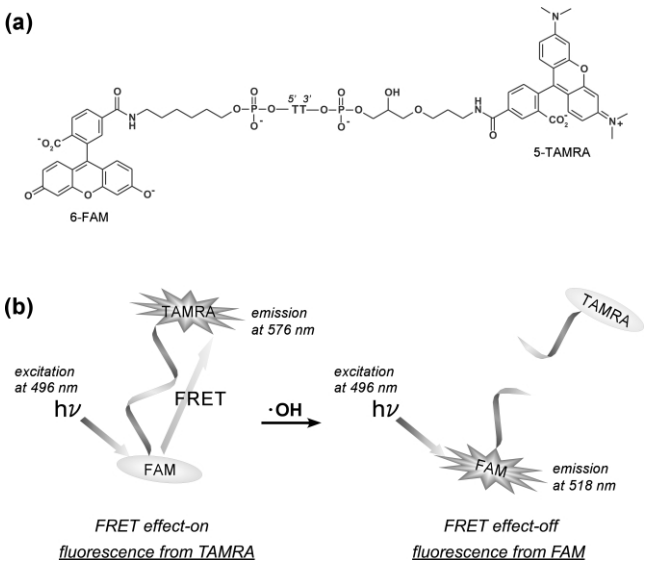
Oxygen-derived species have been implicated as damaging agents in toxicology and pathology.[1] In particular, the hydroxyl radical (·OH) is highly reactive and contributes to ongoing DNA damage in cellular systems. It is now becoming clear that ·OH is involved in many biological processes such as the redox alteration of cell-membrane channels, apoptosis and T cell activation.[2] As a result, the biological roles of ·OH have been the subject of considerable attention.

To elucidate the unknown biological roles of ·OH, an accurate and selective method to detect ·OH in various biological systems would be desirable. Though an electron paramagnetic resonance (EPR) method using spin trapping agents such as 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) is a major technique that is currently used for detecting ·OH,[3] the method cannot be applied to the real-time imaging of ·OH in single cell due to the low space resolution. On the other hand, a fluorimetric detection method using a fluorescent molecular probe should be ideal for this purpose with respect to sensitivity as well as space resolution since fluorescent microprobes with high space resolution are currently available. For example, many types of fluorescent probes for detecting nitric oxide (NO), a reactive radical species that functions as an important messenger molecule in biological systems, have been developed in attempts to clarify the physiological roles of NO.[4] Meanwhile, a few fluorescent probes have been reported for monitoring ·OH production. Makrigiorgos *et al*. synthesized the succinimidyl ester of coumarin-3-carboxylic acid (SECCA) and applied it to the detection of ·OH produced by ionizing radiation.[5] The probe reacts preferably with ·OH and is converted into a fluorescent 7-hydroxycoumarin adduct. Nagano and co-workers recently designed and synthesized 2-[6-(4′-hydroxy)phenoxy-3*H*-xanthen-3-on-9-yl]benzoic acid (HPF) and 2-[6-(4′-amino)phenoxy-3*H*-xanthen-3-on-9-yl]benzoic acid (APF) as novel fluorescence probes for the selective detection of highly reactive oxygen species (hROS) including ·OH and reactive intermediates of peroxidase.[6] Unfortunately, however, a fluorescence probe, which simply changes its fluorescence intensity by reacting with a target molecule, tends to be affected by various factors, such as the probe concentration and environmental conditions (temperature, pH, *etc.*). In order to resolve this difficulty, a ratiometric measurement based on changes in the ratio of fluorescence intensity would be ideal because the change in concentration of the target molecule can be detected independent of such factors. Herein, we wish to report on a novel fluorescent probe that can be excited by visible light and can be used to detect ·OH ratiometrically with a high selectivity.

The cleavage of a DNA strand is one of the major reactions in ·OH chemistry. Meneghini and co-workers reported that single-stranded DNA was broken by ·OH generated by a Fenton-like reaction between intracellular Fe(II) and hydrogen peroxide.[7] Dervan reported on the sequence-specific cleavage of double helical DNA using a homopyrimidine oligodeoxyribonucleotide coupled with an EDTA–Fe(II) complex, from which ·OH is generated under aerobic conditions.[8] From another point of view, the reaction in which DNA is cleaved by ·OH might be used to develop a novel fluorescent probe for the detection of ·OH. Therefore, we designed probe **1** as a novel fluorescent probe: the deoxythymidine dimer, the 5′-end and 3′-end of which were combined with fluorescent 6-carboxyfluorescein (6-FAM) (λ_{ex} = 496 nm, λ_{em} = 518 nm) and with fluorescent 5-carboxytetramethylrhodamine (5-TAMRA) (λ_{ex} = 558 nm, λ_{em} = 576 nm), respectively. When the probe is excited at 496 nm, which is the excitation wavelength for FAM, the probe would emit fluorescence derived from TAMRA, because fluorescence resonance energy transfer (FRET) should take place between the two adjacent fluorophores. However, when the probe is reacted with ·OH and the phosphate linker in the probe is cleaved, the fluorescent signal from FAM would be observed due to the cancellation of the FRET effect (Scheme 1). Therefore, the ratiometric detection of ·OH might be possible using probe **1**, based on the 'on-off' switching of FRET.

