Bioluminescence

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Rational Design and Development of Near-Infrared-Emitting Firefly Luciferins Available In Vivo**

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Bioluminescence, especially that of the firefly, is widely used as an analytical tool in the life sciences,[1] because excitation is unnecessary and target signals can be detected with high sensitivity. However, bioluminescence is generally restricted to the visible region, and overlaps with the absorption spectra of many biological molecules, such as hemoglobin, which makes it difficult to detect signals from deep tissues.[2] So, extending bioluminescence beyond the visible range, especially into the near-infrared (NIR) region, is an important goal.

Researchers have tried to lengthen the emission wavelength of bioluminescence mainly by modifying luciferase. For example, the genetic modification of various kinds of luciferase has been reported,[3] but so far an emission maximum beyond 650 nm has not been achieved. Bioluminescence resonance energy transfer (BRET) has also used to tune emission wavelength: for example, there have been reports of NIR bioluminescence with chemically modified luciferase,[4] far-red luminescence technology using a Cypridina luciferase–organic dye conjugate,[5] and NIR-emitting luciferase using quantum dots (QDs)[6] or quantum rods (QRs).[7] However, it is difficult to apply these systems to in vivo reporter assays or to trace luciferase-expressing cells without antibodies, because they are all based on ex vivo luciferase modification. A NIR-emitting fluorescent protein having an emission maximum over 700 nm was also reported,[8] but has not been characterized as a BRET acceptor.

On the other hand, luciferin modification has been employed to obtain activatable luciferins,[9] but modification of luciferin to tune its bioluminescence spectrum has only been attempted in a few cases,[2a,10] in all of which bioluminescence remained in the visible region. We considered that, in principle, it should be possible to freely tune the bioluminescence spectra by conjugating a BRET acceptor to luciferin by a suitable linker, without the need for manipulation of luciferase (Supporting Information, Figure S1).

We have previously developed functional aminoluciferin (AL) derivatives by attaching functional groups to the amino group of AL,[11] making use of the fact that AL does not lose its luminescence when its amino group is alkylated.[12] Interestingly, we obtained an AL derivative that showed a longer emission wavelength than conventional substrates, owing to the occurrence of BRET (Cy5 COOH–AL; Figure 3a). However, it seemed surprising that Cy5 COOH–AL, which contains a bulky fluorophore conjugated to AL, is luminescent, considering the size of conventional luciferase substrates. Indeed, we found that many AL derivatives with bulky substituents lost their luminescent character (data shown in part in Figure S3). So, we set out to establish a new design strategy to obtain luminescent AL–fluorophore conjugates. Such a strategy should make it possible to develop a range of AL derivatives with various functional groups. In particular, we hoped to develop luciferins that emit in the NIR range (especially over 700 nm, the most desirable window for biological applications). Such NIR-emitting luciferins are expected to be especially suitable for cellular imaging and in vivo imaging.

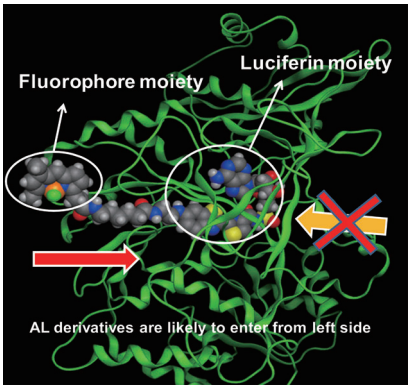

Figure 1. Result of the docking simulation of an AL derivative with luciferase from *Photinus pyralis*.

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To establish a rational design strategy, we first conducted a docking simulation of AL derivatives with firefly luciferase using the MOE software package (Figure 1). The crystal structure of firefly luciferase has been reported by several groups, including Conti et al.[13] (PDB:1 LCI; first report but without substrate) and Auld et al.[14] (PDB:3IES; complex with luciferase inhibitor). Among them, we chose the one reported by Nakatsu et al.[15] (PDB: 2DIS; complex of luciferase from *Luciola cruciate*, Japanese name: Genji-botaru, with a high-energy intermediate analogue, DLSA) for our docking study because of the structural similarity of the substrates. Therefore, we used this crystal structure as a scaffold and changed the amino acid sequence to that of *Photinus pyralis*, which was used in our work. We fixed the peptide backbone of luciferase and the DLSA (aminoluciferin) moiety, and searched for the most stable conformation, freely rotating the side chains of amino acids and the fluorophore-linker moiety of the substrate (Figure 1).

