DOI: 10.1002/cbic.200700718

BODIPY-Based Fluorescent Redox Potential Sensors that Utilize Reversible Redox Properties of Flavin

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Living cells are continuously exposed to various stresses, such as reactive oxygen species (ROS), reactive nitrogen intermediates (RNI), UV light, ionizing radiation, and metal ions.[1] In controlling intracellular redox systems (e.g., redox potential, $E^{0\prime}$, of cytosols are −0.28 to −0.22 V vs. SHE at pH 7.0), thiol–disulfide equilibria of intracellular thiols, such as glutathione (GSH) and thioredoxin, play critical roles in controlling the whole redox status and regulating structures and functions of proteins.[2] For example, more than 95% of GSH exists as a reduced form in cells and the depletion of GSH levels induces oxidative stresses. In order to understand the mechanisms involved in oxidative signals, antioxidant defense systems, and related intracellular phenomena, fluorescence sensing systems that respond to environmental redox potential are considered to afford a potentially powerful methodology. However, most fluorescent probes of ROS and RNI[3–5] irreversibly react with these species and examples of sensors that reversibly respond to thiol–disulfide equilibria have been limited.[6]

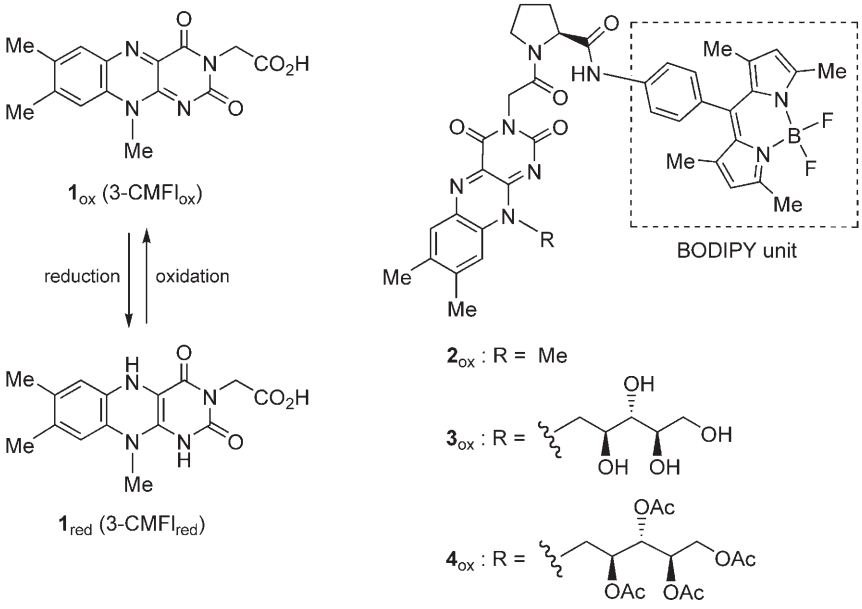
Our strategy for fluorescence sensing of thiol–disulfide equilibria is to utilize flavins, which are well studied cofactors or photoreceptors utilized in natural flavoproteins, including dehydrogenases, oxygenases, DNA photolyases, and thioredoxin reductases.[7] Recently, we reported an artificial DNA photolyase[8] that consist of a Zn^{2+}-1,4,7,10-tetraazacyclododecane complex[9] as binding site for *cis–syn* thymine photodimer (T[*c*,*s*]T) and a 3-carboxymethyllumiflavin ($\mathbf{1}_{ox}$) unit as photosensitizer. Interestingly, the redox potential of $\mathbf{1}_{ox}$ (−0.44 V vs. Ag/AgCl or −0.24 V vs. SHE; Table 1) is close to that of intracellular thiols, so that the flavin moiety of $\mathbf{1}_{ox}$ is reduced by thiols, such as dithiothreitol (DTT), in aqueous solution.[8,10] Because $\mathbf{1}_{ox}$ emits modest fluorescence (quantum yield of emission (Φ_f) = 0.16; Table 1) and its reduced form, $\mathbf{1}_{red}$, is almost nonfluorescent, we postulated that $\mathbf{1}_{ox}$ could be a reversible fluorescent sensor that responds to thiol–disulfide equilibria. However, cell staining with **1** and its ethyl ester was unsuccessful, possibly due to their rather high hydrophilicity and low quantum yields of emission.

