Electronic Supplementary Information (ESI)

Luciferase–Rose Bengal Conjugates for Singlet Oxygen Generation by Bioluminescence Resonant Energy Transfer

*Seonghoon Kim, HyeongChan Jo, Mijeong Jeon, Myung-Gyu Choi, Sei Kwang Hahn, Seok Hyun Yun**

Experimental Procedures

Materials Rose Bengal (dye content 95%), 6-bromohexanoic acid (97%), protease-free bovine serum albumin (BSA, ≥98%), N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), and N-Hydroxysuccinimide (NHS) were purchased from Sigma-Aldrich. Water-soluble native coelenterazine (CTZ) for *in vivo* applications was purchased from NanoLight Technology. DBCO-NHS ester and azido-PEG4-NHS ester, which are click chemistry reagents, and cell penetrating peptides (CPP, JBS-Proteoducin, CPP-C01L) cocktails were from Jena Bioscience. Singlet Oxygen Sensor Green (SOSG, ≥90%) was purchased from Invitrogen. 2'7'-dichlorodihydrofluorescein diacetate (DCFDA, OxiSelect Intracellular ROS Assay Kit) was obtained from Cell Biolabs. All other solvents and reagents were purchased from Sigma-Aldrich, and used without further purification.

Preparation of RLuc8.6 Mammalian expression plasmids containing RLuc8.6 (pcDNA-RLuc8.6-535) and bacterial expression plasmids containing RLuc8 (pBAD-RLuc8) plasmid were kindly supplied by Dr. Gambhir in Stanford University.[1] pBAD-RLuc8.6-535 plasmid was synthesized by replacing RLuc8 sequence by RLuc8.6 sequence. NcoI and SalI were used as a cloning enzyme. pBAD-RLuc8.6-535 plasmid was transfected to the LMG 194 strain. Proteins produced in E.coli were purified and extracted by immobilized metal ion affinity chromatography and anion exchange chromatography. RLuc8.6 proteins (35 kDa) were analyzed by SDS-PAGE.

Synthesis of RB-HA and RB-NHS RB-NHS was synthesized by reacting RB-hexanoic acid (RB-HA) with NHS ester (Supp. Fig. S2a). RB-HA was prepared by slightly modified Neckers's methods.[2] Briefly, RB sodium salt (1.00 g, 0.983 mmol) and 6-bromohexanoic acid (0.556 g, 2.85 mmol) reacted in a round bottom flask (50 ml) containing 50% of acetone (10 mL) and 50% of distilled water (10mL) by refluxing (80 °C, 24 h). Cooled solution was acidified with 5% aqueous H_2SO_4, and then extracted and washed by chloroform to remove NaBr salts and other unreacted chemicals. The resulting red oil was dried under reduced pressure and loaded on silica gel flash chromatography column (70:30:1, hexane:ethyl acetate:acetic acid). Desired red solid was analyzed by TLC (Rf at 0.24, ethyl acetate:methanol 88:12), ^{1}H NMR, and mass spectroscopy (ESI+, m/z calculated 1108.557, found 1108.552), and matched with references.[2] RB-NHS easter was synthesized by reacting the obtained red solid with three equivalent of EDC (1-ethyl-3-(dimethylaminopropyl) carbodiimide) and 4 equivalent of N-hydroxysuccinimide (NHS) in dimethylformamide (DMF) under stirring overnight at room temperature.[3] DMF was removed in low pressure at 80 °C. The residue was extracted and washed by chloroform and H_2O. Organic layer was concentrated to 5 ml of chloroform. The organic solution was precipitated with 100 ml of ice-cold diethyl ether. Pure RB-NHS clusters were obtained by drying the precipitate. RB-NHS was analyzed by mass spectroscopy (ESI+, m/z calculated, 1181.576; found 1181.572) and matched with references.[3]

Preparation of RB-BSA conjugates BSA (10 mg, 66 kDa) dissolved in phosphate-buffered saline (PBS, 1 ml, pH 7.4), and various molar ratio of RB-NHS in DMSO (10 µl) was mixed and reacted for 2 hours at room temperature. The solution was centrifuged at 5,000 RPM for 5 min to precipitate aggregates. Supernatant was carried to a 50-kDa MWCO centrifugal filter device. The supernatant was then concentrated and re-dispersed with buffer to remove unreacted RB-NHS. Conjugation ratio of RB-BSA was optimized and analyzed by absorption and fluorescence spectroscopy.

