



# Development of a Sensitive Bioluminogenic Probe for Imaging Highly Reactive Oxygen Species in Living Rats

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**Abstract:** A sensitive bioluminogenic probe for highly reactive oxygen species (hROS),  $SO_3H$ -APL, was developed based on the concept of dual control of bioluminescence emission by means of bioluminescent enzyme-induced electron transfer (BioLeT) and modulation of cell-membrane permeability. This probe enables non-invasive visualization of physiologically relevant amounts of hROS generated deep inside the body of living rats for the first time. It is expected to serve as a practical analytical tool for investigating a wide range of biological functions of hROS in vivo. The design concept should be applicable to other in vivo bioluminogenic probes.

**R**eactive oxygen species (ROS) mediate a wide variety of biological events and human diseases.<sup>[1]</sup> Among them, highly reactive oxygen species (hROS) such as hypochlorite ( $ClO^-$ ), peroxyxynitrite ( $ONOO^-$ ), and OH radical ( $\cdot OH$ ) are very strong oxidants that can directly oxidize nucleic acids, proteins, and lipids, and they are thought to play important roles in living systems.<sup>[2]</sup> Despite their importance, however, the precise roles of hROS in vivo remain elusive.<sup>[2]</sup>

One reason for this is the lack of sensitive methods for the detection of hROS in vivo. For in vitro or in cellulo monitoring of hROS, fluorescence probes<sup>[3]</sup> are generally very useful, but in the case of in vivo detection, the fluorescence signal is

subject to interference from tissue autofluorescence and absorption/scattering of excitation light, which results in poor signal-to-noise ratios and non-quantitative responses.<sup>[4]</sup> Two-photon excitation can overcome these problems, but the observation range is restricted. By contrast, bioluminogenic probes do not suffer from these disadvantages, and indeed, bioluminogenic probes for hydrogen peroxide ( $H_2O_2$ ) can detect  $H_2O_2$  with high sensitivity in vivo.<sup>[5]</sup> However, there has so far been no bioluminogenic probe for hROS.

We recently reported a new class of bioluminogenic probes based on the bioluminescent enzyme-induced electron transfer (BioLeT) mechanism.<sup>[6]</sup> Since several fluorogenic probes for hROS have been developed by utilizing photo-induced electron transfer (PeT),<sup>[3c,d]</sup> which is an analogous phenomenon to BioLeT, we hypothesized it would be possible to develop a bioluminogenic probe for hROS based on BioLeT.

Therefore, we designed a new bioluminogenic probe for hROS, aminophenoxyethyl-aminoluciferin (APL). We expected that the alkyloxyaniline moiety would act as an electron donor for BioLeT (HOMO energy level:  $-4.72$  eV, which is sufficiently high for quenching)<sup>[6]</sup> and that hROS would cleave this luminescence quencher to produce hydroxyethyl-aminoluciferin (HE-AL), which is strongly luminescent (Figure 1a). As expected, the bioluminescence of APL was sufficiently quenched, while HE-AL exhibited about 100-fold higher bioluminescence (Figure 1a and Figure S1 in the Supporting Information).

We confirmed that APL generates HE-AL upon reaction with hROS (Figure S2) and can be used to selectively and quantitatively monitor exogenously added hROS in vitro (Figures S3, S11), in cellulo (Figure S4), and in vivo (luciferase transgenic (luc-Tg) rats;<sup>[7]</sup> Figure 1b and Figure S5). The probe was reactive to  $ClO^-$ ,  $ONOO^-$ ,  $\cdot OH$ , and  $H_2O_2$ /HRP but not to milder ROS such as  $H_2O_2$ ,  $O_2^-$ , and NO under our experimental conditions. Furthermore,  $H_2O_2$ , which could be generated during the luciferin/luciferase reaction,<sup>[8]</sup> did not affect the performance of the probe. The detection sensitivity of exogenously added hROS under pseudo-tissue (Figure S6) and in the peritoneal cavity of luc-Tg rats (Figure S7) was much higher than that with the fluorescent probe 4-COOH MMSiR,<sup>[3a]</sup> even though the excitation and emission wavelengths of 4-COOH MMSiR are optimized in the near-infrared (NIR) region for efficient in vivo imaging.<sup>[9]</sup> There was a 1.1-fold increase with fluorescence with 4-COOH MMSiR compared to 7.9-fold increase in bioluminescence with APL in rat peritoneal cavity after injection of 5  $\mu mol$  of NaOCl.