As shown in Figure 1, we found that in the most stable conformation the linker moiety penetrates through the luciferase molecule, and motion of the fluorophore and linker moiety of the substrate in the luciferase complex would appear to be highly restricted. If an AL derivative entered from the right side and the adenosine moiety is placed like a cap at the entrance of the pocket, as indicated by the orange arrow in the figure, the bulky fluorophore moiety would have to pass through the luciferase molecule, which seems very unlikely. Therefore, based on this simulation, we hypothesized that AL derivatives enter from the left side, as indicated by the red arrow. If this is the case, a flexible linker might be desirable, so that the fluorophore does not hinder the access of the luciferin moiety to the active site of the luciferase. We considered that it might be possible to employ relatively bulky fluorophores, if we conjugated them by appropriate linkers.

Taking these points into consideration, we next examined the structure–luminescence intensity relationship of a number of AL derivatives. (Figure 2; see also the Supporting Information, Figures S2 and S3) To simplify the discussion, here we focus on several AL–fluorophore conjugates in which BRET does not occur because of the short absorption wavelength of the fluorophores. The relative luminescence intensity of each compound was compared at three minutes after the start of the reaction (see Figure S6 for the choice of this time).

At first, fluorescein piperazine–AL was not luminescent (Figure 2b). We also confirmed that fluorescein piperazine–AL was not consumed by firefly luciferase (Figure S2). These results support the idea that short and rigid linkers are unsuitable, as we had expected from the docking simulation with the crystal structure. Next, we focused on BODIPY FL–AL and BODIPY FL X–AL, which differ only in the length of the linkers. BODIPY FL X–AL, which has a longer linker, was brighter than BODIPY FL–AL (Figure 2b), and this tendency was also observed in other substrates (Figure S3). Therefore, relatively long linkers appear to be favorable. Focusing on 8-phenyl BODIPY X–AL, we found that luminescence was retained if an appropriate linker was selected (Figure 2b), even though the fluorophore is relatively bulky (the BODIPY moiety and the benzene moiety conjugated at the 8 position of BODIPY are orthogonal), which is consistent with our hypothesis that the fluorophore moiety does not pass through the luciferase molecule. We also found that the nature of the fluorophore is not critical and that excessively hydrophobic linkers and polyethylene glycol (PEG) linkers conjugated near the luminophore are not desirable (Figure S3). These results are consistent with the docking simulation and the above discussion.

Therefore, our strategy of obtaining a range of AL–fluorophore conjugates by using appropriate linkers (Figure S3) to conjugate various kinds of fluorophores, including bulky ones, to AL with retention of luminescence appears to be feasible.

Based on these findings, we designed and synthesized AL derivatives bearing the NIR fluorophores Cy5, BODIPY 650/665, SiR700,[16] and Cy7 as BRET acceptors to cover a broad NIR window (Figure 3a). As expected, all the substrates showed NIR bioluminescence (Figure 3b; see also the Supporting Information, Figure S6). BRET efficiency was very high (Figure S4), even if the overlap of the absorption spectrum of the acceptor and the emission spectrum of the original AL was small, this is probably because the BRET donor and acceptor are sufficiently close to each other (the BRET process is intramolecular). The BRET efficiency of our system is comparable to those of reported BRET systems using luciferase modified with NIR dye,[4] QDs,[6] or QRs,[7]