Synthesis of (RB-BSA)-PEG-Rluc8.6 (LucRB) conjugates RB-BSA (15 µM, 1 ml) was reacted with 5 molar excess of Azido-PEG_4-NHS ester (388.37 g/mol) in PBS for 2 hours at room temperature. Similarly, Rluc8.6 proteins were functionalized with two molar excess of DBCO-NHS ester (402.4 g/mol) in PBS for 2 hours. Unreacted linkers were separated from RB-BSA-PEG-Azido and DBCO-RLuc8.6 solutions by passing it through

ready-to-use desalting column (PD10, GE healthcare). After column purification, RB-BSA-PEG-Azido (15 μM) and DBCO-Rluc8.6 (100 μM) were coupled via click-chemistry in PBS on a plate shaker at 4 °C overnight. The mixture solution was transferred to the 100-kDa MWCO centrifugal filter device to remove uncoupled proteins. Various ratios of BR-PEG-RLuc8.6 (LucRB) conjugates larger than 100-kDa were isolated from the mixture solution by repeated centrifugal filtration and re-dispersion until bioluminescence signal from filtrate became noise level of spectroscope. Centrifugation was performed less that 2 min for each step to prevent dryness and aggregation of the proteins at 4 °C and 3,000 g. Separated conjugates were kept in 4 °C in dark until use. Molecular weight of the LucRB was measured by using 4–15% precast polyacrylamide gels Mini-PROTEAN (Bio-rad), and prestained protein ruler (PageRuler Plus, 10 to 250 kDa, Life Technology). Measurement of size and zeta potential of LucRB was performed by Zetasizer Nano ZS (Malvern Instruments, UK).

Measurement of BL power decay rates The decay rates of BL intensity was measured to estimate the conjugation ratio of RLuc8.6 on LucRB. In the analysis, the concentration of LucRB was assumed to be the same as the concentration of a BSA component. The time-lapse plots of the BL signals emitted from various concentrations of free RLuc8.6 or LucRB (0.4 μM of BSA) solution (PBS 200 μl) mixed with CTZ (1 μg dissolved in PBS 20 μl) were obtained by using a power meter (PM100D, Thorlabs). The measured curves were fitted by a formula of Michaelis-Menten's kinetics of enzyme reaction to obtain the decay constants.

Measurement of BL spectrum CTZ (10 μl, 0.1 μg/μl in PBS) was pre-loaded on a single well in a clear-bottom black-body 384-well plate. LucRB solution (100 μl, 10 nM in PBS) or RLuc8.6 (100 μl, 50 nM in PBS) were injected into the well by using a syringe pump. Bioluminescence emission light was collected from the well for 5 s by an objective lens equipped and its spectrum was measured.

Measurement of ROS by using SOSG Singlet-oxygen sensitive fluorescence probe (SOSG, Invitrogen) was used to measure the amount of singlet oxygen generated by BRET or laser irradiation. It was found that SOSG interacts with CTZ in solution without LucRB. Also, the interaction of SOSG with LucRB causes both the intensity and peak wavelength of SOSG FL to change. To avoid these unwanted interferences, SOSG dissolved in methanol (10 μl, 2 mM) was pre-mixed with BSA dissolved in PBS (1 ml, 5 mg/ml) for 2 hours before ROS measurement to increase SOSG stability. LucRB solution (2 μM, 1 ml) was added into SOSG solution loaded in a cuvette with lid. To each sample, different amount of CTZ (0 to 100 μg) dissolved in 100 μl of PBS was added. After one hour when the BL reaction is completed, the FL intensity of SOSG was measured by using laser excitation (491 nm, 0.1 mW/cm^2, 1 sec exposure). For laser-induced PDT, SOSG FL was measured immediately after laser irradiation (532 nm, 0-16 min, 1 mW/cm^2). Laser beams were illuminated through a side of disposable cuvette (an illumination area was ~1 cm^2) while the sample solution was stirred using a rotating magnetic bar to mix any bleached dyes.

Cell-culture conditions A colon adenocarcinoma cell line CT26 derived from BALB/c mice were cultured in 100-mm cell culture dishes in Roswell Park Memorial Institute (RPMI) 1640 medium (Life technologies) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin solution, and incubated at 37 °C under 5% CO_2. The cells were sub-cultured about 2 times per week with trypsin-EDTA. For cytotoxicity studies, CT26 cells were seeded in 96-well plates at a density of 2 x 10^4 cells per well for most experiments except for imaging, for which cells were seeded in the same well plates at a density of 2 x 10^3 cells per well. For all cases, cells were cultured for 24 hours before experiments.

Encapsulation of LucRB by CPP Cell-penetrating peptide cocktail (CPP) was dissolved in sterile and oxygen free PBS (1.25 ml) and sonicated for 5 min. Insoluble disulfides compound were eliminated by centrifugation at 10^4 g for 5 min. 1 μl CPP stock solution per μg of LucRB was mixed thoroughly by repeated pipetting and incubated for 1 hour at room temperature before use.