Probe **1** was custom-made by the Hokkaido System Science Co. Ltd. (Hokkaido, Japan). When a 200 mM phosphate buffer solution (pH 7.4) containing probe **1** at a concentration of 50 μM was excited at 496 nm, a strong fluorescence derived from TAMRA was observed at a wavelength of 576 nm, while a very weak fluorescence derived from FAM was observed at 518 nm. TAMRA is not excited directly at a wavelength of 496 nm. Therefore, the result suggests that the intramolecular FRET from FAM to TAMRA occurred effectively in probe **1**, as expected.

In the next step, the fluorescence properties and reactivity of probe **1** with ·OH were examined. In order to generate ·OH in a sample solution, Fenton's reagent (a solution of $FeSO_4$ and H_2O_2) was used. A 200 mM phosphate buffer solution (pH 7.4) containing probe **1** at a concentration of 50 μM was incubated at 25 °C for 15 min after the addition of the mixed solution of $FeSO_4$ (10–100 mM)



Scheme 1 Chemical structure of probe **1** (a), and schematic illustration of the detection mechanism of ·OH using the probe (b).

† Electronic supplementary information (ESI) available: relation between the concentration of Fenton's reagent and the fluorescence intensity ratio at 518 to 576 nm for probe **1**. See http://www.rsc.org/suppdata/cc/b3/b314204c/

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and H_2O_2 (100 nM–1 M) (total volume of the sample was 4 μL). Each sample solution was diluted with 200 mM phosphate buffer (pH 7.4) to a final volume of 500 μL, and a fluorescence spectrum was obtained for the diluted sample solution. As shown in Fig. 1, after the incubation of probe **1** with Fenton's reagent, the fluorescence intensity at 576 nm (I_{576}) derived from TAMRA decreased, while the fluorescence intensity at 518 nm (I_{518}) derived from FAM increased. When a 200 mM phosphate buffer (pH 7.4) containing FAM or TAMRA was incubated with Fenton's reagent, as was done for the probe **1** solution, no significant change in the fluorescence spectrum was observed. These results suggest that the linker for the two fluorophores in probe **1** was cleaved by a reaction with ·OH, generated from the Fenton's reagent and, as a result, the intramolecular FRET was cancelled because the two fluorophores were not able to interact with each other. The change in the fluorescence intensity from probe **1** increased with an increase in the concentration of Fenton's reagent. Fig. S1 (ESI†) shows the relationship between the fluorescent ratio (I_{518}/I_{576}) of a solution of probe **1** and the concentration of $FeSO_4$ in the added Fenton's reagent. As can be seen from Fig. S1, a good linear correlation was observed between them in the concentration of range less than 100 μM. Therefore, probe **1** can be used for the quantitative determination of ·OH. At concentration ranges higher than 100 μM, the ratio of fluorescence intensity is saturated indicating that nearly all of probe **1** in the sample solution had reacted with ·OH and the linker of the probe was cleaved. In the case that a probe **1** solution at lower concentrations (1–25 μM) was used for the reaction with Fenton's reagent (100 μM $FeSO_4$, 1 mM H_2O_2), the increase of fluorescent ratio (I_{518}/I_{576}) was also observed.

The selectivity of probe **1** for ·OH is very important, especially when probe **1** is applied to *in vivo* cellular systems. In the case of the *in vitro* experiment, probe **1**, fortunately, showed no significant fluorescence changes when DNase I was added. The length of the DNA chain in probe **1** is very short and the native structural property of DNA may be absent in probe **1** as the result of modification by the two fluorophores. Namely, the enzyme, DNase I, was not able to recognize probe **1** as a substrate. No significant fluorescent changes were observed when probe **1** was treated with 3T3 cell lysates (total protein concentration: 1 mg mL^{-1}). The result suggested that probe **1** was not cleaved by various kinds of nuclease, which were contained in the cell lysates. In addition, probe **1** also showed no significant fluorescence changes when other reactive oxygen species such as peroxynitrite ($ONOO^-$), superoxide ($O_2^{-\cdot}$), hydrogen peroxide (H_2O_2), nitric oxide (NO), alkylperoxyl radical ($ROO^{\cdot}$), or hypochlorite (OCl^-)‡ were added. Therefore, it can be concluded that probe **1** has good selectivity for ·OH.

