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A Thiol-Reactive Fluorescence Probe Based on Donor-Excited Photoinduced Electron Transfer: Key Role of Ortho Substitution

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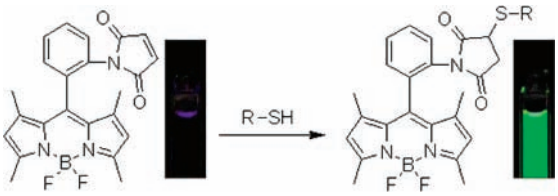
ABSTRACT



We designed and synthesized a novel thiol-reactive fluorescence probe based on the BODIPY fluorophore. The fluorescence of this probe is strongly quenched by donor-excited photoinduced electron transfer (d-PeT) from BODIPY to maleimide, but after reaction with thiol, the fluorescence of BODIPY is restored, affording a 350-fold intensity increase.

Thiols play key biological roles, for example, in maintaining the higher-order structures of proteins, in glutathione (GSH) conjugation via glutathione S-transferase (GST), and in regulation of the intracellular redox state through the equilibrium of GSH and GSSG. Because of their nucleophilicity, thiol moieties have been used as targets for protein labeling via conjugation with electrophilic functional groups, such as haloacetamide.[1] Many thiol-reactive fluorescence probes and tags, including *N*-(*p*-(2-benzimidazolyl)phenyl)-maleimide (BIPM),[2] *N*-(*p*-(7-diethylamino-4-methylcoumarin-3-yl)phenyl)maleimide (CPM),[3] and fluorescein-5-maleimide,[4] have been developed. Most of them are excited in the ultraviolet region, except fluorescein-5-maleimide, which is excitable with visible light. However, fluorescein-5-maleimide has relatively strong fluorescence even before reaction with thiol. Maeda et al. reported a thiol-reactive fluorescence probe (BESthio)[5] which has almost no fluorescence before reaction with thiol and is excited with visible light, but it does not conjugate with thiol and simply releases fluorescein after reaction with thiol. Here, we report a novel thiol-conjugating fluorescence probe which is excited with visible light and has almost no fluorescence before reaction with thiol.

Existing thiol-reactive fluorescence probes and tags have a maleimide moiety as the thiol-reactive group, and the longer their excitation wavelength is, the stronger is their background fluorescence before conjugation with thiol. We

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considered that the fluorescence of the unreacted probe might be strongly quenched by utilizing the maleimide moiety as an electron acceptor for donor-excited photoinduced electron transfer (d-PeT).[6] In the PeT process, the distance from the electron donor to the electron acceptor is a very important parameter, so we considered that ortho substitution would be most appropriate; this is in contrast to the para substitution in most existing fluorescence probes for thiol.

To test our hypothesis that ortho substitution would provide strong quenching in the unreacted fluorescence probe, we synthesized ortho-, meta- and para-substituted maleimide derivatives of BODIPY (**1**, **2**, and **3**) via conventional routes.[7] BODIPY has strong fluorescence, which is independent of solvent and pH, in contrast to the case of fluorescein. We then measured the fluorescence quantum yields of these three compounds, and the results are shown in Figure 1. The fluorescence spectra are shown in Figure

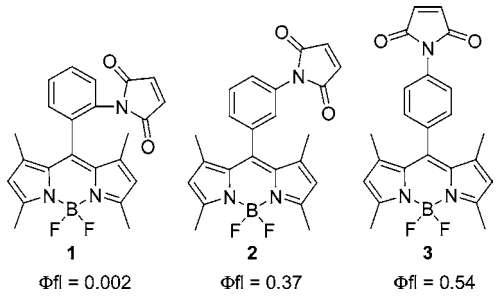

Figure 1. Structures and the fluorescence quantum yields of compounds **1**, **2**, and **3**. The fluorescence quantum yields were determined on the basis of fluorescein (0.85) in 0.1 M NaOH(aq) as a standard, and were measured in DMSO.