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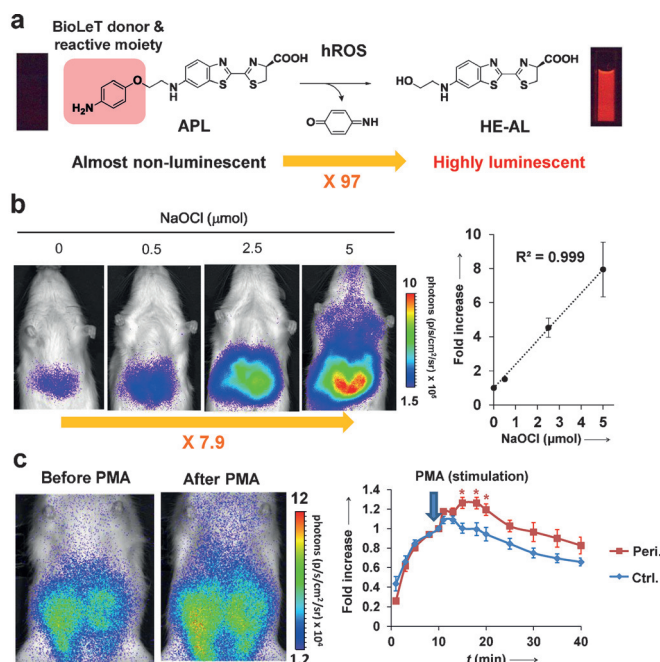
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**Figure 1.** Evaluation and application of APL. a) Design of APL based on the BioLeT mechanism. b) Bioluminescence imaging (BLI) of luc-Tg rats injected ip with APL and 0–5 μmol NaOCl. c) BLI of hROS in an acute peritonitis model of luc-Tg rats. Average photons from the abdomen were measured at each time point under control conditions (Ctrl.: zymosan-/PMA-) or after induction of peritonitis (Peri.: +/+). The probe (1 μmol in 900 μL of PBS) was injected ip at 0 min and PMA (30 μg for Peri., 0 μg for Ctrl. in 1 mL of PBS) was injected ip at 10 min. The right-hand image (after PMA) was taken 18 min after probe injection. Luminescence intensity was normalized to that immediately before PMA injection. Statistical analysis was conducted with Welch's t-test (\* $P < 0.05$ ).  $n = 8$  for Ctrl.,  $n = 3$  for Peri.. Error bars represent  $\pm$  SEM. Note that the scale of L.I. in Figure 1b is 10-fold different from that in Figure 1c.

