

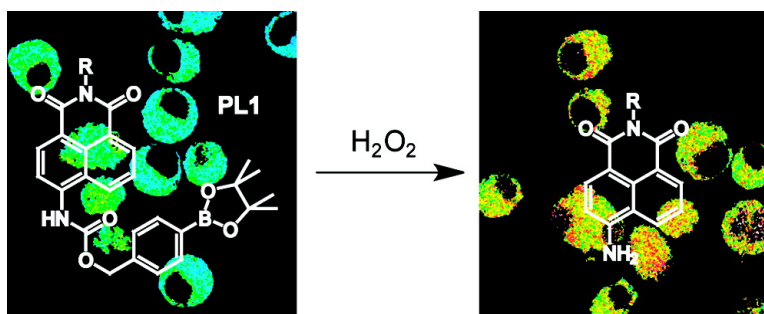
Communication

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## An ICT-Based Approach to Ratiometric Fluorescence Imaging of Hydrogen Peroxide Produced in Living Cells

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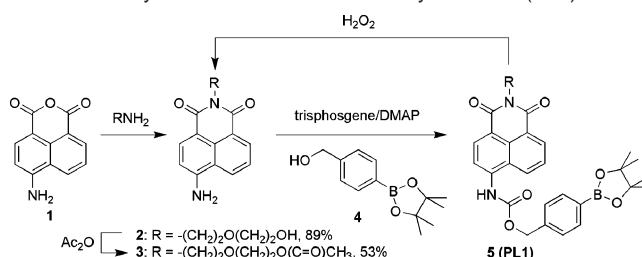
The chemistry of hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) in living systems is receiving increasing attention owing to its diverse contributions as a marker for oxidative stress and damage events associated with aging and disease,<sup>1</sup> as a second messenger for cellular signal transduction,<sup>2–4</sup> or as a killing agent generated by immune cells to combat microbial invasion.<sup>5</sup> A key step toward elucidating the complex biological roles of this reactive oxygen metabolite is the development of new methods to monitor dynamic changes in peroxide concentrations generated within localized regions of the cell, as spatial and temporal variations in cellular  $\text{H}_2\text{O}_2$  flows can lead to dramatically different physiological or pathological consequences. Small-molecule reporters offer one approach to meet this need,<sup>6</sup> and indicators that give a turn-on emission increase in response to  $\text{H}_2\text{O}_2$  have been reported and applied for the study of peroxide biology.<sup>7–15</sup>

Despite advances in the development of new synthetic reagents, a limitation of intensity-based probes is that variabilities in excitation and emission efficiency, sample environment, and probe distribution pose potential problems for use in quantitative measurements. In contrast, ratiometric probes provide the practical advantage of built-in corrections for such variabilities by allowing simultaneous detection of two signals resulting from reacted and unreacted forms of the probe in the same sample.<sup>16</sup> We now present the synthesis and application of Peroxy Lucifer 1 (PL1), a new type of ratiometric fluorescent reporter for imaging  $\text{H}_2\text{O}_2$  produced in living systems. Owing to its dual emission readout, PL1 is capable of visualizing highly localized changes in  $\text{H}_2\text{O}_2$  concentrations generated by live cells in response to phagocytic stimulation while retaining the ability to spatially monitor relative  $[\text{H}_2\text{O}_2]$  fluxes throughout the rest of the sample.

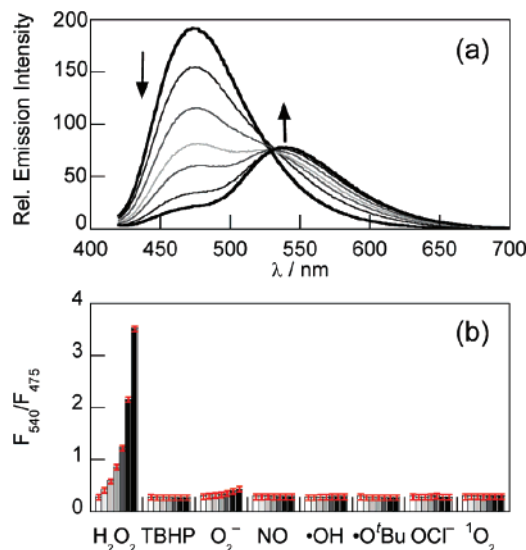
Our approach to ratiometric fluorescence detection of cellular  $\text{H}_2\text{O}_2$  relies on controlling internal charge transfer (ICT) within a dye platform to promote a change in its emission color upon reaction with  $\text{H}_2\text{O}_2$ . Specifically, modulation of the electron-donating 4-amino donor on a 1,8-naphthalimide (e.g., Lucifer Yellow) affects both ICT and emission color, as making this substituent more electron-deficient results in ICT-induced blue shifts in emission maxima. We reasoned that modifying the 4-amino donor into a more electron-withdrawing carbamate group that could be specifically decaged by  $\text{H}_2\text{O}_2$  back to the amine would provide a switch for ratiometric detection of  $\text{H}_2\text{O}_2$ , a strategy inspired by indicators for pH,<sup>17</sup> anions,<sup>18</sup> metal ions,<sup>19,20</sup> and sugars.<sup>21</sup> On the basis of this design, the synthesis of Peroxy Lucifer 1 (PL1) is shown in Scheme 1.