On the basis of these results, we designed and synthesized bora-3a,4a-diaza-*s*-indacene (BODIPY)-based fluorophores **2**, **3**,

Table 1. Electrochemical and photochemical properties of redox potential sensors and flavins in HEPES (100 mM, pH 7.0) containing DMSO (30%) at 25 °C.

	$E^{0\prime}$ [V][a]	$\Phi_{f\,red}$[b]	$\Phi_{f\,ox}$[b] (λ_{max} 512 nm)[c]	I_{ox}/I_{red}
riboflavin	−0.41	~0	0.15	>50
1 (3-CMFl)	−0.44	~0	0.16	>50
2	−0.44	0.05	0.31	10
3	−0.41	0.07	0.35	9

[a] Potential vs. Ag/AgCl in 100 mM HEPES (pH 7.0) containing 30% DMSO. Typical cyclic voltammetric curves of **1** and **2** are shown in Figure S1. [b] Values relative to Φ_f of fluorescein (Φ_f = 0.90) in 0.1 M NaOH. [c] Ratio of fluorescence intensity of the oxidized form (I_{ox}) to that of the reduced form (I_{red}) at 512 nm (λ_{ex} = 450 nm).



Scheme 1. Structures of fluorescent sensors that respond to redox potential.

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and **4**, which consist of flavin as a controller of photochemical properties of BODIPY,[11] and L-proline as a linker to connect the flavin and BODIPY units.[12,13] It was hypothesized that emission of BODIPY would be strong when the flavin of **2** was in an oxidized form, and would be suppressed by electron transfer when the flavin was reduced (Scheme 1).

The redox potentials ($E^{0\prime}$) of **2** and **3** are almost identical to those of **1** and riboflavin, as determined by cyclic voltammetry (CV) experiments (Table 1 and Figure S1 in the Supporting Information). UV absorption spectra of the oxidized form of **2** ($\mathbf{2}_{ox}$) in 100 mM HEPES (pH 7.0) containing 30% DMSO (Figure S2) are nearly the sum of UV absorption of $\mathbf{1}_{ox}$ and L-prolyl-BODIPY (for the structure, see Scheme S1); this indicates negligible electronic interactions between the oxidized flavin and BODIPY units. In contrast, in UV absorption spectra of the reduced form of **2** ($\mathbf{2}_{red}$), a small shoulder was observed at 530–540 nm; this suggests some interaction between BODIPY and the reduced form of flavin.[14]

A plain curve in Figure 1A displays the fluorescence emission of $\mathbf{2}_{ox}$ (5 μM). Upon reduction with $Na_2S_2O_4$ (1 mM), fluorescence emission of **2** at 512 nm was considerably quenched (Figure 1A, dashed curve). The ratio of emission intensity of $\mathbf{2}_{ox}$ and $\mathbf{2}_{red}$, I_{ox}/I_{red}, at 512 nm was approximately 10 (Table 1).[15,16] Reoxidation of $\mathbf{2}_{red}$ with H_2O_2 (10 mM) or by exposure to air restored its emission to almost the same intensity as that of $\mathbf{2}_{ox}$; this implies a reversible response to redox status in solution.

It was found that the molar absorption coefficient (ε) of **2** at 450 nm changed between −0.42 V and −0.36 V vs. SHE in DTT-based redox buffers ($E^{0\prime}$ of DTT is −0.31 V vs. SHE at pH 7.0 and 25 °C) according to the change in redox of aqueous solutions (Figure S4).[17] The redox potential fluorescence emission profile of **2** at 512 nm (λ_{ex} = 450 nm; Figure 1B) gave a sigmoidal curve between −0.40 V and −0.36 V vs. SHE, which is about ninefold increase in emission. From this curve, the midpoint potential for **2** was determined to be approximately −0.38 V vs. SHE,[18] which is close to that of **1** (ca. −0.39 V vs. SHE) under the same conditions (Figure S5).

