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New Class of Bioluminogenic Probe Based on Bioluminescent Enzyme-Induced Electron Transfer: BioLeT

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Supporting Information

ABSTRACT: Bioluminescence imaging (BLI) has advantages for investigating biological phenomena in deep tissues of living animals, but few design strategies are available for functional bioluminescent substrates. We propose a new design strategy (designated as bioluminescent enzyme-induced electron transfer: BioLeT) for luciferin-based bioluminescence probes. Luminescence measurements of a series of aminoluciferin derivatives confirmed that bioluminescence can be controlled by means of BioLeT. Based on this concept, we developed bioluminescence probes for nitric oxide that enabled quantitative and sensitive detection even in vivo. Our design strategy should be applicable to develop a wide range of practically useful bioluminogenic probes.

In vivo optical imaging is a powerful tool to study complex physiological and pathological processes in living organisms,[1,2] and bioluminescence imaging (BLI), which utilizes genetically encodable luciferase as an internal light source, has a number of advantages for this purpose. Unlike fluorescence imaging, which requires external illumination, a bioluminescence signal generated deep inside the body can be detected with high signal-to-noise ratio and is unaffected by tissue absorption/scattering of excitation light or background autofluorescence of tissues. So, the luciferin-luciferase system, especially that of firefly, has been widely used in BLI to monitor gene expression, to track cells, and to access tumor progression in in vivo systems.[3−5] Functional BLI, which can be achieved by off/on switching of bioluminescence in response to a specific biological phenomenon, can provide even more information. For example, advances in luciferase engineering technology, such as split luciferase complementation[6,7] and bioluminescence resonance energy transfer,[8,9] have made it possible to quantify protein−protein interactions and to detect biomolecules by bioluminescence. Chemical probes utilizing bioluminescence have also been used to visualize various molecular activities such as those of β-galactosidase,[10] β-lactamase,[11] and proteases,[12,13] as well as hydrogen peroxide (H_2O_2),[14,15] in vivo.

However, rational design strategies for bioluminescence probes are currently quite limited compared to those for fluorescence probes. Various principles have been applied to rational development of fluorescence probes, including photoinduced electron transfer (PeT),[16,17] Förster resonance energy transfer (FRET),[17,18] spirocyclization,[19,20] and intramolecular charge transfer,[21,22] whereas currently available rational design strategies for bioluminescence probes rely mostly on the "caged luciferin" concept, i.e., release or generation of D-luciferin (native firefly luciferin) or aminoluciferin (AL) after reaction of the probe with the target molecule. Probes based on this idea include 6′-*O*-ether analogues of D-luciferin[10,11,14] and others[12,13,23−25] (Figure S1 and also see SI for another bioluminescence probe). Thus, the limitations of current design strategies continue to hamper the development of bioluminescence probes, despite the potential advantages of such probes, and it is generally agreed that new rational design strategies are urgently needed.[26,27] Here, we report a novel principle for controlling the luminescence properties of AL derivatives by utilizing electron transfer from a benzene moiety to the excited luminophore (Figure 1A). Based on this idea, we designed, synthesized, and evaluated bioluminogenic probes for nitric oxide (NO). Our design concept should be applicable to develop a wide range of practically useful bioluminogenic probes.

In order to develop a flexible design strategy for bioluminescence probes, we focused on electron transfer as an off/on switching mechanism, since PeT-based switching has been a versatile design strategy for fluorescence probes targeting a wide range of biomolecules, including NO,[17] singlet oxygen,[17] and metal ions.[16] The principle of PeT is that an electron-transfer process to or from the excited state of a fluorophore diminishes the fluorescence from the singlet excited state (Figure 1A, upper). As a working hypothesis, we considered that the luminescence of luciferase substrates would be similarly modulated, since both fluorophore and bioluminophore take singlet excited states, although they differ in the driving force, i.e., light illumination or enzymatic reaction (Figure 1A, lower). Therefore, we investigated the feasibility of employing electron