We assessed the spectroscopic properties of PL1 under physiological-like conditions (20 mM HEPES, pH 7.4). In the absence of  $\text{H}_2\text{O}_2$ , PL1 displays one major absorption band centered at 375 nm ( $\epsilon = 9600 \text{ M}^{-1}\text{cm}^{-1}$ ) with a corresponding blue-colored fluorescence maximum at 475 nm ( $\Phi = 0.38$ ). The relative blue shift of these absorption and emission features compared to other Lucifer-type dyes is consistent with ICT involving the relatively

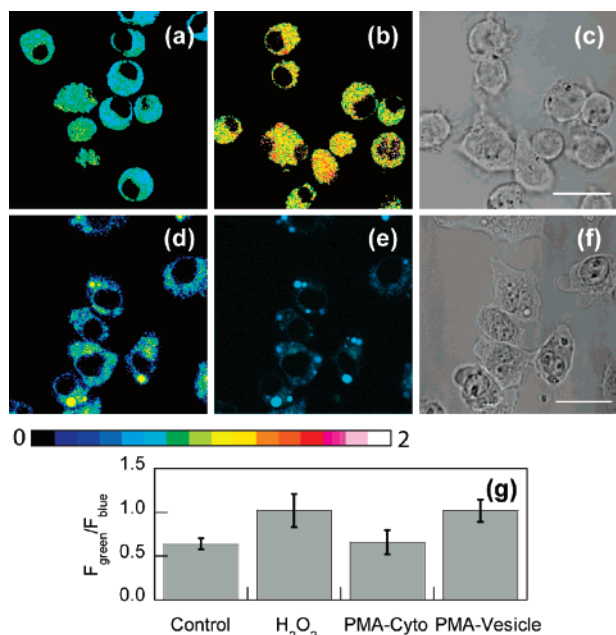
**Scheme 1.** Synthesis and Action of Peroxy Lucifer 1 (PL1)



electron-poor carbamate donor. Reaction of PL1 with  $\text{H}_2\text{O}_2$  triggers chemoselective cleavage of a boronate-based carbamate protecting group to deliver the green fluorescent aminonaphthalimide dye **3** as characterized by its absorption ( $\lambda_{\text{abs}} = 435 \text{ nm}$ ,  $\epsilon = 8600 \text{ M}^{-1}\text{cm}^{-1}$ ) and emission ( $\lambda_{\text{em}} = 540 \text{ nm}$ ,  $\Phi = 0.11$ ) spectra, respectively (Figure 1a). The ratio of amine- to carbamate-derived emission intensities ( $F_{540}/F_{475}$ ) upon excitation at 410 nm varies from 0.3 in the absence of  $\text{H}_2\text{O}_2$  to 3.6 after complete conversion to **3**, a ca. 12-fold emission ratio change. Under pseudo-first-order conditions (1  $\mu\text{M}$  PL1 and 1 mM  $\text{H}_2\text{O}_2$ ), the observed rate constant for  $\text{H}_2\text{O}_2$  deprotection is  $k_{\text{obs}} = 8.8 \times 10^{-4} \text{ s}^{-1}$ . The ratiometric emission response of PL1 is highly selective for  $\text{H}_2\text{O}_2$  over other reactive oxygen species (Figure 1b). Compared to reactions with  $\text{H}_2\text{O}_2$ , the ratio of carbamate- to amine-substituted naphthalimides does not change appreciably with *tert*-butylhydroperoxide, hypochlorite,



**Figure 1.** (a) Fluorescence response of 5  $\mu\text{M}$  PL1 to 200  $\mu\text{M}$   $\text{H}_2\text{O}_2$ . Spectra were acquired before and 15, 30, 45, 60, 90, and 120 min after  $\text{H}_2\text{O}_2$  was added. (b) Fluorescence responses of 5  $\mu\text{M}$  PL1 to various reactive oxygen species (ROS) at 200  $\mu\text{M}$ . Bars represent emission intensity ratios  $F_{540}/F_{475}$  at 0, 15, 30, 45, 60, 90, and 120 min after addition of each ROS. Data were acquired at 25  $^\circ\text{C}$  in 20 mM HEPES, pH 7.4, with excitation  $\lambda = 410 \text{ nm}$ .