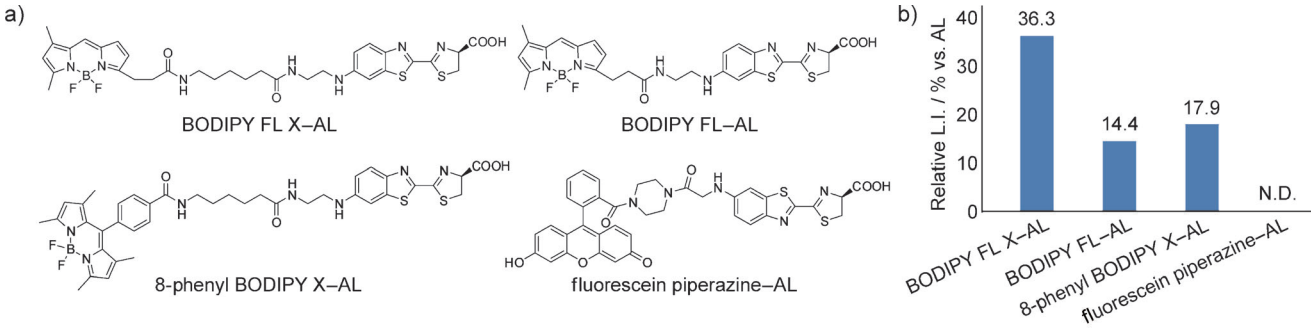

Figure 2. Example of the structure–luminescence intensity relationship of AL–fluorophore conjugates. a) Structure of each substrate. b) Comparison of relative luminescence intensity of each substrate (% vs. AL). Luminescence intensity was compared at 3 min after the addition of luciferase. Luminescence intensity of AL is about 30% of that of D-luciferin. N.D. = not determined.

