TEMPO-based Redox-sensitive Fluorescent Probes and Their Applications to Evaluating Intracellular Redox Status in Living Cells

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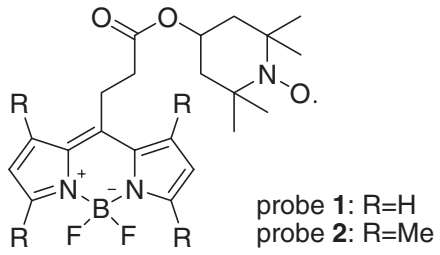
Two redox-sensitive fluorescent probes were developed for evaluating the intracellular redox status of living cells as a complement to the widely used dye 2′,7′-dichlorodihydrofluorescein (DCFH).

A reducing intracellular environment is necessary for living cells. There are many redox couples existing in living cells, and they work together with antioxidant enzyme systems to maintain the balance of the intracellular redox status.[1] It is becoming clear that the redox status plays important roles in many cellular processes, such as DNA synthesis, transcriptional activation, enzyme kinetics, protein folding, and transport.[2] Disorder of the cellular redox status is thought to be associated with various pathological conditions. Fluorescent probes are promising tools for the exploration of the world inside cells. So far, many fluorescent probes have been designed to detect various reactive oxygen species (ROS),[3] but few probes are effective for evaluating the reducing intracellular environment.[4] In living cells or tissues, the intracellular ROS are usually scavenged by antioxidants or enzyme systems of the cells rather than being trapped by probes when they are used at low concentration or they are not sufficiently reactive. DCFH is a commonest probe used by biologists to reflect the levels of ROS in living cells. Caution must be taken when interpreting the results from this kind of probe, because so many aspects other than ROS can lead to the fluorescence intensity increase.[5] Therefore, it is necessary to develop new probes as a supplement in order to achieve reliable results.

We herein report two redox-sensitive fluorescent probes **1** and **2** (Scheme 1), which were easily synthesized (see Supporting Information).[6] These two probes were designed on the basis of the fluorophore–nitroxide mechanism. This kind of probe molecule is composed of a nitroxide moiety and a fluorophore.[7] They can serve as fluorescent probes for radicals and redox-active species.[8] Some of them have been used for analysis of vitamin C (VC)[9] and for investigation of antioxidant capability.[10] Stable nitroxyl radicals can be applied as spin labels.[11] They can be rapidly reduced in living cells and tissues into hydroxylamines[12] and have been used as contrast agents for magnetic resonance imaging[13] and as probes for the intracellular redox status of tumors.[14] While a fluorophore and a nitroxide are linked at an appropriate distance, the fluorescence of the fluorophore can be effectively quenched by the nitroxide moiety. However, when the nitroxide moiety is reduced to hydroxylamine by antioxidants or living cells, the fluorescence extensively increases, providing information about the redox environment of the cells.



Scheme 1.

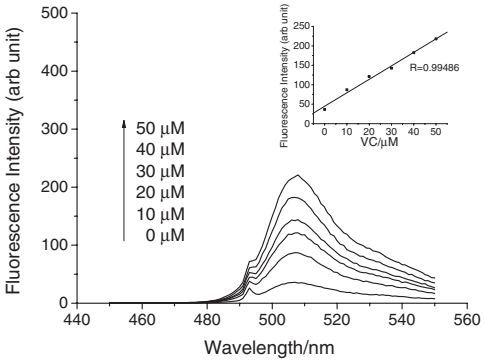


Figure 1. Fluorescence spectra of probe **1** (final concentration: 10 μM) in 0.1 M phosphate buffered saline (PBS, pH 7.4) recorded 30 min after the addition of VC (ranging from 0–50 μM). The fluorescence intensity was determined with excitation at 491 nm. Inset: Fluorescence intensity ($\lambda_{ex} = 491$ nm, $\lambda_{em} = 507$ nm) plotted against the concentration of VC.

First, we investigated the reactivity of probe **1** toward common antioxidants in biological systems, such as VC and glutathione (GSH). The probe was low-fluorescent before reacting with VC, but the fluorescence intensity increased dramatically upon addition of VC. As indicated in Figure 1, a linear correlation exists between the fluorescence intensity and the VC concentration (ranging from 0 to 50 μM).[15] We also determined the reaction rate constants of the probes with VC,[6] which exhibited their sensitivity to the change of redox state.