**Figure 2.** Confocal, ratiometric fluorescence images of live RAW 264.7 macrophage cells. Images displayed in pseudocolor represent the ratio of emission intensities collected in optical windows between 535–600 (green) and 430–495 nm (blue), respectively, upon two-photon excitation at 820 nm. (a) Cells incubated with 5  $\mu\text{M}$  PL1 for 15 min at 37  $^\circ\text{C}$ . (b) PL1-loaded cells after treatment with 100  $\mu\text{M}$   $\text{H}_2\text{O}_2$  for 60 min at 37  $^\circ\text{C}$ . (c) Brightfield images of cells in panel (b) with 20  $\mu\text{m}$  scale bar. (d) PL1-loaded cells after treatment with PMA (1  $\mu\text{g}/\text{mL}$ ) for 30 min at 37  $^\circ\text{C}$ . (e) Cells in panel (d) showing only the green emission channel. (f) Brightfield image of cells in panels (d) and (e) with 20  $\mu\text{m}$  scale bar. (g) Relative  $F_{\text{green}}/F_{\text{blue}}$  ratios displayed by cells loaded with PL1 only (control) and PL1-stained cells treated with  $\text{H}_2\text{O}_2$  or PMA (cytoplasm vs vesicle).

superoxide, singlet oxygen, nitric oxide, hydroxyl radical, and *tert*-butoxy radical.

We next sought to apply PL1 for ratiometric fluorescence imaging of  $\text{H}_2\text{O}_2$  in live biological samples. Two-photon confocal fluorescence images (820 nm excitation) taken of RAW 264.7 macrophages incubated with 5  $\mu\text{M}$  PL1 indicate that the dye is cell-permeable (Figure 2a). The ratio image constructed from 535 to 600 nm (green) and from 430 to 495 nm (blue) fluorescence collection windows using ImageJ software gave an average emission ratio value of 0.6. Treatment of PL1-loaded cells with 100  $\mu\text{M}$   $\text{H}_2\text{O}_2$  for 60 min triggers an increase in this ratio to ca. 1.0 (Figure 2b), consistent with  $\text{H}_2\text{O}_2$ -mediated boronate cleavage occurring within these cells. Macrophages incubated with PL1 only show negligible changes in emission ratio, and nuclear staining, flow cytometry, and trypan blue assays establish that the cells are viable throughout the imaging experiments (Figure S4). Experiments in HEK cells give similar results (Figure S3).

We then tested the ability of PL1 to detect endogenous bursts of  $\text{H}_2\text{O}_2$  produced within living cells by stimulating PL1-loaded macrophages with phorbol myristate acetate (PMA) to induce phagocytosis-associated  $\text{H}_2\text{O}_2$  generation.<sup>22,23</sup> The data in Figure 2d and e show clear increases in green-to-blue emission ratios localized within the phagocytic vesicles (>1.0) compared to other intracellular regions (0.6) upon PMA treatment, consistent with macrophage production of  $\text{H}_2\text{O}_2$  to combat this insult. These data

establish that PL1 is capable of live-cell imaging of  $\text{H}_2\text{O}_2$  at natural immune response levels. Moreover, the ratiometric readout provided by this probe allows for detection of highly localized changes in  $\text{H}_2\text{O}_2$  concentrations at phagocytic sites while at the same time visualizing [ $\text{H}_2\text{O}_2$ ] variations throughout the rest of the cytoplasm.

In summary, we have presented the synthesis, properties, and live-cell evaluation of a new type of ratiometric fluorescent probe for  $\text{H}_2\text{O}_2$ . PL1 features a chemospecific  $\text{H}_2\text{O}_2$  switch to modulate ICT with a marked blue-to-green emission color change. Experiments in macrophages show that PL1 can visualize  $\text{H}_2\text{O}_2$  produced in living cells by ratiometric imaging. We are now using PL1 and related chemical tools to identify and quantify endogenous sources of biological  $\text{H}_2\text{O}_2$ , as well as expanding this general ICT design concept to detection of other important analytes in living systems.

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**Supporting Information Available:** Synthetic and experimental details. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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