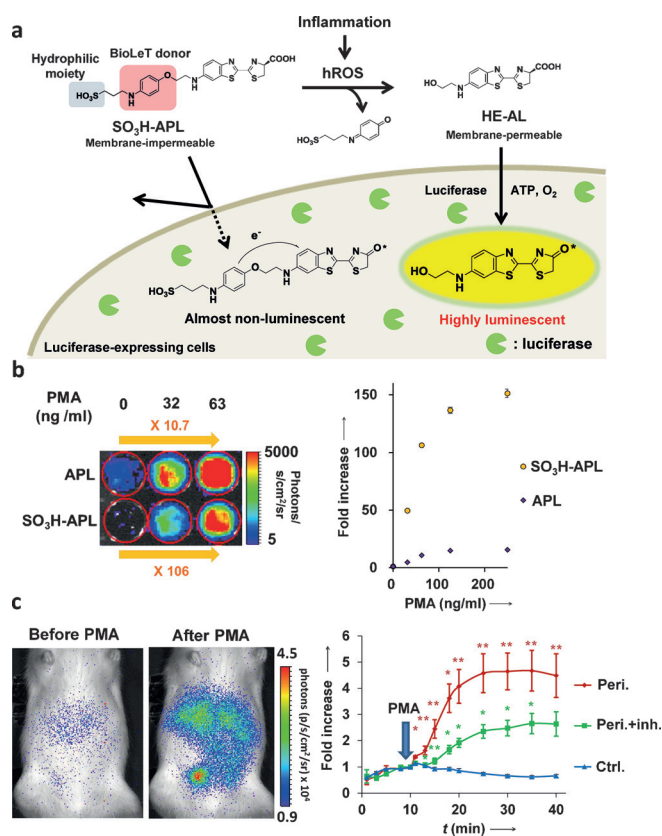
Motivated by these promising results, we next examined the ability of APL to visualize production of biologically relevant amounts of hROS in an acute peritonitis model<sup>[10]</sup> in luc-Tg rats. In order to induce migration of neutrophils to the peritoneal cavity, zymosan from *Saccharomyces cerevisiae* was administered through intraperitoneal (ip) injection. After 5 h, APL was injected ip, followed by bioluminescence imaging before/after ip injection of phorbol 12-myristate 13-acetate (PMA), a well-known, potent stimulator of the respiratory burst of neutrophils. However, although we observed a luminescence increase in the abdominal cavity after PMA stimulation, the activation ratio of the signal was only 1.3-fold (Figure 1c, S8), which was worrisome for practical usage. Therefore, it appeared that a probe with much higher sensitivity would be needed to clearly visualize small amounts of hROS in vivo.

In order to develop a probe with improved sensitivity, we carefully considered why we had obtained such a low activation ratio with APL in vivo. We thought that the key issue was the inevitable background bioluminescence signal of the probe itself, since APL can permeate through the cell membrane to encounter luciferase and ATP and emit

photons, even though it is well quenched by BioLeT. Therefore, in order to further reduce the background signal and to improve the in vivo performance of the probe, we focused on our previous finding that aminoluciferin derivatives with low cell-membrane permeability emit fewer photons in cellulo.<sup>[11]</sup> Specifically, we hypothesized that precise control of the membrane permeability of the substrate (see below) could be used as a new ON/OFF switching mechanism for bioluminescence. To our knowledge, changing the passive cell permeability of the substrate has never previously been used directly as a switching mechanism (see note S1 for further discussion and consideration of other reports).<sup>[12]</sup> We thus developed SO<sub>3</sub>H-APL by extending a sulfoalkyl moiety from the amino group of the aminophenoxy moiety of APL (Figure 2a). We anticipated that unreacted SO<sub>3</sub>H-APL would predominantly show extracellular localization owing to its membrane-impermeable character arising from the hydrophilic, negatively charged sulfonate moiety, and so would have little chance to encounter luciferase/ATP, which exist exclusively inside the cells, to emit photons. Even if a small fraction of SO<sub>3</sub>H-APL is internalized into cells, background luminescence would still be suppressed by BioLeT. Then, upon reaction with hROS, the membrane permeability and luminescence quantum efficiency would be increased simultaneously, thereby producing a dramatic luminescence activation. Indeed, we found that while SO<sub>3</sub>H-APL showed similar in vitro luminescence properties and hROS reactivity to APL (Figure S9–S11), it showed far higher luminescence activation in cellulo (Figure 2b and Figure S12), which enabled us to visualize hROS secreted from luciferase-expressing neutrophils with a remarkable activation ratio (Figure 2b).

A subcellular localization study confirmed that the intracellular concentration of SO<sub>3</sub>H-APL was indeed much smaller than that of APL (Figure S13). It is important to note that a small but significant fraction of the SO<sub>3</sub>H-APL was still present intracellularly (Figure S14), and therefore the role of BioLeT as a quenching mechanism remains important.