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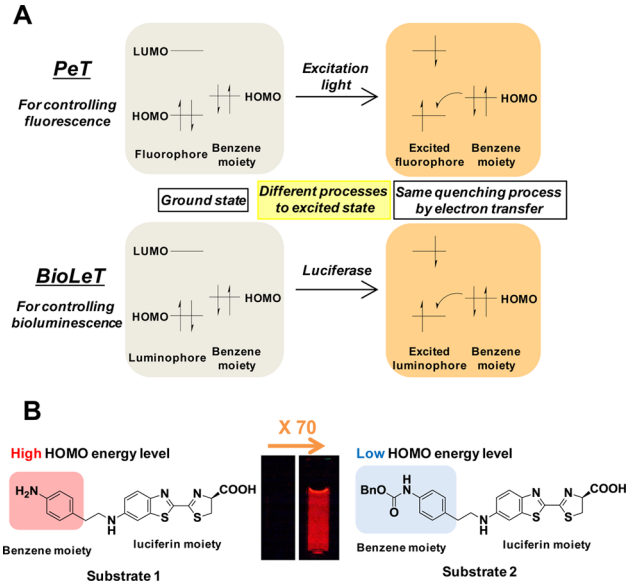

Figure 1. Concept of quenching of the excited state of a luminophore by BioLeT. (A) Schematic diagram of a fluorescent molecule and a bioluminescence substrate bearing a benzene moiety with a high HOMO energy level. The excited luminophore can be quenched by BioLeT, just as the excited fluorophore is quenched by PeT. (B) Chemical structures of substrate **1** and **2** and comparison of luminescence upon reaction with luciferase.

transfer to the excited state of a luminophore as a principle for controlling luminescence.

In our studies of PeT,[28,29] we showed that the rate of electron transfer is strongly dependent on the HOMO energy level of the electron donor. To examine whether the luminescence of luciferin analogues could be similarly quenched through an electron-transfer process, we first prepared substrate **1** and its urethane derivative **2** by introducing an electron-donating aniline moiety or a less electron-donating anilide moiety into the AL scaffold (Figure 1B, Scheme S1). AL was selected because a range of functionalities can be introduced at the 6′-*N* position without loss of the luminescence (Figure S1).[30−32] Upon reaction with luciferase from *Photinus pyralis*, substrate **2** showed 70-fold higher luminescence than **1** (Figure 1B), even though **2** has a bulky additional moiety compared with **1**, and **1** was completely consumed by the enzymatic reaction (Figure S2). These results indicated that substrate **1** reacted with luciferase to yield an excited-state intermediate but did not emit strong bioluminescence, presumably because the electron-transfer process occurs much faster than the light-emitting process. This supports the idea that luciferase-dependent luminescence can be modulated by means of an electron-transfer process. As there is no previous report of electron transfer to the excited state of a luminophore, we designated this process as bioluminescent enzyme-induced electron transfer (BioLeT).

Next, in order to confirm that BioLeT really exists and to examine whether it is feasible to rationally design bioluminescence probes by utilizing this phenomenon, we prepared a series of AL derivatives bearing benzene moieties with various HOMO energy levels (Figures 2A and S3, Schemes S2−5) and examined the correlation between HOMO energy level and luminescence intensity. Indeed, the luminescence intensity of the substrates varied greatly depending on the HOMO energy level of the benzene moiety (Figures

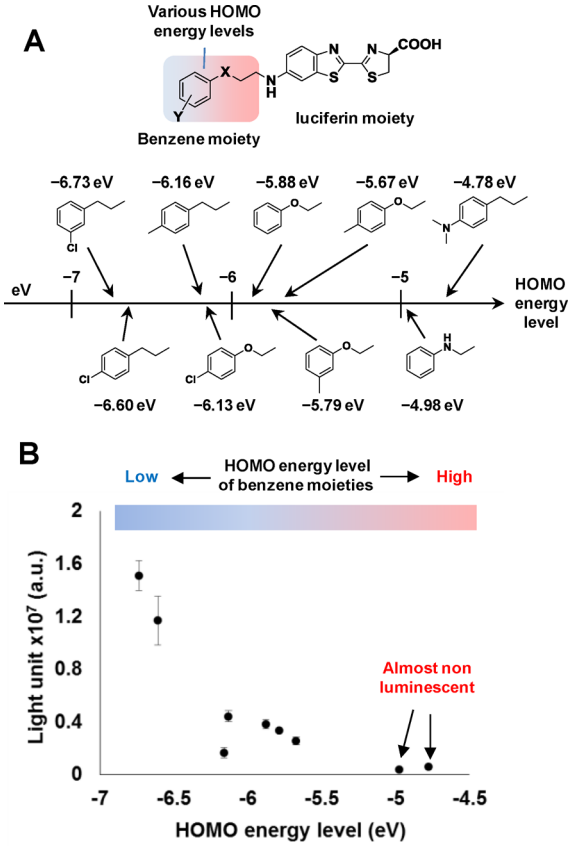

Figure 2. Verification of BioLeT with AL derivatives. (A) Chemical structures of AL derivatives used for verification of the occurrence of BioLeT. (B) Relationship of HOMO energy level of the benzene moiety and luminescence intensity of the substrates. Error bars represent ± SD (n = 3).