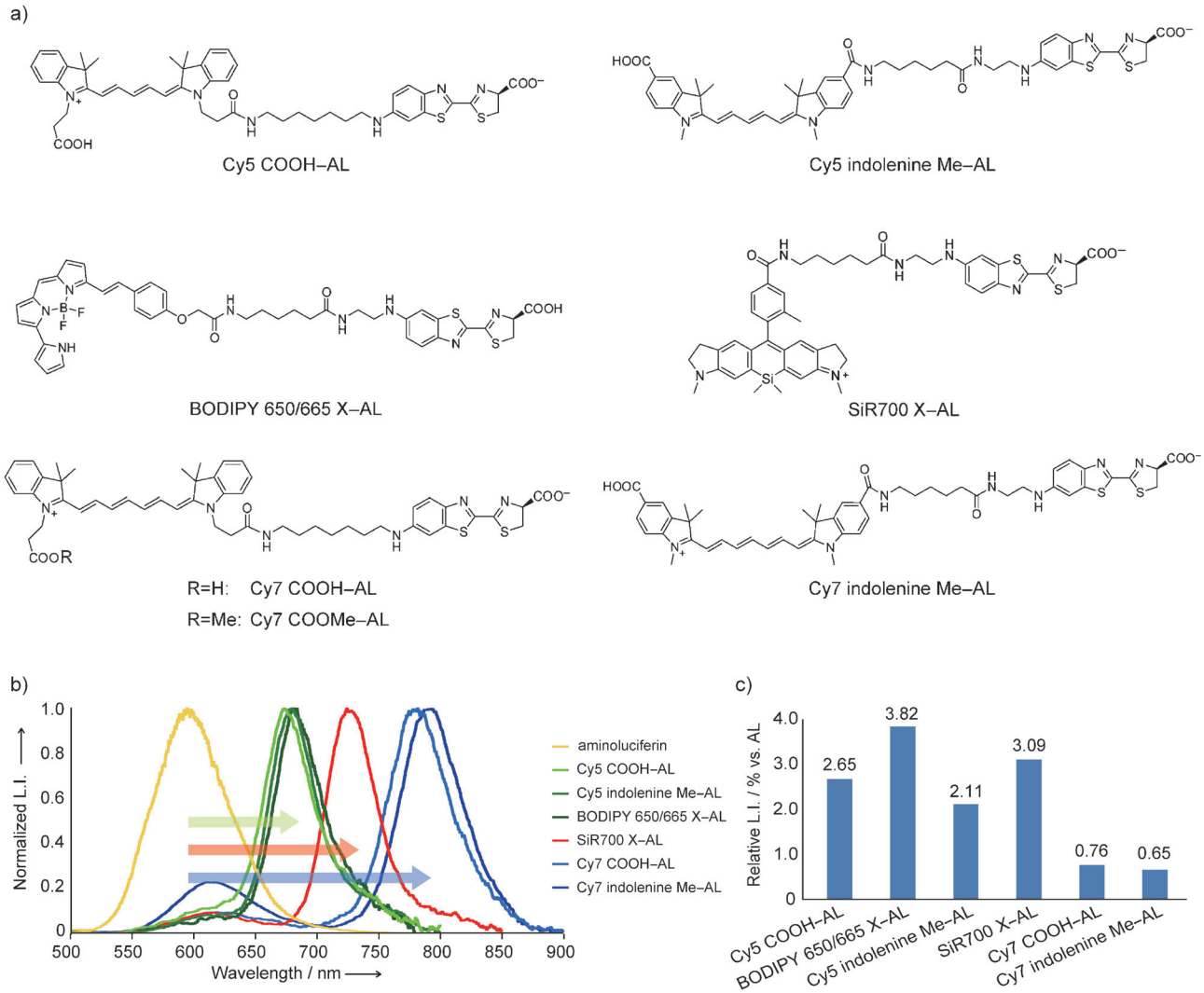

Figure 3. NIR-emitting luciferins and their properties. a) Structure of each substrate. b) Normalized emission spectra of each substrate. c) Comparison of relative luminescence intensity of NIR-light-emitting luciferins. Luminescence intensity was compared at 3 min after the addition of luciferase.

which have been optimized to achieve a high BRET efficiency, but which require ex vivo luciferase modification. We could freely tune the bioluminescence emission spectra by using appropriate NIR fluorophores. Remarkably, the maximal emission wavelength among substrates having Cy7 as a BRET acceptor reached almost 800 nm, which is extraordinarily long compared to those of conventional substrates and other reported substrates.[2a,10] We also confirmed that NIR bioluminescence in the wavelength range emitted from these substrates showed excellent tissue penetration by means of phantom experiments using pseudo tissue (Figure S5).

Next, we applied these NIR-emitting luciferins to living cells. A representative substrate, BODIPY 650/665 X–AL, showed NIR bioluminescence in cells expressing luciferase in the cytosol (Figure 4a), which is consistent with the fact that BODIPY 650/665 X–AL is cell-membrane-permeable and is distributed in the cytosol (Figure S7). On the other hand, Cy7 COOH–AL was membrane-impermeable and not luminescent in a cell-based assay (data not shown), so we esterified the carboxyl group. The methyl-esterified substrate, Cy7 COOMe–AL (Figure 3a), showed almost the same luminescence properties as Cy7 COOH–AL in vitro (Figure S6), even though the net charge of the cyanine moiety is different from that of other cyanine-based substrates (net charge of the cyanine moiety at physiological pH of Cy7 COOMe–AL was +1, others 0), and also showed NIR bioluminescence in luciferase-expressing cells (Figure 4b), in which luciferase is localized to the mitochondria, matching the intracellular localization of the substrates (Figure S7). It should be noted that luminescence from this substrate in cells expressing luciferase in the cytosol was very weak (data not shown); therefore, it is important to match the subcellular localization of the substrate and luciferase, and it is not adequate to simply use a cell-membrane-permeable substrate.

Finally, we examined the suitability of these NIR-emitting luciferins for in vivo imaging (Figure 4c,d). We transplanted luciferase-expressing cells subcutaneously in living mice, and tried to detect NIR bioluminescence from the cells using