Finally, we assessed the performance of SO<sub>3</sub>H-APL for the visualization of hROS secreted from neutrophils in the luc-Tg rat acute peritonitis model. We found that the luminescence signal in the abdominal cavity increased markedly after PMA injection (Figure 2c, red line; see Figure S15a for quantification of fold increase of luminescence and Note S2 for a comment on the luminescence site), thus confirming that our concept of reducing background luminescence to increase probe sensitivity by controlling the membrane permeability of the probe does indeed work well (see also Figure S15b for the raw luminescence values and further discussion). We also observed a clear inhibitory effect of ebselen (Figure 2c, green line), a scavenger of various ROS,<sup>[13]</sup> thereby confirming that hROS were indeed produced in this rat model. It is especially noteworthy that SO<sub>3</sub>H-APL could clearly visualize biologically relevant amounts of hROS without the need to open the peritoneal cavity or shave the fur in this relatively large animal model, in which all of the tissues are much thicker than those of mice (Figure S16), because this indicates that SO<sub>3</sub>H-APL could allow time-lapse/repeated non-invasive in vivo imaging of physiologically relevant levels



**Figure 2.** Evaluation and application of  $\text{SO}_3\text{H-APL}$ . a) Probe design of  $\text{SO}_3\text{H-APL}$  based on precise control of both BioLeT and cell-membrane permeability of the substrate. b) BLI of hROS generated from luciferase-expressing neutrophils in a 96-well plate stimulated with different concentrations of PMA. Each red circle in the image represents one well of a 96-well plate. Error bars represent  $\pm$  SEM ( $n=3$ ). c) BLI of hROS in an acute peritonitis model of luc-Tg rats performed according to the same method as in Figure 1c (in the peritonitis plus hROS inhibitor condition (Peri. + inh.), ebbsen 0.5  $\mu\text{mol}$  was also added to PMA solution). The right-hand image (after PMA) was taken 30 min after probe injection. Statistical analysis was conducted with Welch's t-test ( $*P<0.05$ ,  $**P<0.01$ : for Peri. (red) the comparison is with Ctrl., for Peri. + inh. (green), the comparison is with Peri.).  $n=4$  for Ctrl.,  $n=5$  for Peri.,  $n=3$  for Peri. + inh., error bars represent  $\pm$  SEM.

of hROS in deep tissues. This has previously been impossible even with the sensitive NIR fluorescence probe 4-COOH MMSiR, which required laparotomy to detect signals even from much larger amounts of exogenously added hROS (Figure S7).

As regards biodistribution, the ip-injected probe remained in the peritoneal cavity of luc-Tg rat for at least 30 min (Figure S17), as was also the case for D-luciferin (native substrate) and AL, but the intravenous (iv)-injected probe was distributed throughout the whole body (Figure S18), thus suggesting that this probe would be potentially available for hROS imaging anywhere in the body.

We have thus succeeded in developing  $\text{SO}_3\text{H-APL}$ , a highly sensitive bioluminogenic probe for hROS, by utilizing dual control of bioluminescence emission by means of BioLeT and modulation of cell-membrane permeability. Even APL was greatly superior to a NIR fluorescence probe

for in vivo imaging, and the further improvement afforded by  $\text{SO}_3\text{H-APL}$  makes it the first probe that can detect physiological amounts of hROS clearly and non-invasively in inflammation or disease models in deep tissues. Application of the membrane-impermeable  $\text{SO}_3\text{H}$  moiety to control the passive membrane permeability of the probe as an additional ON/OFF switching modality, which is generally not available for fluorescence probes, could be useful for developing other bioluminogenic probes. We believe this work will extend the applicability of luciferin/luciferase systems by providing both a highly sensitive tool for investigating the roles of hROS in vivo, as well as a new design strategy for highly activatable in vivo bioluminogenic probes.

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