2. The para-substituted probe **3** is strongly fluorescent, while the meta-substituted probe **2** shows somewhat weaker, though still strong, fluorescence. However, the ortho-substituted probe **1** shows almost no fluorescence, as we had hoped. This result clearly supports our hypothesis that the closer the distance between the electron donor (in this case, BODIPY) and the electron acceptor (in this case, maleimide), the more strongly the fluorescence will be quenched.

Next, we examined whether the fluorescence of the BODIPY moiety of *o*-maleimideBODIPY is restored after reaction with thiol. The fluorescence intensity of *o*-maleimideBODIPY increased linearly upon the addition of *N*-acetylcysteine (NAC) (Figure 3). The putative electron flows before and after reaction of the probe are shown schematically in Figure 4.

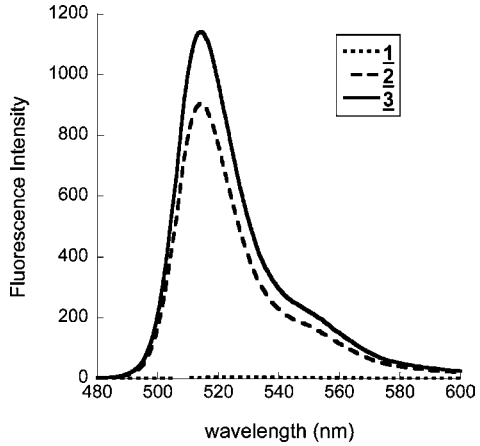

Figure 2. Fluorescence spectra of maleimideBODIPYs **1**, **2**, and **3**. The probe concentration was1 μM in DMSO and the excitation wavelength was 505 nm.

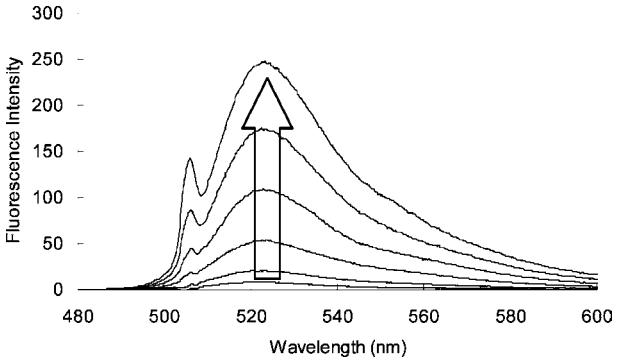

Figure 3. Fluorescence enhancement of *o*-maleimideBODIPY upon reaction with thiol. Concentrations of 0.2, 0.4, 0.6, 0.8, and 1.0 μM of NAC were reacted with 1.0 μM *o*-maleimideBODIPY, in 0.1 M sodium phosphate buffer (pH 7.4). The excitation wavelangth was 505 nm.

Then, we compared the fluorescence enhancements obtained with fluorescein-5-maleimide and with our new probe, *o*-maleimideBODIPY, upon reaction with NAC (Figure 5). Before reaction, *o*-maleimideBODIPY is almost nonfluorescent, while fluorescein-5-maleimide is fluorescent. The increase of the fluorescence quantum yield of *o*-maleimideBODIPY upon reaction is more than 350-fold (from 0.002 to 0.73), while that of fluorescein-5-maleimide is only about 10-fold (from 0.06 to 0.64). Such a high signal-to-noise ratio (350-fold) has been never achieved with any other thiol-reactive fluorescence probe which can be excited with long-wavelength light.

