Luciferase–Rose Bengal conjugates for singlet oxygen generation by bioluminescence resonance energy transfer†

Seonghoon Kim, ‡ a,b HyeongChan Jo, b Mijeong Jeon, b,d Myung-Gyu Choi, b,c Sei Kwang Hahn b,d and Seok-Hyun Yun a,b†

Conjugates of Rose Bengal and Renilla luciferase generated singlet oxygen upon binding with coelenterazine via bioluminescence resonance energy transfer (BRET). Since the applications of conventional PDT have been limited to superficial lesions due to the limited light penetration in tissue, BRET activated PDT which does not require external light illumination may overcome the limitations of conventional PDT.

Photodynamic therapy (PDT) is used clinically to treat dermatologic lesions, retinal diseases, and epithelial tumors.1,2 PDT employs photosensitizer (PS) molecules and uses light to activate drugs to generate reactive oxygen species (ROS), such as singlet oxygen, and free radicals. These photochemical products can kill target cells and destruct tissues. This toxicity mechanism is different from the cytotoxicity mechanisms of chemotherapy, radiation therapy and immunotherapy. The difference makes PDT an attractive option for stand-alone or combinatorial therapy. Another distinct aspect of PDT comes from the light-induced activation of photosensitizers. This provides an advantage of spatial and temporal controllability of drug activation. However, the need for light also limits the applications of PDT, because of the light’s shallow penetration depth in tissues. To date, clinical PDT has been adopted to treat diseases in the skin and retina, which physicians can readily approach with a light source, or in epithelial layers of endoscopically accessible sites such as gastrointestinal tracts. To enhance the therapeutic depth of PDT, considerable efforts have been made in developing PS3 molecules with action spectra in the near-infrared (NIR) range and up-conversion nanoparticles4 that absorb NIR photons and deliver energy to conventional PS drugs. Yet, the limited optical penetration depth (<5 mm) even in the NIR range leaves many diseases out of reach.

To solve this problem, researchers have sought new methods capable of remotely activating PS agents in deep tissues. One such approach is to use Cherenkov radiation produced by beta particles during radioactive decay.5 However, the potential toxicity of radio-active isotopes and inorganic photosensitive nanoparticles, such as TiO2, needs to be addressed for clinical translation.6 Another approach is to use Förster resonance energy transfer from chemiluminescent or bioluminescent molecules. Yuan et al. demonstrated antimicrobial PDT by employing luminol and electrostatically-bound cationic oligo(p-phenylene vinylene).7 Hsu et al. demonstrated cancer therapy by using self-illuminating quantum dots conjugated with mutant Renilla luciferases.8 Kim et al. extended this approach and demonstrated local therapy of cancer cells in draining lymph nodes in mice.9 While these experiments support the feasibility of remotely activated PDT, the long-term toxicity of luminol and quantum dots raise concern about their potential for clinical translation. Furthermore, because donors (luciferases) and acceptors (PSs) are administered separately, bioluminescence resonance energy transfer (BRET) occurs only when they are located close to each other within 5–10 nm. Chemical conjugation of bioluminescent enzymes and PS drugs could solve this problem, but such BRET pairs have not been demonstrated.

Here, we report the conjugation of a luciferase and a PS, for the first time to our knowledge. In this work, we have used mutant Renilla luciferases 8.6 (RLuc8.6)10 and Rose Bengal (RB) dyes to form BRET pairs (Fig. S1, ESI†), where the emission peak of RLuc8.6 at 535 nm is well matched with an RB’s absorption peak at 550 nm. RB is an efficient PS with a high quantum efficiency of 0.7–0.8 (measured in aqueous media) in the generation of singlet oxygen.11 We have investigated the efficiency of singlet oxygen generation and the ROS-induced cytotoxicity of the luciferase–RB conjugates for BRET-induced PDT in direct comparison with laser-induced activation used in conventional PDT.
Our initial scheme for conjugating RLuc8.6 and RB via a short linker retained bioluminescence (BL) capability but failed to achieve efficient BRET to RB (Fig. S2, ESI†) presumably due to the quenching of the RB. To solve this problem, we used bovine serum albumin (BSA) as a central piece to which RLuc8.6 and RB were conjugated. The rationales for this design were to provide space between RB and RLuc8.6 to evade quenching and enhance the BRET efficiency by attaching multiple RLuc8.6 molecules in each complex. When the mixing ratio of RB to BSA was 5:1, the highest FL intensity was measured from RB–BSA conjugates (Fig. S3a, ESI†). The actual conjugation ratio was estimated to be 2.2:1 by comparing the absorbance of purified RB–BSA conjugates to the absorbance of unpurified simple mixtures of RB–NHS and BSA (Fig. S3b, ESI†). RLuc8.6 was linked to the RB–BSA conjugate by Cu-free click reaction to form an RB–BSA–PEG₃–RLuc8.6 conjugate, hereinafter called LucRB (Scheme 1). A polyacrylamide gel electrophoresis of LucRB purified with a 100 kDa filter showed bands near 140, 180, 210 and 250 kDa, respectively, corresponding to 2, 3, 4 and 5 RLuc8.6 molecules per construct (Fig. S3c, ESI†). The LucRB conjugates with heterogeneous molecular weights were used in the experiments without further purification. The hydrodynamic size and zeta potential of LucRB were measured to be 11 nm and −6.6 mV, respectively.