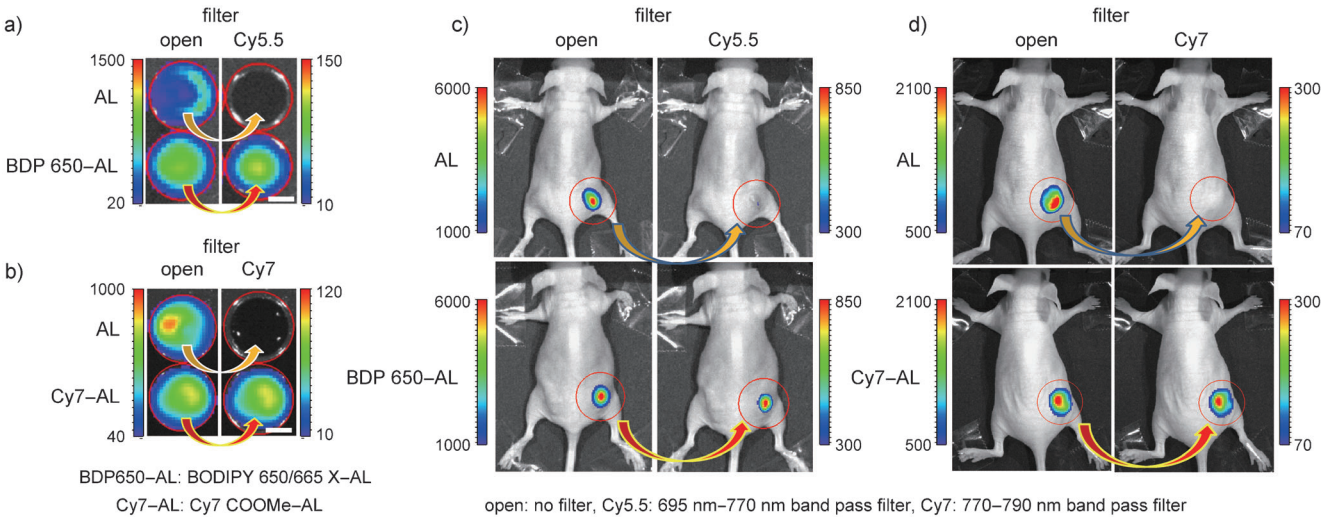

Figure 4. NIR bioluminescence in living cells and living mice. a) NIR bioluminescence from BODIPY 650/665 X–AL in Colon26-luc2 cells. b) NIR bioluminescence from Cy7 COOMe–AL in HEK293-luc2-mito cells. Cells expressing luciferase were seeded in 96-well plates (scale bar: 3 mm) and luminescence intensity of AL and NIR-emitting luciferins was adjusted for cell number (a,b). c) NIR bioluminescence from BODIPY 650/665 X–AL in Colon26-luc2 cells transplanted into living mice. The substrate was administered by intratumoral injection. d) NIR bioluminescence from Cy7 COOMe–AL in HEK293-luc2-mito cells in living mice. The substrate was injected subcutaneously together with luciferase-expressing cells. Luminescence intensity of AL and NIR-emitting luciferins was adjusted for exposure time (c,d).

BODIPY 650/665 X–AL and Cy7 COOMe–AL. We found that NIR emission from both substrates could be detected in vivo. By using band-pass filters, we established that the emission spectra of NIR bioluminescence emitted from living cells and living mice were almost the same as in vitro (Figure S8 a,b). Thus, substrates developed according to our design strategy are suitable for in vivo NIR-bioluminescence imaging.

In conclusion, we have developed BRET-based NIR-emitting luciferins by utilizing a new design strategy based on the results of docking simulation and studies of structure–luminescence intensity relationships. As far as we know, this is the first report of NIR bioluminescence obtained in living cells and living animals without the need for the manipulation of luciferase. Our results indicate that compounds synthesized according to this strategy have the ability to provide NIR bioluminescence signals from deep tissues. A remaining issue is that luminescence from these substrates was rather weak (about 0.6–4 % of that of AL in vitro (Figure 3 c) and about 0.1–0.4 % in cells; Figure S8c), probably because of their restricted intracellular delivery, as well as their relatively weak luminescence in vitro. However, we think further optimization of the structure of the substrates, as well as the optimization of luciferase by point mutation may overcome these drawbacks. For example, the quantum efficiency (QE) of the fluorescence of the BRET acceptor we used in this study was relatively low (Figure S4), so it should be possible to increase the luminescence intensity if we could incorporate other dyes that have higher QEs as the BRET acceptor. Further optimization of linkers (such as the position of amide bonds, or hydrophobicity) might also make it possible to improve both luminescence intensity in vitro and intracellular delivery. As for point mutation of luciferase, Ultra-Glo (Promega) is reported to be capable of highly sustained light emission with AL derivatives,[12b,c] and Harwood et al. have reported a mutant firefly luciferase that efficiently utilizes AL derivatives,[17] so it might also be possible to increase the luminescence intensity of our NIR-emitting luciferins by using such mutant luciferases. We should point out that the deficiencies in our present system are counterbalanced by the uniquely long emission wavelengths obtainable with these compounds.

Although we used NIR fluorophores to achieve NIR emission in this report, our findings suggest that, in principle, any kind of chromophore could be conjugated to AL by a suitable linker, which opens up the possibility of obtaining new bioluminescent functional molecules that can be used in deep tissue in living animals. In other words, if other photofunctional groups are used as BRET acceptors, it should be possible to induce the associated functions without excitation in living animals.

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