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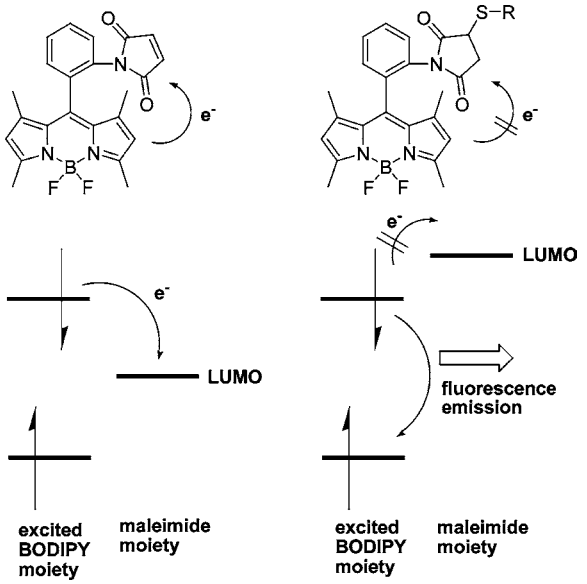

Figure 4. Schematic illustration of d-PeT from excited BODIPY to maleimide, in the unreacted probe, but not in the thiol-reacted probe.

We next applied our new fluorescence probe for labeling of bovine serum albumin (BSA). The result of SDS-PAGE analysis is shown in Figure 6. Our novel fluorescence probe, *o*-maleimideBODIPY, clearly and quantitatively labeled BSA at very low concentration. Detection with such high sensitivity cannot be achieved with Coomassie brilliant blue, which is an absorptiometry-based technique, or with BESthio, which cannot conjugate with thiol.

In conclusion, we have developed a novel approach to the design of fluorescence probes, by arranging the fluorophore and the quencher in an ortho relationship on a benzene ring so that the PeT process can occur easily in the unreacted probe. On the basis of this strategy, we developed a novel

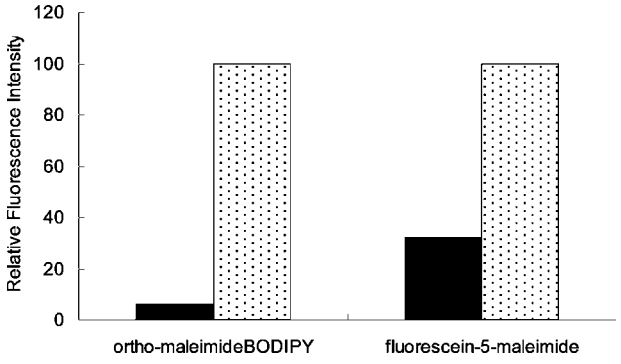

Figure 5. Fluorescence enhancements of 1 μM *o*-maleimide-BODIPY and fluorescein-5-maleimide upon reaction with NAC in 0.1 M sodium phosphate buffer (pH 7.4) containing 0.1% DMSO as a cosolvent. The black columns indicate the relative fluorescence intensity without NAC and the dotted columns indicate the relative fluorescence intensity 10 min after the addition of 0.5 μM NAC.

Figure 6. Fluorescence image of protein bands in SDS-PAGE gel, obtained with a FluorImager595 (Amersham), with excition at 488 nm. Concentrations of 5, 10, 50, and 100 μg/mL BSA in sodium phosphate buffer containing 5% DMSO as a cosolvent (0.1 M, pH 7.4) were labeled with 30 μM *o*-maleimideBODIPY at 37 °C. After 60 min, the samples were treated with SDS and β−mercaptoethanol, vortexed, and heated at 95 °C for 5 min before being loaded on the gel for electrophoresis.

thiol-reactive fluorescence probe, *o*-maleimideBODIPY, in which the fluorescence is strongly quenched by d-PeT. The strong fluorescence of BODIPY is restored after reaction with thiol, resulting in an extremely high signal-to-noise ratio. Some existing maleimide-based fluorescence probes have almost no fluorescence before reaction with thiol,[8] but they require excitation in the ultraviolet range and their fluorescence after reaction with thiol is not so strong. Our probe can be excited with visible light and has an extremely high signal-to-noise ratio as a result of rational design utilizing PeT. This probe was confirmed to be useful for detecting extremely low concentrations of protein in the gel after SDS-PAGE.

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Supporting Information Available: Synthesis, experimental details, and characterization of BODIPY derivatives. This material is available free of charge via the Internet at http://pubs.acs.org.

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