ESR signal of probe **1** (final concentration: 10 μM) in 0.1 M PBS (pH 7.4) was recorded 0, 10, 20, and 30 min after the addition of 100 μM VC. As shown in Figure 2, the intensity of the ESR signal decreased with time. The reaction between probe **1** and GSH was very slow.[12] 24 h after the addition of 0–50 μM GSH to the probe **1** solution, a concentration-depended increase of the fluorescent intensity was observed (Figure 3). Addition of cells also led to the increase of fluorescence intensity of the probe solution (Figure S4).[6]

Next, we applied the new dyes to living cells. Dichlorofluorescin diacetate (DCFH-DA) was used to reflect the total ROS levels in the cells. A group of human hepatocellular carcinoma (HepG2) cells were treated with 100 μM H_2O_2 for 30 min before the addition of dyes. Cytometry results both from probe **1** and from DCFH exhibited an increase of oxidative stress of the HepG2 cells (Figure 4). So these dyes could be useful probes for flow cytometry which allows direct characterization and

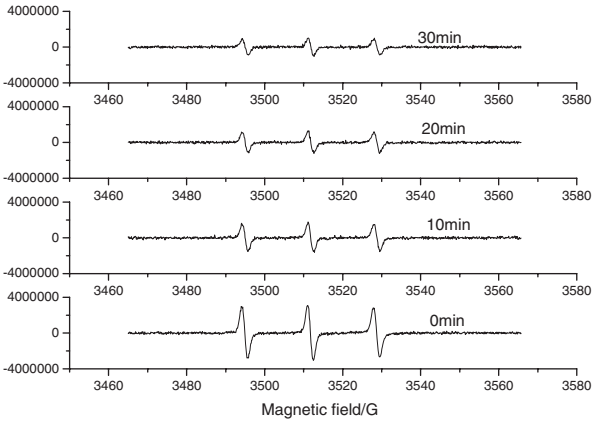


Figure 2. ESR intensity of probe **1** (final concentration: 10 μM) in 0.1 M PBS (pH 7.4) recorded 0, 10, 20, and 30 min after the addition of 100 μM VC.

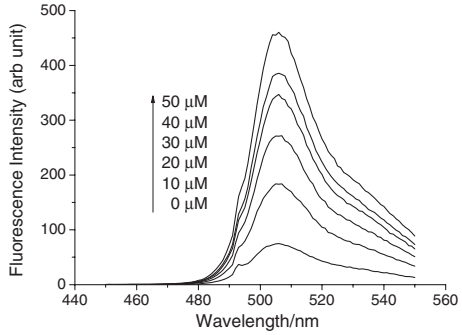


Figure 3. Fluorescence spectra of probe **1** (final concentration: 10 μM) in 0.1 M PBS (pH 7.4) recorded 24 h after the addition of 0–50 μM GSH.

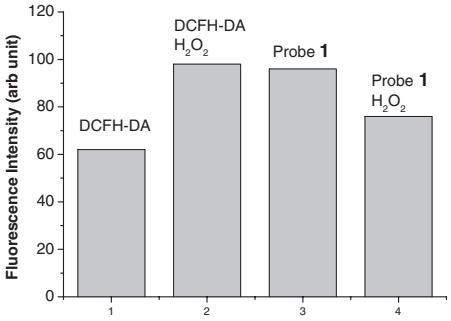


Figure 4. Relative fluorescence intensity of HepG2 cells incubated with DCFH-DA or probe **1** obtained by flow cytometry. From left to right: HepG2 cells treated with PBS (DCFH-DA); HepG2 cells treated with 100 μM H_2O_2 (DCFH-DA H_2O_2); HepG2 cells treated with PBS (Probe **1**); HepG2 cells treated with 100 μM H_2O_2 (Probe **1** H_2O_2).

analysis of several parameters and functions of intact living cells in a few seconds.

We chose probe **2** for microscopy imaging experiments because it is more stable under irradiation than probe **1**. Consistent with the cytometry results, HepG2 cells without treatment with H_2O_2 gave a brighter image than those treated with 100 μM H_2O_2 (Figure 5).

In conclusion, we developed two new fluorescent probes **1** and **2**, which were sensitive to reducing environment. In living cells, the nitroxide moiety of the probes is reduced to hydroxylamine quickly and becomes highly fluorescent. The newly synthesized probes were used to evaluate antioxidant capability of antioxidants, VC and GSH, and the redox status of living cells. Now we are investigating the changes of the redox status of some cancer cells using the present probes.

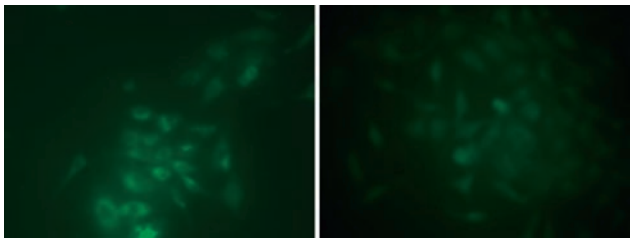


Figure 5. Fluorescence images of live HepG2 cells. (a) Fluorescence image of HepG2 cells incubated with 5 μM probe **2** for 10 min at 25 °C. (b) Fluorescence image of HepG2 cells which were treated with 100 μM H_2O_2 for 5 min at 25 °C before stained with probe **2** for 10 min at 25 °C. Excitation was provided at 488 nm.

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6 Supporting Information is available electronically on the CSJ-Journal Web site, http://www.csj.jp/journals/chem-lett/index.html. Additionally, CCDC-716606 contains the supplementary crystallographic data for compound **2**. These data can be obtained free of charge from The Cambridge Crystallographic Data Center via www.ccdc.cam.ac.uk/data_request/cif.
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15 It was also found that the concentration of VC (at 2–10 mM) is proportional to the rate constant of the increase in fluorescence.

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