In vitro BRET PDT Cells were plated in 96-well plates (2 x 10^4 cells/well) one day prior to the experiment. After removing media, cells were incubated in FBS-free fresh media (200 μl) containing CPP-encapsulated LucRB (CPP-LucRB, 100 μM) or RB (5, 10, 20, or 40 μM) in incubator for 12 hours and 1 hour, respectively. Plates were washed twice with 200 μl of Dulbecco`s Phosphate Buffered Saline (DPBS). For BRET-PDT groups, 200 μl of media containing *in vivo* CTZ (5 or 10 μg) was added and incubated for 12 hours.

Laser-induced PDT Conventional PDT was conducted by illuminating an expanded beam of 532-nm continuous-wave output from a laser (Dual Calypso, Cobolt) onto cells in culture-well plates at an irradiance level of 10 mW/cm^2 for 1 min. The cells were incubated in RB (10, 30, 50 μM) containing media for one hour and washed twice before

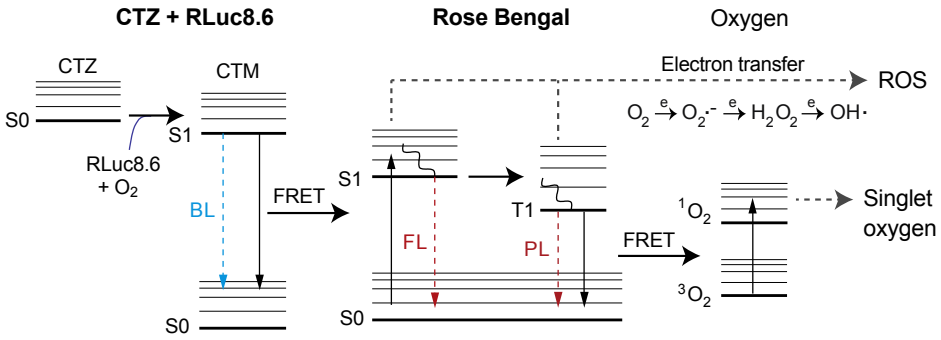
laser irradiation. Inhibition of ROS generation was observed by using media containing NaN_3 (1 mM). Viability of CT26 cells was determined by the MTT assay (Vybrant MTT cell proliferation assay kit, Invitrogen).

MTT cytotoxicity assay MTT assay was carried out according to the following protocols. After conducting PDT, cells in plates were incubated for 12 hours, and then the media was changed to 200 μl of FBS-free media. 20 μl of 5 mg/ml MTT dissolved in PBS was transferred to each well. The plate was incubated for 1 hour. After removing 200 μl of media from the well, 50 μl of DMSO was added to each well and mixed thoroughly with a pipette to dissolve violet color formazan crystals completely. The plate was incubated for another 10 min. Absorbance at 540 nm was measured by using a multi-well plate reader (Spectramax, plus384, Molecular Devices).

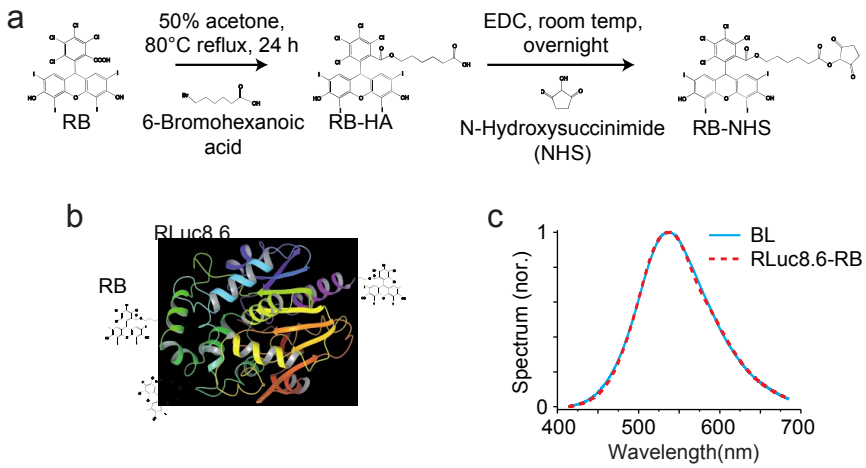
Measurement of intracellular ROS Oxidative stress in cells were imaged by using cell-permeant, 2',7'-dichlorodihydrofluorescein diacetate (DCFDA, Oxiselect intracellular ROS assay kit, Cell Biolabs Inc.) as a fluorescent probe. CT26 cells were seeded in 96-well plates at a density of 2 x 10^4 cells per well one day before experiments. The cells were stained with LucRB or RB using the same protocol as in MTT experiments. DCFDA dissolved in DMSO (2 μl, 500 μM) was added in 200 μl of media and incubated for 30 min. After washing each well twice with PBS, the cells were treated with CTZ solution (for BRET-PDT) or laser irradiation (conventional PDT). After 30 min measured from the beginning of the therapy, FL images of RB and DCFDA were obtained from red fluorescent protein (RFP) and green fluorescent protein (GFP) channels, respectively. The amounts of ROS in cells were quantitatively analyzed by flow cytometer (FACSCalibur, Becton Dickinson, San Diego, CA).

