Strong red fluorescent probes suitable for detecting hydrogen peroxide generated by mice peritoneal macrophages†

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This paper reports the synthesis, fluorescence properties, and biological applications of naphthofluorescein disulfonate (NFDS-1), as a red fluorescence imaging probe to detect intracellular H_2O_2.

The far-visible and the near-IR spectral regions (600–1000 nm), where only a few classes of molecules exhibit significant absorption, and they do not contribute to the fluorescence signal, are areas of low background fluorescence interference in biological systems.[1,2] The features of the particular spectral region make it ideal for using a fluorogenic probe to detect reactive oxygen species (ROS) in living cells. However, as far as we know, only one probe in this region has been reported so far, for detecting nitric oxide.[2]

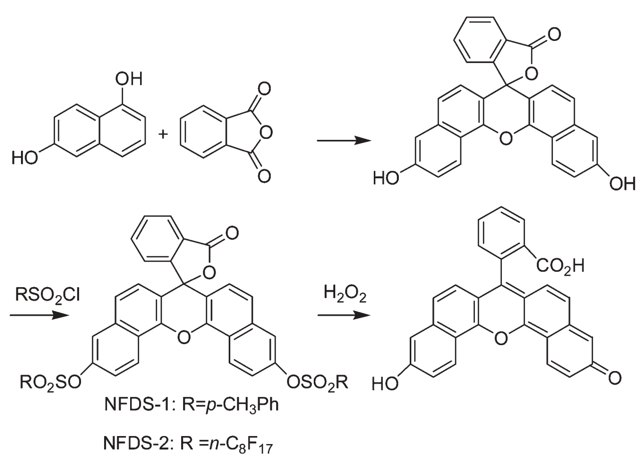
In the current work, we aim to develop molecular probes that could be used to detect reactive oxygen species in this spectral region. Reactive oxygen species such as superoxide radical anion, hydrogen peroxide, hydroxyl radical, nitric oxide and peroxynitrite play a vital role in physiology.[3] A rapid rise in intracellular oxidant levels under oxidative stress could cause damage to biological molecules and result in various diseases.[4–6] Hydrogen peroxide is the precursor to other ROS, and its homeostasis can have diverse physiological and pathological consequences.[7] However, how this damage occurs is insufficiently understood even in the simplest eukaryotic organisms.[8]

In order to explore the role of hydrogen peroxide in toxicology and human diseases, it is necessary to establish an accurate way of detecting the ROS, especially in living cells. Many probes such as dihydro-analogues of fluorescent dyes [*e.g.* dichlorofluorescin diacetate (DCFDA), dihydrorhodamine 123,[9,10] phosphine-based fluorophores,[11,12] lanthanide coordination complexes,[13] and chromophores with ROS-cleavable protecting groups[14,15]] have been developed in recent years. DCFDA is the most popular one, and has been used frequently to detect cell-derived H_2O_2,[9] but it suffers from a major drawback in that it is poorly selective toward H_2O_2 owing to its autoxidation and reaction with other ROS or peroxidase.

Recently, an interesting method for H_2O_2 detection was developed based on the selective H_2O_2-mediated transformation of monopentafluorobenzenesulfonyl fluorescein to fluorescein.[15] The method relies on simple deprotection, not on oxidation, which allows the highly specific and peroxidase-independent detection of H_2O_2 under the complicated oxidative circumstances. However, there are two disadvantages in the fluorescein-based probe: the weak fluorescence of the probe itself and the high background fluorescence interference in biological systems reduced measurable sensitivity (the detection limit is 92.3 nmol or higher). *In vivo*, hydrogen peroxide concentrations are usually considered to be in the lower nanomolar range. Therefore, it is necessary to develop probes that have higher selectivity and enough sensitivity for H_2O_2.

We designed and synthesized naphthofluorescein disulfonates (NFDS-**1**, NFDS-**2**, Scheme 1) as fluorescence imaging probes for intracellular H_2O_2, which were characterized with elemental analysis, IR, and ^{1}H NMR. NFDS are closed, colorless, and non-fluorescent lactones. Upon treatment with H_2O_2, hydrolytic deprotection of NFDS would subsequently generate open, colored, and fluorescent products.