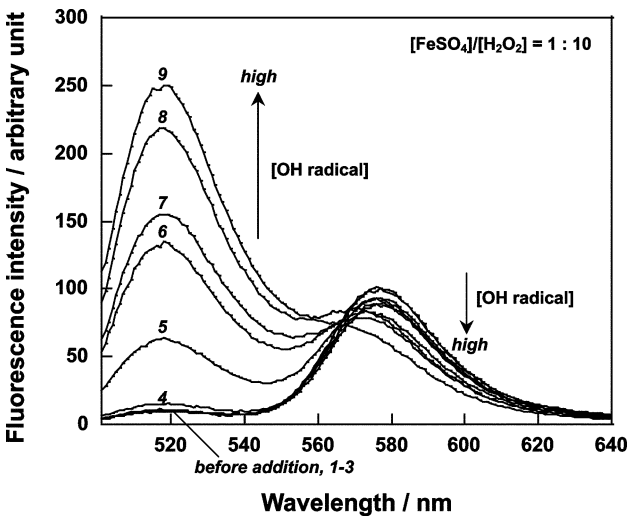

Fig. 1 Fluorescence spectra of probe **1** after the addition of Fenton's reagent at various concentrations. Fenton's reagent {[$FeSO_4$]/[H_2O_2]: (1) 10 nM/100 nM, (2) 100 nM/1 μM, (3) 1 μM/10 μM, (4) 10 μM/100μM, (5) 50 μM/500 μM, (6) 100 μM/1 mM, (7) 1 mM/10 mM, (8) 10 mM/100 mM, (9) 100 mM/1 M} was added to a solution of probe **1** (50 μM) at the total volume of 4 μL and incubated at 25 °C for 15min. The samples were diluted with 200 mM phosphate buffer (pH 7.4) to a final volume of 500 μL and the fluorescence spectra of the probe solutions were obtained by excitation at a wavelength of 496 nm.

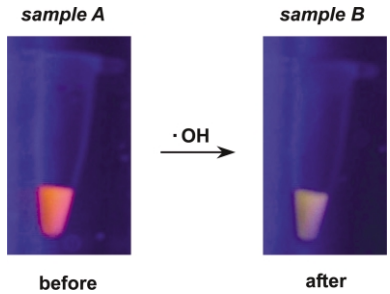

Fig. 2 Photo of probe **1** solution before (sample A) and after (sample B) the generation of ·OH. Sample A was prepared by diluting probe **1** (100 μM, 4 μL) with a 200 mM phosphate buffer solution (pH 7.4) to a final volume of 40 μL. Sample B was prepared by adding Fenton's reagent (20 mM $FeSO_4$, 200 mM H_2O_2) to the probe **1** solution (100 μM, 4 μL) and by incubating at 25 °C for 15 min, and then by diluting with a 200 mM phosphate buffer solution (pH 7.4) to a final volume of 40 μL. The photo was taken by irradiating the samples at a wavelength of 365 nm by means of a UV transilluminator.

Fig. 2 shows the fluorescent images for a probe **1** solution before and after the generation of ·OH. The red fluorescence from probe **1** in 200 mM phosphate buffer (pH 7.4) turned to yellow upon the addition of the Fenton's reagent, when the probe **1** solution was excited at 365 nm with a UV transilluminator (Photo-Print 2000 XDSP, Cosmo Bio). As shown in Fig. 2, the generation of ·OH can be readily visualized using probe **1**. Therefore, probe **1** would be expected to be suitable for the real-time imaging of ·OH in living cells.

In conclusion, the selective and ratiometric detection of ·OH was achieved by using a novel fluorescent probe, probe **1**. The probe reported here represents the first example, in which the ratiometric detection of ·OH is realized. We are currently applying the probe to the real-time imaging of ·OH in living cells, and our results will be reported in the near future.

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Notes and references

‡ The following reagents were used for donors of reactive oxygen species:[6] SIN-1 for $ONOO^-$, KO_2 for $O_2^{-\cdot}$, NOC7 for NO, 2,2-azobis(2-amidinopropane)dihydrochloride for $ROO^{\cdot}$, NaOCl for OCl^-.

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