HaloTag Protein-Mediated Site-Specific Conjugation of Bioluminescent Proteins to Quantum Dots

Yan Zhang, Min-kyung So, Andreas M. Loening, Hequan Yao, Sanjiv S. Gambhir, and Jianghong Rao

Molecular Imaging Program at Stanford, Department of Radiology
Stanford University School of Medicine
1201 Welch Road, Stanford, California 94305-5484 (USA)

Materials. Quantum dots were purchased from Quantum Dot Corp. (Hayward, California). QD@COOH (655 nm) has typical CdSe/ZnS core-shell structures coated with polymers and endowed with carboxylate groups. The quantum yield is 83%, determined in 50 mM borate buffer. The coupling reagent 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) was obtained from Fluka. Coelenterazine, the substrate for Luc8, was purchased from Prolume (Pinetop, Arizona). All other chemicals and solvents were from Sigma-Aldrich. Water was purified with Milli-Q biocel (Millipore Corp.). pH2 (HaloTag) plasmid was from Promega. NanoSep 100K filters for QD purification were from Pall, Life Science.

Scheme S1. Reagent and conditions: a. Boc₂O/EtOH, 0 °C, 2 hr; b. NaH/DMF-THF and 6-chloro-1-iodohexane; c. TFA in DCM, then K₂CO₃, MeOH; d. NaH/DMF-THF and iodoacetic acid sodium salt; e. N-Hydroxysuccinimide and DCC in DCM; f. DIPEA/THF; g. TFA in DCM, then K₂CO₃, MeOH.

Preparation of 2: Under argon, to a solution of 2-(2-aminoethoxy)ethanol (1.05 g, 10 mmol) in anhydrous EtOH (20 ml) was added Boc₂O (2.2 g, 10 mmol) at 0 °C. After stirring at room temperature for 2 h, the reaction mixture was diluted with CH₂Cl₂, washed with brine and the organic layer was dried over Na₂SO₄. The solvent was removed to get the residue 2 as colorless oil (2.05 g, 100%), which was used for the next step without further purification. ¹H NMR: (400MHz, CDCl₃) δ 5.07 (br, 1H), 3.74 (m, 2H), 3.60 (m, 4H), 3.33 (m, 2H), 2.69 (m, 1H), 1.45 (s, 9H).

Preparation of 3: To a solution of 2 (1.44 g, 7.0 mmol) in THF (10 ml) and DMF (5 ml) at 0 °C was added NaH (300 mg, 80% in mineral oil, 9.4 mmol). After stirring at 0 °C for 30 min, 6-chloro-1-iodohexane (1.5 ml, 10.5 mmol) was added to the above solution. The reaction mixture was stirred overnight and quenched with saturated NH₄Cl. The mixture was extracted with EtOAc (100 ml), washed with H₂O and Brine, and dried over Na₂SO₄. Filtration, concentration and purification by column chromatography provided colorless oil (1.5 g, 66%). ¹H NMR (CDCl₃, 400MHz) δ 5.06 (br, 1H), 3.62 (m, 2H), 3.55 (m, 2H), 2,69 (m, 1H), 1.45 (s, 9H).
3.47 (m, 2H), 3.33 (m, 2H), 1.78 (m, 2H), 1.62 (m, 2H), 1.5-1.3 (m, 4H), 1.43 (m, 9H); ESI-MS: 345.8 [M+Na]^+, 223.8 [M-Boc]^+; calc. 323.86 [M]^+. To a solution of the above product (218 mg, 0.7 mmol) in 5 ml of anhydrous CH₂Cl₂ at 0 °C was added TFA (0.6 ml). After stirring for 2h, the solvent was removed and the residue was treated with anhydrous K₂CO₃ (276 mg, 2 mmol) in MeOH (5 ml). The mixture was filtered and the filtrate was concentrated to obtain 3 (110 mg, 83%) as colorless oil. ¹H NMR (CDCl₃, 400MHz) δ 3.85 (m, 2H), 3.50 (m, 8H), 2.95 (m, 2H), 1.78 (m, 2H), 1.60 (m, 2H), 1.5-1.3 (m, 4H); LC-MS: 223.8 [M]^+; calc. 223.74 [M]^+.

Preparation of 4: Under argon, 80% of NaH (160 mg, 5mmol) was added to a solution of 2 (1.02 g, 5 mmol) in anhydrous DMF( 5 ml) at 0 °C. After stirring at 0 °C for 30min, ICH₂COONa (1.1g, 5 mmol) was added to the reaction mixture. The reaction mixture was stirred for 4 h and quenched with 1N of HCl. The mixture was extracted with CH₂Cl₂. The organic layer was washed with brine and dried over Na₂SO₄. The solvent was removed to yield the free acid. N-hydroxysuccinimide (1.0 g, 5 mmol) and the free acid were dissolved in CH₂Cl₂ (10 ml) and cooled to 0 °C. To the resultant solution was added DCC (1.2 g, 5.1 mmol), then the reaction mixture was warmed to room temperature and stirred overnight. The reaction mixture was diluted with hexanes and filtered. The filtrate was concentrated under reduced pressure and afforded the NHS ester 4 as crude colorless oil.