The NFDS-**2** was chosen for the following reasons: First, owing to the effect of perfluorooctanesulfonic acid, a surfactant generated from the hydrolysis of NFDS-**2** in the reactive circumstances, the excitation and emission spectra of the product, naphthofluorescein, generated from reaction of NFDS-**2** with H_2O_2 would have a red shift compared with NFDS-**1**, theoretically. Second, the perfluoro-octane chain would enhance the reactivity of the NFDS-**2** toward H_2O_2 owing to the lipophilic characteristics of fluorine atoms and hydrogen peroxide.



Scheme 1 The synthesis of fluorescent probes and their reaction with H_2O_2.

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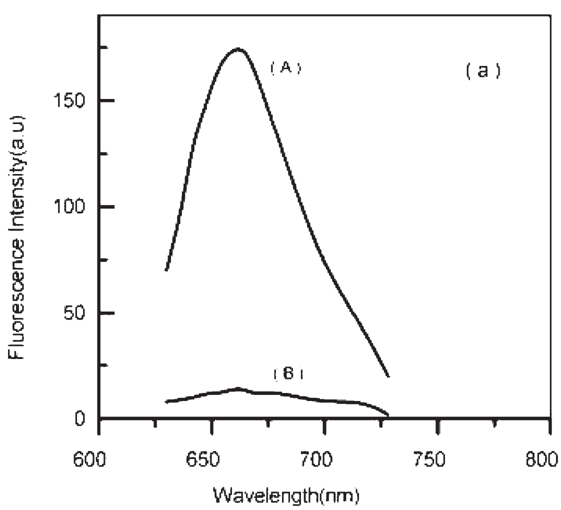
NFDS-**1** and NFDS-**2** were evaluated under chemical circumstances. A solution of NFDS-**1** (50 μM) or NFDS-**2** (50 μM) in DMSO was diluted 10 times with 2-[4-(hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) buffer (0.1 M, pH 7.4). Treatment of each of the solutions (5 μM) containing the probe compounds with H_2O_2 (1 μM) at 37 °C for 40 min gave fluorescence responses, Fig. 1. With different concentrations of H_2O_2 we obtained the NFDS-**1** linear calibration curve from 6.0×10^{-9} to 4.0×10^{-6} M and the detection limit (81.5 pM) which was lower than for reported probes (92.3 nM).[15] We believe that the lower detection limit of NFDS-**1** for H_2O_2 was based on its low background interference and the non-fluorescence of the probe itself. The experiments also showed that the fluorescence property of the product generated from reaction of NFDS-**2** with H_2O_2 is not stable enough owing to the concentration change of the surfactant, perfluorooctanesulfonic acid, generated from the hydrolysis of NFDS-**2**, as shown in Fig. 1(b). Therefore, in the following experiments, we have focused our attention on NFDS-**1**.



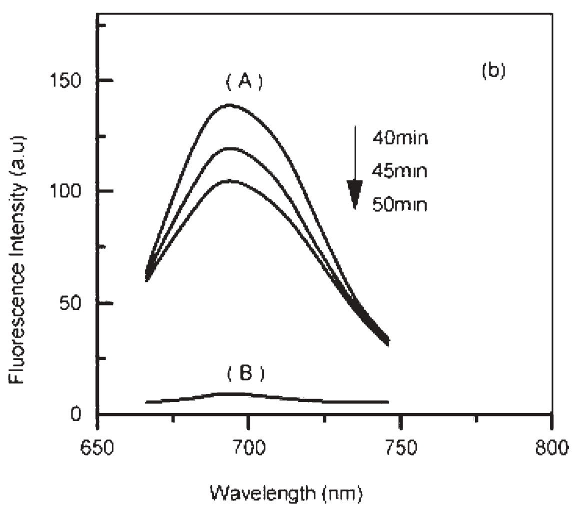


Fig. 1 (a) Emission spectra of 5 μM NFDS-**1** with 1 μM H_2O_2 (A) and blank (B) in HEPES (pH = 7.4) at 37 °C for 40 min (λ_{em} = 662 nm). (b) Emission spectra of 5 μM NFDS-**2** with 1 μM H_2O_2 (A) at 37 °C for 40 min, 45 min and 50 min, and blank (B) at 37 °C for 40 min (λ_{em} = 692 nm).