While RLuc8.6 is colorless, LucRB solution has a pink color owing to RB (Fig. 1a). When CTZ was administered, LucRB produced significantly lower BL emission intensity compared to RLuc8.6 solution. This is because of the BRET to RB, and RB has a lower fluorescence quantum yield (QY) of ~5% compared to RLuc8.6’s high BL QY of ~50% (Fig. 1a). To confirm the enzymatic activity of RLuc8.6 in LucRB, we measured the time-lapse curves of BL intensity using a large-area detector (Fig. 1b, inset). We found that the total BL energy integrated over the entire emission time was linearly proportional to the amount of CTZ (up to 10 μg) but independent of sample concentration. The exponential decay rates of BL emission from free RLuc8.6 measured as a function of concentration followed the Michaelis-Mentens kinetic model of the substrate–enzyme reaction (Fig. 1b). The BL decay rate of LucRB at a concentration of 0.4 μM was equivalent to the BL decay rate of RLuc8.6 solution at a concentration of 1.51 μM. This means that the average number of RLuc8.6 in a single LucRB molecule was about 3.8.

RLuc8.6 has a broad BL spectrum that overlaps well with the absorption spectrum of RB (Fig. 1c). By comparison, the BL spectrum of LucRB showed an additional shoulder around 580 nm (Fig. 1d). The difference of the LucRB and RLuc8.6 spectra corresponded to the FL emission spectrum of RB (Fig. 1c and d). The BRET ratio, defined as the FL emission energy from the acceptor (RB) to the BL emission energy of the donor (RLuc8.6), is measured to be 7.5%. The relative QYs of free RB (0.05) and LucRB (0.02) were estimated by comparison with the FL intensity of Rhodamine 6G (QY, 0.95) upon 532 nm excitation in PBS solution. We estimated energy transfer efficiency by comparison with the total BL energy from RLuc8.6 (50 μM) and transferred energy to RB (23.2 μJ) calculated by dividing RB fluorescence energy (B–A, 0.44 μJ) of LucRB by the LucRB’s QY. The calculated BRET efficiency is 46.4% (= 23.2/50 μJ). In summary, 46.4% of the total optical energy is transferred to RB, and 2% of the transferred energy is emitted as FL, while the rest contributes to the generation of singlet oxygen and ROS.

To quantify singlet oxygens generated by the excitation of LucRB, we used a Singlet Oxygen Sensor Green (SOSG) dye whose FL emission is increased by singlet oxygen.12,13 We found that the
LucRB is a red fluorophore that can generate singlet oxygen when excited by light. In the presence of a photosensitizer, such as CTZ, it can produce ROS (reactive oxygen species) upon laser excitation. The degradation of LucRB molecules by the generated ROS signals at high CTZ concentrations may be in part due to saturated uptake above 65 μM of LucRB. These data indicate that the intracellular concentration of LucRB is negligible (Fig. 3b(iii)), which confirms that the cytotoxicity mechanism is mediated by BRET-induced ROS. For the same LucRB concentration (100 μM) we performed laser-excited PDT at an optical fluence of 600 mJ cm⁻² (532 nm, 10 mW cm⁻², 1 min) and achieved a similar level (40%) of cell death (Fig. 3c(i)). Laser-induced PDT was performed on cells incubated with bare RB molecules at varying concentrations. The cell death ratio by laser-excited RB at 10 μM (equivalent intracellular RB concentration to 100 μM LucRB) was about 40% (Fig. 3c(ii)), again the same as above. Therefore, these results further support that the cytotoxicity mechanism of LucRB is due to BRET-induced ROS generation. With higher RB concentrations of 30 and 50 μM, the cell death ratio increased to 65 and 75%, respectively. This result is encouraging as it indicates that higher intracellular concentrations of LucRB might produce stronger cytotoxicity.

We performed confocal fluorescence imaging of cells using 2',7'-dichlorofluorescein diacetate (DCFDA), an ROS indicator. CT26 cells incubated with LucRB showed red FL from LucRB but very low green FL from DCF, indicating low intracellular ROS levels (Fig. 4a). As a positive control, cells incubated with hydrogen peroxide showed strong green FL from oxidized DCF (Fig. 4b). As another positive control, cells incubated with bare RB (10 μM) and treated with laser light (532 nm, 10 mW cm⁻², 1 min) showed a strong increase in green FL.
The mechanism of PDT based on ROS generation from LucRB is distinctively different from chemotheraphy and radiation therapy. No cross-resistance between BRET-induced PDT and chemotherapy has been known. Conventional PDT has been shown to be effective against radio-resistant and chemo-resistant cells and, also, may sensitize resistant cells to chemotherapy. Therefore, BRET-PDT has potential for combination therapy with chemo- and radio-therapy. Unlike conventional chemo agents, LucRB agents are non-toxic on their own until activated by the administration of CTZ. This temporal switch allows BRET PDT to be performed at optimal timing when the most preferred bio-distribution of the drug and maximal therapeutic outcome can be achieved.

The relatively low intracellular uptake efficiency of LucRB limited the amount of cytotoxicity. Other known delivery methods, such as liposomal delivery, may increase the intracellular uptake efficiency and thus improve the therapeutic potential. Conjugation of LucRB to antibodies against specific cell-surface biomarkers may enhance targeting and cytotoxicity to tumor cells. Finally, other possible combinations of different types of luciferases, photosensitizers, and substrates appear to have potential and are worth investigation for BRET-PDT.

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References