Figure 2 displays the results of dual staining of HeLa cells with $\mathbf{2}_{ox}$ and MitoTracker Red CMXRos dye,[3] which is a mitochondrion-selective dye. After incubation of HeLa cells with $\mathbf{2}_{ox}$ (1 μM) and Mitotracker (20 nM) for 30 min at 37 °C, phase-contrast microscopic images of viable cells were analyzed (Figure 2A) and bright, punctate staining patterns were observed (Figure 2B); this indicates that $\mathbf{2}_{ox}$ permeated the cellular membrane. The merged image of Figure 2B and the staining patterns of Mitotracker (Figure 2C) indicated that $\mathbf{2}_{ox}$ is located in the cytosol, and to some extent in the nucleolus and/or perinuclear region as well as in mitochondria (Figure 2D); these experiments were repeated at least three times. After treatment with $Na_2S_2O_4$ (10 mM), intracellular fluorescence decreased considerably; this implies that $\mathbf{2}_{ox}$ was reduced to $\mathbf{2}_{red}$ (Figure 2E). Retreatment of the cells shown in Figure 2E with H_2O_2 (10 mM) restored the bright fluorescence (Figure 2F) to the same extent as observed in Figure 2B; this demonstrates that **2** reversibly responds to the redox potential in living cells. We also tried fluorescent staining of HeLa cells with $\mathbf{3}_{ox}$.[19,20] However, only very weak fluorescent images were observed

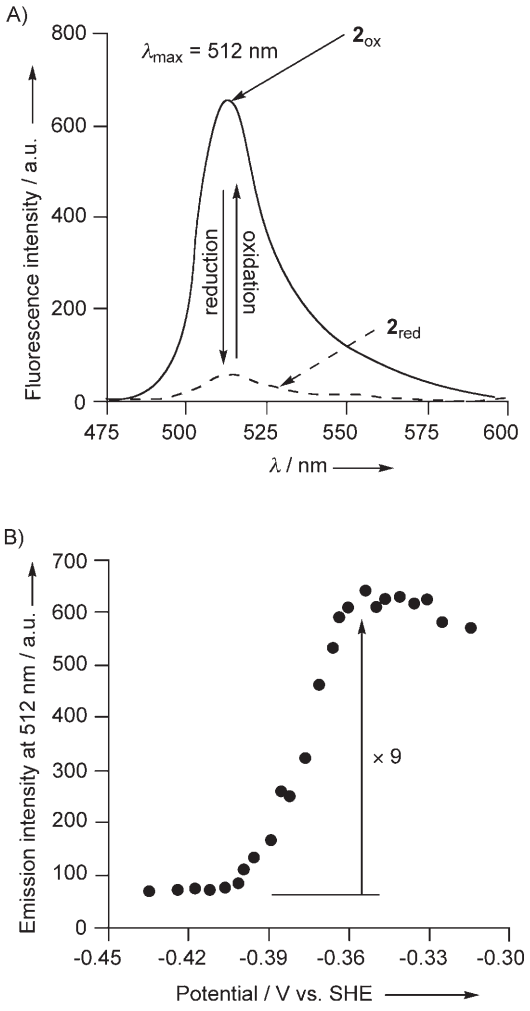

Figure 1. A) Fluorescence emission spectra (λ_{ex} = 450 nm) of $\mathbf{2}_{ox}$ (——) and $\mathbf{2}_{red}$ (-----) in 100 mM HEPES buffer (pH 7.0) containing 30% DMSO at 25 °C; [**2**]: 5 μM; a.u.: arbitrary units. B) Change in fluorescence emission intensity at 512 nm of **2** (5 μM) as a function of the redox potential of the buffer solution. Experiments were performed in buffer solution (100 mM; HEPES was used for pH 7.0, 7.4, and 8.0, and TAPS for pH 8.6) containing 30% DMSO, DTT_{red}, and DTT_{ox} ($[DTT_{red}] + [DTT_{ox}] = 50$ mM).