2B and S3c), while the shapes of their bioluminescence spectra and the K_m values of the substrates were almost identical (Figure S3b,c). As in the case of fluorophores (Figure S4), the luminescence was quenched as the HOMO energy level of the benzene moiety increased, which strongly supports the occurrence of BioLeT-dependent quenching. Further, since the AL derivatives were almost non-luminescent when the HOMO energy level of the benzene moiety was more than −5 eV, it should be possible to predict whether or not the luminescence of substrates would be strongly quenched by BioLeT by calculation of the HOMO energy level of the benzene moieties (see SI for further discussion about Figure 2B).

In order to validate BioLeT as a principle for developing a new class of bioluminescence probes, we aimed to develop a novel bioluminescent probe by using BioLeT as a luminescence switching mechanism. For this, we focused on NO as target molecule, since it is well established that a diaminobenzene group reacts with NO under air to form benzotriazole, resulting in a substantial change of the HOMO energy level of the benzene moiety.[33,34] We designed and synthesized diaminophenylpropyl-AL (DAL) as shown in Figure 3A and Scheme S6. We also prepared DAL-T, which is the putative reaction product of DAL with NO, as a reference substrate. The HOMO energy levels of the diaminophenyl moiety and benzotriazole moiety were calculated to be −4.68 and −6.22 eV, respectively, which indicated that bioluminescence of DAL would be

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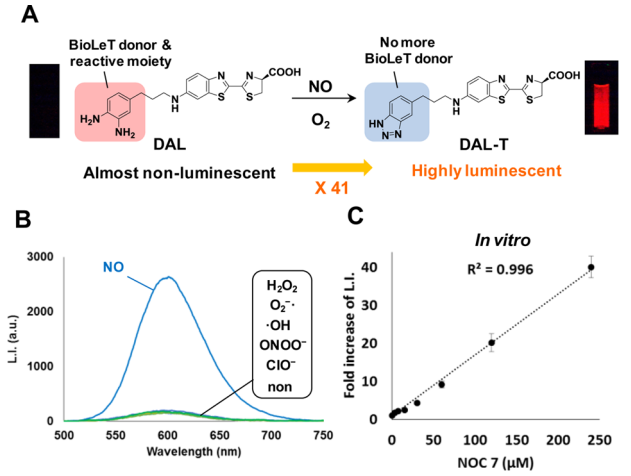

Figure 3. Development of DAL, a bioluminescence probe for NO. (A) Molecular design of DAL based on the BioLeT mechanism. (B) Selectivity of DAL among various ROS. (C) Quantification of NO released from a NO donor using DAL in vitro (error bars represent ± SD n = 4).

sufficiently quenched by BioLeT, while that of DAL-T would not be quenched, resulting in recovery of the characteristic luminescence properties of DAL upon reaction with NO.

As expected, the luminescence intensity of DAL was strongly quenched, while that of DAL-T was not, and the increase of DAL-T luminescence compared to that of DAL was about 41-fold (Figures 3A and S5). We confirmed that the rates of consumption of DAL and DAL-T by luciferase were almost the same (Figure S6). These findings strongly support the idea that the difference of luminescence intensity of DAL and DAL-T was indeed derived from the difference of luminescence quantum efficiency from the singlet excited state, which should be regulated by BioLeT. We also confirmed that effective quenching of bioluminescence was reduced when a diamino-benzene moiety was conjugated at a remote site from the luminophore (Figure S7 and Scheme S7), which again supports the operation of BioLeT, because the efficacy of quenching of the singlet excited state by electron transfer is known to decrease with increasing distance between the electron donor and the acceptor[28] and such a large difference in efficacy of the quenching in this range of distance (<1 nm) is characteristic of electron-transfer-based quenching.