The reactivity of NFDS-**1** toward various ROS, reductant, glutathione (GSH), and esterase was studied in detail. The fluorescent response from the solution of NFDS-**1** (10 μM, in HEPES buffer) with H_2O_2 (2 μM) at 662 nm with excitation at 602 nm after incubation at 37 °C for 40 min was compared to those of reactions with NaOCl, t-BuOOH, esterase, 1,4-hydroquinone, GSH, 3-(aminopropyl)-1-hydroxy-3-isopropyl-2-oxo-1-triazene (NOC-5), 3-morpholinosydnonimine (SIN-1), and ascorbic acid (Vc) (final 10 μM each), and with superoxide radical anion or hydroxyl radical. Superoxide radical anions were generated by the enzymatic reaction of hypoxanthine (HPX, 1 ml, 10 μM) with xanthine oxidase (XO, 0.2 ml, 1 U ml^{-1}) or KO_2 (10 μM). The Fenton reaction between H_2O_2 (5 μM) and Fe^{2+} ion (10 μM) was used as the source of $^{\bullet}OH$. All results are summarized in Table 1. The experiments showed that NFDS-**1** provided a highly specific fluorescent response toward H_2O_2, while giving a small response to ascorbic acid, glutathione, esterase, and other ROS, especially to hydroxyl radical. We suggest that the observed selectivity of NFDS-**1** for H_2O_2 over more oxidizing ROS is based on simple deprotection, not on an oxidative mechanism.

We next assessed the reaction of NFDS-**1** for H_2O_2 generated in living cells. Separated mice peritoneal macrophages (PM) were seeded onto a glass slide. Then the cells were loaded with NFDS-**1** (10 μM, DMSO–HEPES buffer, pH 7.4) by incubation at 37 °C for 30 min and showed negligible intracellular background fluorescence, Fig. 2(a). Probe-loaded macrophages were stimulated with phorbol myristate acetate (PMA: 2 ng ml^{-1}) at 37 °C for 10 min, and a strong signal was observed, Fig. 2(b). In addition, prompt fluorescent increases in probe-loaded macrophages treated with exogenous H_2O_2 (10 or 100 nM, respectively) were also observed, Fig. 2(c) and (d). Brightfield transmission measurements after NFDS-**1** incubation and 100 nM H_2O_2 addition confirm that the cells are viable throughout the imaging experiments, Fig. 2(e). These data establish that NFDS-**1**, a small lipophilic molecule, is membrane-permeable, and can respond to nanomolar change in H_2O_2 concentration within living cells, while native cellular species such as GSH and ascorbic acid, as shown in Fig. 2(a), do not contribute to the fluorescence imaging.

In conclusion, we have presented in this article the synthesis, fluorescence properties, and biological applications of NFDS-**1**, as a fluorescence imaging probe to detect intracellular H_2O_2. This naphthofluorescein-based reagent features high selectivity for H_2O_2 over other intracellular ROS and some biological compounds, a wide dynamic response range and low detection

Table 1 Relative fluorescence intensity (RFI) observed upon reaction of NFDS-**1** with various ROS, and biological compounds

Compounds	RFI[a]	Compounds	RFI[a]
Blank	10	$ONOO^-$ (SIN-1)	8
H_2O_2	155	t-BuOOH	35
$O_2^{-\bullet}$ (XO/HPX)	20	1,4-hydroquinone	8
$O_2^{-\bullet}$ (KO_2)	42	GSH	8
$^{\bullet}OH$	5	Ascorbic acid	11
^{-}OCl	34	Esterase	10
$NO^{\bullet}$ (NOC-5)	7		

[a] All data obtained at 662 nm after incubation at 37 °C for 40 min.