(Figure 2G), possibly due to the poor cell-permeability of **3**. In contrast, $\mathbf{4}_{ox}$, in which all the hydroxyl groups are acetylated, afforded bright fluorescent images (Figure 2H); this indicates that **4** was more cell-membrane permeable than **3**. Even after 24 h treatment with 1 μM $\mathbf{2}_{ox}$ or $\mathbf{4}_{ox}$, most cells (90%) were viable; this implies that these sensors cause negligible cell damage.[21,22]

In summary, we have presented the BODIPY-based fluorescent sensors **2**, **3**, and **4**, the fluorescent intensities of which can be controlled by the redox status of a flavin unit; these sensors can reversibly respond to thiol–disulfide equilibria in solutions and in living cells. This methodology could produce useful sensing systems for redox potentials in bioorganic, bioinorganic, analytical, and medicinal chemistry, and cell biology.

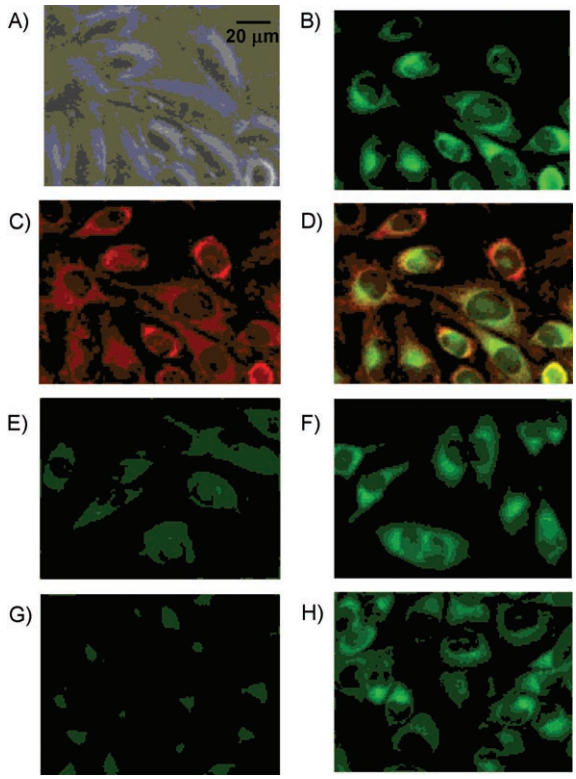

Figure 2. Microscopic images of HeLa cells. A) Phase contrast image of HeLa cells stained with 1 μM $\mathbf{2}_{ox}$ and 20 nM Mitotracker for 30 min at 37 °C. B) Fluorescent image of HeLa cells stained with 1 μM $\mathbf{2}_{ox}$ and 20 nM Mitotracker for 30 min at 37 °C (irradiated with visible light at 475 nm to observe **2**). C) Fluorescent image of HeLa cells stained with 1 μM $\mathbf{2}_{ox}$ and 20 nM Mitotracker for 30 min at 37 °C (irradiated with visible light at 562 nm to observe Mitotracker). D) Composite of Figures 2B and 2C. E) Fluorescent image of HeLa cells after addition of 10 mM $Na_2S_2O_4$ to the cells shown in Figure 2B. F) Fluorescent image of HeLa cells after addition of 10 mM H_2O_2 to the cells shown in Figure 2E. G) Fluorescent image of HeLa cells stained with 1 μM $\mathbf{3}_{ox}$ for 30 min at 37 °C (irradiation at 475 nm). H) Fluorescent image of HeLa cells stained with 1 μM $\mathbf{4}_{ox}$ for 30 min at 37 °C (irradiation at 475 nm; exposure time was 0.67 s for all images).

Acknowledgements

S.A. is grateful for a Grant-in-Aid from the Ministry of Education, Science, and Culture in Japan and for grants from the Naito Foundation (Japan), the Sumitomo Foundation (Japan), and the Japan Science and Technology Agency (JST).

Keywords: biosensors · emission spectroscopy · flavins · redox chemistry

[1] a) *Oxidative Stress, Disease, and Cancer* (Ed.: K. K. Singh), Imperial College Press, London, **2005**; b) *Redox Regulation of Cell Signaling and Its Clinical Application* (Eds.: L. Packer, J. Yodoi), Marcel Dekker, New York, **1999**.