Preparation of 1: The crude NHS ester 4 was dissolved in DMF, and to this solution was added Halo ligand 3 (0.3 g) and DIPEA (0.3 ml, 2 mmol). The reaction mixture was stirred overnight and quenched with saturated NH₄Cl. The reaction mixture was extracted
with CH$_2$Cl$_2$. The combined organic layers were washed with brine and dried over Na$_2$SO$_4$. The solvent was concentrated and the residue was purified by flash chromatography to get colorless oil (150 mg, 24%). $^1$HNMR: (400MHz, CDCl$_3$) δ 7.30 (br, 1H), 4.02(s, 2H), 3.74-3.40 (m, 18H), 3.35 (m, 2H), 2.76 (m, 2H), 2.60 (m, 2H), 1.50-1.30 (s, 4H), 1.45(s, 9H). LC-MS: m/z 469.3 [M+1]$^+$; calc. 468.3 [M]$^+$. The above product (120 mg, 0.26 mmol) was dissolved in CH$_2$Cl$_2$ and treated with TFA (1 mL) at room temperature for 2h. The solvent was removed and the residue was treated with anhydrous K$_2$CO$_3$ (276 mg, 2 mmol) in MeOH (5 ml). The mixture was filtered and the filtrate was concentrated to obtain 1 (80 mg, 85%) as colorless oil. $^1$HNMR: (400 MHz, CDCl$_3$) δ 3.92 (s, 2H), 3.70-3.20 (m, 18H), 2.09(m, 2H), 2.64 (m, 2H), 2.50 (m, 2H), 1.40-1.20 (s, 4H). LC-MS: m/z 369.2 [M+1]$^+$; calc. 368.2 [M]$^+$.

2. Quantum dots conjugation and purification

**Preparation of QD@1**: 40 pmol of QD@COOH (655nm), 40 nmol of HaloTag ligand 1 (in the case of QD to ligand at a ratio of 1:1000), 16 nmol of EDC were added to pH 7.4 borate buffer and mixed well to a final volume of 40 ul. The incubation solution was kept at room temperature for 1 h. Then the mixture was transferred to a pre-washed 100K NanoSep filter. Centrifuge at 5000 rpm at 4 degree until 98% of the buffer was removed. Then added 200ul of pH 8.5 borate buffer to the filter and wash the quantum dots thoroughly. The buffer was removed by repeating centrifugation at 5000 rpm for three times. The conjugate QD@1 on the filter was recovered with pH 7.4 borate buffer and the final concentration of QD@1 was determined from the emission at 655 nm using the original QD@COOH as reference.
Preparation of QD@1-HTP-Luc8: 5 pmol of QD@1 and 100 pmol of HTP-Luc8 (in the case that the ratio of QD@1 to HTP-Luc8 is 1:20) in pH 7.4 borate buffer were mixed well and incubated at 37 °C for 30 minutes. Then the mixture was transferred to 100K NanoSep filter which was pre-washed with cold pH 7.4 borate buffer and kept at 4 °C. The conjugate solution was centrifuged at 7000 rpm at 4 °C to remove most of the buffer containing free HTP-Luc8. The washing-centrifugation process was repeated until there was no free luminescent protein present in the filtrate. The purified QD@1-HTP-Luc8 was recovered with 60 ul of cold pH 7.4 buffer for luminescence and BRET measurement.


PCR was used to obtain luc8, containing a NcoI straddling the initial methionine from plasmid pBAD-RLuc8 and attach a 3’ BamHI site. The halotag gene, which contains a BamHI site in codons 2 and 3, was obtained by PCR from plasmid pHT2, and a 3’ SalI site was attached. These two products were digested and ligated with a SalI/NcoI digested pBAD/Myc-His A plasmid (Invitrogen), which adds a C-terminal 6xHis tag and a stop codon, resulting in plasmid pBAD-Luc8-HaloTag. E. coli LMG194 cells containing this plasmid were grown to an OD_{600} of 0.7, induced with 0.2% arabinose, grown 12 h at 32 °C, pelleted, and frozen. Cells were lysed by thawing in wash buffer (WB: 300 mM NaCl, 20 mM HEPES, 20 mM imidazole, pH 8) containing 1 mg/ml lysozyme, 10 ug/ml RNAse A, and 5 µg/ml DNAse I, slowly mixing for 1 h, and sonicating. Lysates were clarified by centrifugation and allowed to bind to nickel affinity resin (Ni-NTA Superflow, Qiagen) for 1 h at 4 °C with gentle mixing. After washing with WB, protein
was eluted with elution buffer (300 mM NaCl, 20 mM HEPES, 250 mM imidazole, pH 8), and diluted with anion exchange start buffer (10 mM NaCl, 10 mM Tris pH 8). Protein was further purified by anion exchange chromatography (Source 15Q resin, GE/Amersham), followed by gel filtration chromatography run with borate buffer.

4. Bioluminescence and BRET measurement.

Fluorescence and bioluminescence emission spectra were collected with a Fluoro Max-3 (JOBIN YVON Inc., New Jersey); in the case of bioluminescence, the excitation light was blocked, and emission spectra were corrected with a correction file provided by the company. 1 µg of the substrate, coelenterazine, was used for the bioluminescence measurement. The enzymatic activity of Luc8 and the fusion protein HTP-Luc8 was measured with a 20/20® Luminometer (Turner Biosystems, Inc.).