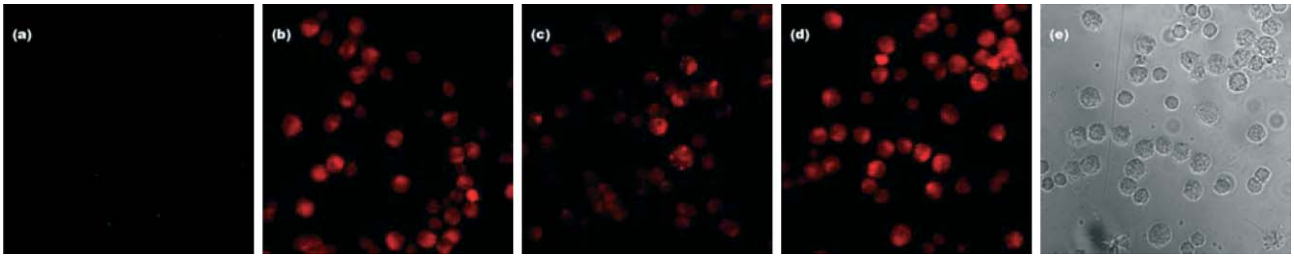


Fig. 2 Confocal fluorescence and phase contrast images of live PM. (a) Fluorescence image of PM incubated with 10 μM NFDS-**1** at 37 °C for 30 min. (b) Fluorescence image of probe-stained PM stimulated with PMA (2 ng ml^{-1}) at 37 °C for 10 min. (c) Fluorescence image of probe-stained PM treated with H_2O_2 (10 nM) at 37 °C for 10 min. (d) Fluorescence image of probe-stained PM treated with H_2O_2 (100 nM) at 37 °C for 10 min. (e) Brightfield image of live PM after NFDS-**1** incubation and 100 nM H_2O_2 addition to confirm viability (Scale bar = 10 μM).

limit owing to its nonredox mechanism, and far-visible excitation and near-IR fluorescence emission profiles to minimize cell and tissue damage while avoiding native fluorescence from native cellular species. Furthermore, we have demonstrated the value of this probe by measuring living cell-derived H_2O_2 and the nanomolar concentration of exogenous H_2O_2 within living macrophages. We found that NFDS-**1** is an excellent fluorescence reagent in detecting intracellular H_2O_2 and can respond to nanomolar change in H_2O_2 concentrations within living cells. Current efforts are devoted toward applying NFDS-**1** in different biological systems to explore its potential applications. We believe that such a naphthofluorescein-based fluorescent dye will have an important application in detecting oxidative stress through direct intracellular imaging.

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Notes and references

1 G. Patonay and M. D. Antoine, *Anal. Chem.*, 1991, **63**, 6, 321.
2 E. Sasaki, H. Kojima, H. Nishimatsu, Y. Urano, K. Kikuchi, Y. Hirata and T. Nagano, *J. Am. Chem. Soc.*, 2005, **127**, 3684.
3 T. Finkel and N. J. Holbrook, *Nature*, 2000, **408**, 239.
4 H. Wiseman and B. Halliwell, *Biochem. J.*, 1996, **313**, 17.
5 J. M. McCord, *Science*, 1974, **185**, 529.
6 K. Dobashi, B. Ghosh, J. K. Orak, I. Singh and A. K. Singh, *Mol. Cell. Biochem.*, 2000, **205**, 1.
7 B. Halliwell and J. M. C. Gutteridge, *Free Radicals in Biology and Medicine*, 3rd edn.; Clarendon Press: Oxford, UK, 1999.
8 T. Finkel, *Curr. Opin. Cell Biol.*, 2003, **15**, 247.
9 A. S. Keston, *Anal. Biochem.*, 1965, **11**, 6.
10 S. L. Hempel, G. R. Buettner, Y. Q. O'Malley, D. A. Wessels and D. M. Flaherty, *Free Radical Biol. Med.*, 1999, **27**, 146.
11 K. Akasaka, T. Suzuki, H. Ohrui and H. Meguro, *Anal. Lett.*, 1987, **20**, 731.
12 M. Onoda, S. Uchiyama, A. Endo, H. Tokuyama, T. Santa and K. Imai, *Org. Lett.*, 2003, **5**, 1459.
13 O. S. Wolfbeis, A. Dürkop, M. Wu and Z. Lin, *Angew. Chem., Int. Ed.*, 2002, **41**, 4495.
14 M. C. Y. Chang, A. Pralle, E. Y. Isacoff and C. J. Chang, *J. Am. Chem. Soc.*, 2004, **126**, 15392.
15 H. Maeda, Y. Fukuyasu, S. Yoshida, M. Fukuda, K. Saeki, H. Matsuno, Y. Yamauchi, Y. Yoshida, K. Hirata and K. Miyamoto, *Angew. Chem., Int. Ed.*, 2004, **43**, 2389.