[2] a) C. M. Grant, *Mol. Microbiol.* **2001**, *39*, 533–541; b) J. F. Curtin, M. Donovan, T. G. Cotter, *J. Immunol. Methods* **2002**, *265*, 49–72.

[3] R. P. Haugland, *Handbook of Fluorescent Probes and Research Products*, 9th ed., Molecular Probes, Eugene, **2002**.

[4] a) K. Tanaka, T. Miura, N. Umezawa, Y. Urano, K. Kikuchi, T. Higuchi, T. Nagano, *J. Am. Chem. Soc.* **2001**, *123*, 2530–2536; b) K. Setsukinai, Y. Urano, K. Kakinuma, H. J. Majima, T. Nagano, *J. Biol. Chem.* **2003**, *278*, 3170–3175; c) H. Maeda, Y. Fukuyasu, S. Yoshida, M. Fukuda, K. Saeki, H. Matsuno, Y. Yamauchi, K. Yoshida, K. Hirata, K. Miyamoto, *Angew. Chem.* **2004**, *116*, 2443–2445; *Angew. Chem. Int. Ed.* **2004**, *43*, 2389–2391; d) A. E. Albers, V. S. Okreglak, C. J. Chang, *J. Am. Chem. Soc.* **2006**, *128*, 9640–9641.

[5] a) T. Nagano, T. Yoshimura, *Chem. Rev.* **2002**, *102*, 1235–1269; b) Y. Gabe, Y. Urano, K. Kikuchi, H. Kojima, T. Nagano, *J. Am. Chem. Soc.* **2004**, *126*, 3357–3367; c) E. Sasaki, H. Kojima, H. Nishimatsu, Y. Urano, K. Kikuchi, Y. Hirata, T. Nagano, *J. Am. Chem. Soc.* **2005**, *127*, 3684–3685; d) T. Ueno, Y. Urano, H. Kojima, T. Nagano, *J. Am. Chem. Soc.* **2006**, *128*, 10640–10641.

[6] a) G. T. Hanson, R. Aggeler, D. Oglesbee, M. Cannon, R. A. Capaldi, R. Y. Tsien, S. J. Remington, *J. Biol. Chem.* **2004**, *279*, 13044–13053; b) C. T. Dooley, T. M. Dore, G. T. Hanson, W. Y. Jackson, S. J. Remington, R. Y. Tsien, *J. Biol. Chem.* **2004**, *279*, 22284–22293; c) K. Lee, V. Dzubeck, L. Latshaw, J. P. Schneider, *J. Am. Chem. Soc.* **2004**, *126*, 13616–13617; d) P. K. Pullela, T. Chiku, M. J. Carvan, III, D. S. Sem, *Anal. Biochem.* **2006**, *352*, 265–273; e) E. W. Miller, S. X. Bian, C. J. Chang, *J. Am. Chem. Soc.* **2007**, *129*, 3458–3459.

[7] E. Silva, A. M. Edwards, *Flavins: Photochemistry and Photobiology*, RSC, Cambridge, **2006**.

[8] Y. Yamada, S. Aoki, *J. Biol. Inorg. Chem.* **2006**, *11*, 1007–1023.

[9] a) E. Kimura, T. Koike, M. Shionoya in *Structure and Bonding, Vol. 89: Metal Sites in Proteins and Models* (Eds.: H. A. O. Hill, J. P. Sadler, A. J. Thompson), Springer, Berlin, **1997**, pp. 1–28; b) S. Aoki, E. Kimura, *Chem. Rev.* **2004**, *104*, 769–787; c) S. Aoki, E. Kimura in *Comprehensive Coordination Chemistry II: Biocoordination Chemistry* (Eds.: L. Que, Jr., W. B. Tolman), Elsevier, Oxford, **2004**, *8*, pp. 601–640.

[10] E. L. Loechler, T. C. Hollocher, *J. Am. Chem. Soc.* **1980**, *102*, 7312–7321.