Next, we examined detection of NO with DAL in vitro. DAL showed a large luminescence increase upon addition of NO-bubbled solution (Figure S8) or NOC7 (Figure 3B), a NO donor that is known to release NO when dissolved in neutral aqueous solvents. LC-MS analysis of the reaction solution demonstrated that DAL was converted to DAL-T as the sole product (Figure S9), providing direct evidence that DAL reacts with NO and the reaction product is luminescent. Further, the luminescence increase was linearly correlated with the amount of NOC7 added to the solution (Figure 3C, detection limit: 1.51 μM) and was selective for NO among various ROS (Figure 3B), showing that DAL can quantitatively and selectively detect NO. These ROS did not affect the luminescence intensity of AL under our conditions (Figure S10). We further examined the availability of DAL in cellulo. When HEK293 cells stably expressing luc2 were incubated with a solution of DAL and various concentrations of NOC7, we observed a luminescence signal that depended on the amount of NOC7 (Figure S11). These results demonstrate that DAL can quantitatively detect NO even in cellulo and support its availability for BLI in living systems. Then, we examined the applicability of DAL in transgenic rats ubiquitously expressing firefly luciferase (luc-Tg rat), whose gene is driven under the ROSA26 promoter.[35] NOC has been reported to release NO even in vivo,[36] so we again used NOC7 as a NO donor. At 10 min after i.p. injection of DAL (1 μmol), a freshly prepared NOC7 (20 μmol) solution in PBS was i.p. injected. We found that DAL could detect NO released from a NO donor in the peritoneal cavity of luc-Tg rat (Figure 4, see Figure S12 for

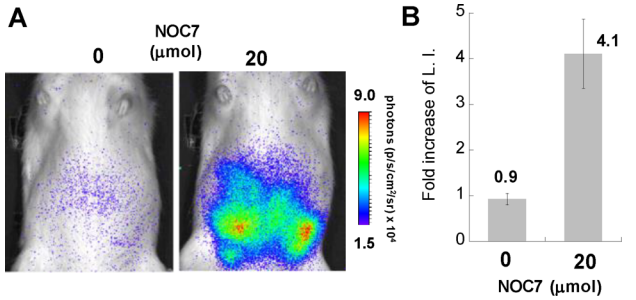

Figure 4. Detection of NO released from NO donor with DAL in vivo. (A) BLI of DAL in luc-Tg rat with NOC7. At 10 min after i.p. injection of 1 μmol DAL, luc-Tg rats were i.p. injected with or without 20 μmol NOC7. The images were taken at 40 min after the injection of NOC7. (B) Quantification of luminescence intensity (L.I.) of DAL from abdomen of luc-Tg rat in (A) (error bars represent ± SEM n = 3). The intensities were normalized by those just before the injection of NOC7.

kinetics). This result directly demonstrates that DAL is applicable for BLI in vivo. Further, when injected i.v., DAL was distributed to the whole body, suggesting that it would be available to monitor NO production throughout the body (Figure S13). It should be noted that a red fluorescence probe for NO, DAR-4M AM, was subject to serious interference by autofluorescence from fur and by absorbance/scattering of skin and tissues (Figure S14). On the other hand, the signal of BLI can be detected even without shaving the fur, and a strong luminescence increase was observed, indicating that functional bioluminescence probes should provide much more reliable data than fluorescence probes in in vivo imaging of rats. Thus, the BioLeT-based strategy enabled us to design and develop a new class of bioluminescence probe for NO, DAL, which could not have been developed based on the caged luciferin strategy.

In conclusion, we present here a new concept for rational design of functional bioluminescence probes, BioLeT, which should enable flexible design of a wide range of functionalized bioluminescence probes for detecting various biomolecules of interest, just as PeT has been used for development of versatile fluorescent probes. The validity of this approach was demonstrated by development of a novel bioluminogenic probe for NO, DAL. For in vivo imaging, bioluminescence-based techniques are well-known to have significant advantages over fluorescence-based ones. We believe that our findings will extend the applicability of luciferin-luciferase systems by providing sensitive tools for investigating a broad range of biological phenomena, especially in vivo.

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■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures, characterization of synthesized compounds, and supplementary data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare the following competing financial interest(s): Luciferase transgenic rats used in this study are in the subject of a Japanese patent (patent number: 5198433).

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