Optical setup of spectroscopy and microscopy The spectral measurement and imaging were carried out by using a home-made optical measurement system. An Xenon arc lamp (750 W, Dongwoo Optron) coupled with single-grating spectrograph (MonoRa DM 200i, Dongwoo Optron) and dual laser (491/532 nm, Dual Calypso, Cobolt) were used as light sources. The systems employed a single-grating spectrograph (focal length 303 mm, Shamrock SR303i, Andor) coupled with an electron-multiplying charge-coupled device (EMCCD, Newton DU-970N-BV, Andor) for spectroscopy and a CCD (Neo sCMOS, Andor) for microscopy imaging. Samples were prepared in either clear-bottom multi-well plates (Corning) or cuvette holders (CVH100, Thorlabs), which are mounted on a motorized XY-stage for multi-well plate (MLS203-1, Thorlabs). An objective lens (10x/20x, Plan Fluor, Nikon) was used to illuminate the sample and collect FL and BL light. Motorized syringe pump (Legato201, KD Scientific) was used to mix coelenterazine and LucRB solution.

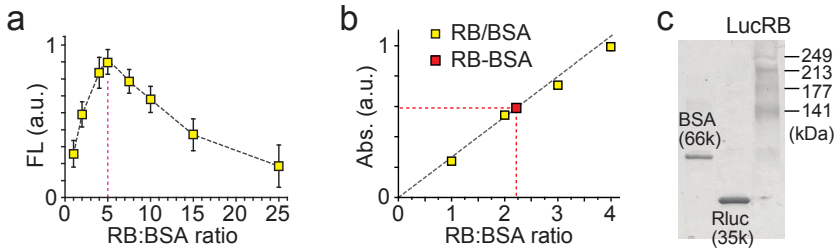
Results and Discussion



Supplementary Figure 1. Energy transfer diagram of BRET and singlet oxygen generation.



Supplementary Figure 2. a, Schematic for a direct conjugation of Rluc8.6 and RB. RB was modified with N-hydroxysuccinimide (NHS) ester[3], and the RB-NHS ester and RB-PEG were conjugated to bare Rluc8.6, respectively. RB-PEG was prepared by conjugation of RB-NHS with a hetero functional polyethylene glycol (carboxy-PEG-amine (CA(PEG)4, Thermo Scientific Pierce) containing 4 polyethylene glycol (PEG) units (~ 2 nm). N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) was used to attach RB-PEG to RLuc8.6. The mixing ratio of RB to RLuc8.6 was varied from 1.2:1 to 5:1. The conjugation was confirmed via UV-Vis spectrum analysis. **b**, A cartoon structure of a Rluc8.6-RB conjugate. **c**, Bioluminescence spectra measured when CTZ was added to a solution of RLuc8.6-RB conjugate (red dashed line) and RLuc8.6 (cyan line). The identical BL emission spectra indicate that the function of RLuc8.6 is largely intact, but the reaction failed to generate fluorescence (FL) emission expected from RB in a spectral range of 560-650 nm. This negative result is attributed to a low BRET efficiency due to quenching of RB molecules.



Supplementary Figure 3. a, Fluorescence (FL) intensities at 585 nm from RB-BSA conjugates made with various RB-NHS/BSA mixing ratios in PBS buffer (pH 7.4). **b**, Absorbance of purified RB-BSA conjugates and RB/BSA mixtures at 550 nm. **c**, Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of RLuc8.6, BSA and LucRB.

References

[1] A. M. Loening, A. M. Wu, S. S. Gambhir, *Nat Methods* **2007**, *4*, 641-643.

[2] aD. Neckers, J. Paczkowski, *Tetrahedron* **1986**, *42*, 4671-4683; bJ. R. Carreon, M. A. Roberts, L. M. Wittenhagen, S. O. Kelley, *Organic letters* **2005**, *7*, 99-102.

[3] K. A. Conlon, M. Berrios, *Journal of photochemistry and photobiology. B, Biology* **2001**, *65*, 22-28.