[11] a) H. Imahori, H. Norieda, H. Yamada, Y. Nishimura, I. Yamazaki, Y. Sakata, S. Fukuzumi, *J. Am. Chem. Soc.* **2001**, *123*, 100–110; b) M. P. Debreczeny, W. A. Svec, M. R. Wasielewski, *New J. Chem.* **1996**, *20*, 815–828; c) R. Ziessel, L. Bonardi, P. Retailleau, G. Ulrich, *J. Org. Chem.* **2006**, *71*, 3093–3102; d) F. Camerel, L. Bonardi, M. Schmutz, R. Ziessel, *J. Am. Chem. Soc.* **2006**, *128*, 4548–4549.

[12] T. Carell, L. T. Burgdorf, L. M. Kundu, M. Cichon, *Curr. Opin. Chem. Biol.* **2001**, *5*, 491–498.

[13] BODIPY–flavin dyads reported by Daub et al., in which BODIPY and flavin are fused directly, showed only weak fluorescence in organic solvents ($\Phi_f = 0.0007$–0.0023 in CH_3CN/toluene): C. Trieflinger, K. Rurak, J. Daub, *Angew. Chem.* **2005**, *117*, 2328–2331; *Angew. Chem. Int. Ed.* **2005**, *44*, 2288–2291.

[14] Negligible interaction between oxidized flavin and BODIPY units of $\mathbf{2}_{ox}$ was observed in the ^{1}H NMR spectra. We could not the measure ^{1}H NMR spectra of $\mathbf{2}_{red}$, because $\mathbf{2}_{red}$ was easily oxidized. We cannot exclude the possibility that the low emission intensity of $\mathbf{2}_{red}$ is due to some interactions, such as π–π stacking between BODIPY and the reduced flavin unit.

[15] We synthesized several analogues that have flavin and BODIPY units connected by different linkers, such as L-pipecolic acid and piperidine-4-carboxylic acid. Among them, **2** was found to have the largest I_{ox}/I_{red} ratio value; details will be described elsewhere.

[16] The I_{ox}/I_{red} values of **2** were dependent on the excitation wavelength (emission at 512 nm) and buffer system used. As shown in Figure S3, the largest I_{ox}/I_{red} (ca. 13-fold) was obtained by excitation at 440 nm in 100 mM HEPES (pH 7.0) containing 30% DMSO. In this work, we chose a common wavelength (450 nm) to excite **1**–**4** for comparison.

[17] Redox potentials of the DTT-based redox buffer were calculated from the Nernst equation, as described in the Supporting Information. Redox equilibria of **2** in DTT-based redox buffer were obtained in 2 h.

[18] The difference between this value (−0.38 V vs. SHE) and the $E^{0\prime}$ value obtained from CV (−0.24 V vs. SHE calculated from −0.44 V vs. Ag/AgCl; Table 1) was attributed to the different electrodes and solvents used in CV.

[19] Fluorescence-titration curve against the redox potential of **3** was similar to that for **2** (Figure S6). The midpoint potential of **3** (ca. −0.34 V vs. SHE) was slightly more positive ($\Delta E^{0\prime} = 40$ mV) than that of **2** (ca. −0.38 V for Figure 2 in the text). This difference was close to the difference in $E^{0\prime}$ values observed in CV ($\Delta E^{0\prime} = 30$ mV) of **2** and **3** listed in Table 1.

[20] Fluorescent intensity of **3** was affected by the percentage of DMSO in the buffer (Figure S7). The percentage of DMSO (10~30%) in the sol-

vent system hardly affected the $E^{0\prime}$ values of **3** in CV experiments (data not shown).

[21] We could not detect the formation of superoxide anion radicals, which might be generated upon photoirradiation of **2**, in aqueous solutions and in living cells, due to lack of appropriate sensors for ROS with a fluorescence emission that can be distinguished from that of **2**.

[22] When $\mathbf{2}_{ox}$ was excited at 450 nm in the presence of Et_3N (50 mM) and DTT_{ox} in HEPES (50 mM) including DMSO (30%), emission of $\mathbf{2}_{ox}$ was slightly (5–10%) quenched (Figure S8), possibly due to photoreduction of the flavin unit.[8] Improvements with respect to this point are now underway.

Received: November 24, 2007
Published online on March 12, 2008

