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

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Quantum dots are fluorescent semiconductor nanocrystals that have attracted much attention as fluorescence imaging probes owing to their unique optical properties such as high quantum yield, high molar extinction coefficients, narrow emission spectra, size-dependent tunable emission, and high photostability.^[1–8] To apply quantum dots to biological detection and imaging applications, quantum dots have to be conjugated to molecules (e.g. peptide ligands, carbohydrate, antibodies, small molecule ligands) that can specifically recognize the biological target under study. Numerous examples have been reported on the use of quantum dots for both in vitro assays and in vivo imaging, but these

quantum dot conjugates are either assembled through non-specific interactions or prepared through site-nonspecific coupling reactions.^[9–18] For example, for proteins, the conjugation typically involves random amide coupling with either amino- or carboxylate-presenting quantum dots.^[9,15,16] Non-specific conjugation chemistry leads to chemical heterogeneity of synthesized conjugates, may compromise the protein activity and even induce aggregations, and is not applicable to specific labeling of target proteins in vivo.^[19] Specific non-covalent interactions between receptors and ligands, such as carbohydrate–lectin and streptavidin–biotin, have been applied to assemble quantum-dot complexes.^[1,20–21] Herein, we report a specific conjugation method that utilizes a genetically engineered hydrolase to covalently immobilize a bioluminescent protein at the quantum-dot surface. This immobilized bioluminescent protein can efficiently produce chemical energy to excite quantum dots through resonance energy transfer.

Our method employs a commercially available, engineered haloalkane dehalogenase, the HaloTag protein (HTP).^[22] The native enzyme is a monomeric protein (MW \approx 33 kDa) that cleaves carbon halogen bonds in aliphatic halogenated compounds.^[22] Upon nucleophilic attack by the chloroalkane to Asp106 in the enzyme, an ester bond is formed between the HaloTag ligand and the protein (Scheme 1). HTP contains a critical mutation in the catalytic triad (His272 to Phe) so that the ester bond formed between HTP and HaloTag ligand cannot be further hydrolyzed (Scheme 1).^[22] HaloTag ligands labeled with small organic dyes, such as coumarin and fluorescein, have been developed for in vivo labeling of target proteins.^[22] Herein we apply this technology for the specific conjugation of proteins to quantum dots.

To take advantage of this specific protein–ligand interaction, quantum dots can be functionalized with HaloTag ligands. A protein target can in turn be genetically fused to HTP at either its N- or C- terminus. The resulting fusion protein can then be conjugated to quantum dots through the reaction between HaloTag ligands and HTP (Scheme 1).

To demonstrate the utility of this method for quantum dot conjugation, we chose a bioluminescent protein, *Renilla* luciferase, as our target. We have recently demonstrated that when *Renilla* luciferase is conjugated to quantum dots, bioluminescence resonance energy transfer (BRET) can take place.^[23] Such quantum dot conjugates can emit light without light excitation and offer greatly improved sensitivity for in vivo imaging. With *Renilla* luciferase as the target protein for the conjugation, the conjugation reaction can be conveniently evaluated from the BRET emission of the quantum dots—a measure of both the conjugation chemistry and the function of the conjugated luciferase.

A stabilized mutant of *Renilla* luciferase (Luc8) was genetically fused to the N terminus of the HTP and expressed to obtain the fusion protein HTP–Luc8. The C terminus of HTP–Luc8 contained a 6 \times His tag to facilitate its purification. Gel electrophoresis analysis indicated that the molecular weight of the fusion protein was consistent with the expected value, approximately 70 kDa (Figure 1a). The bioluminescence activity of the fusion protein was estimated to be 1.2 \times

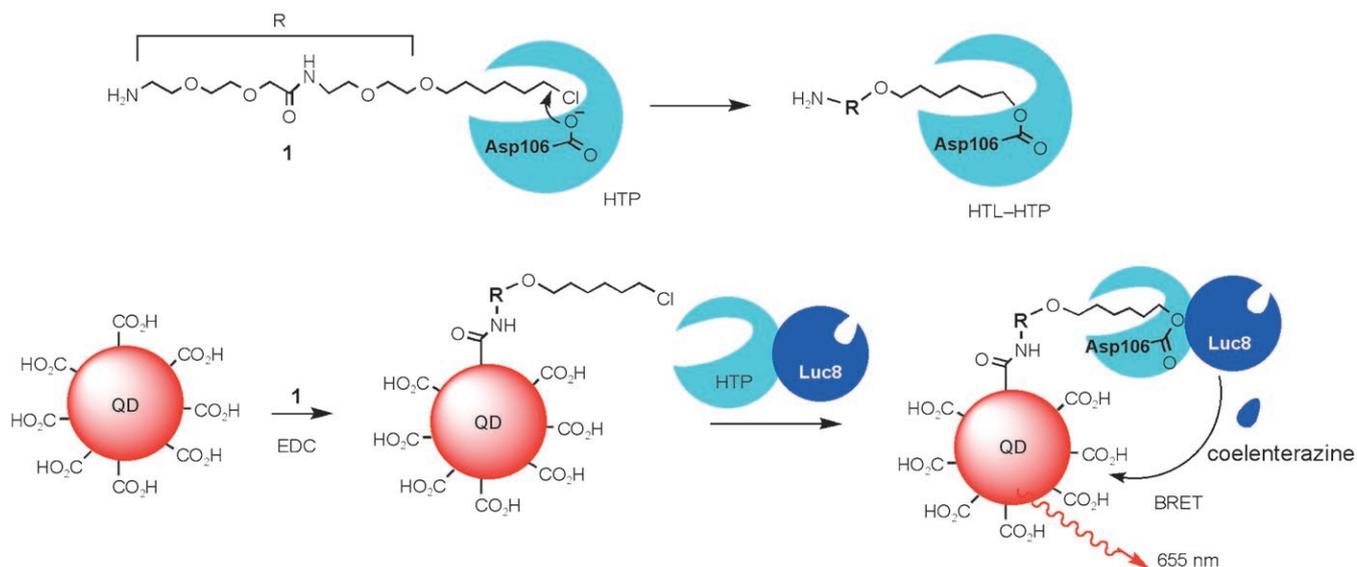
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Scheme 1. Schematic of the specific conjugation of proteins to quantum dots mediated by the HaloTag protein and its ligand. HTL = HaloTag ligand.

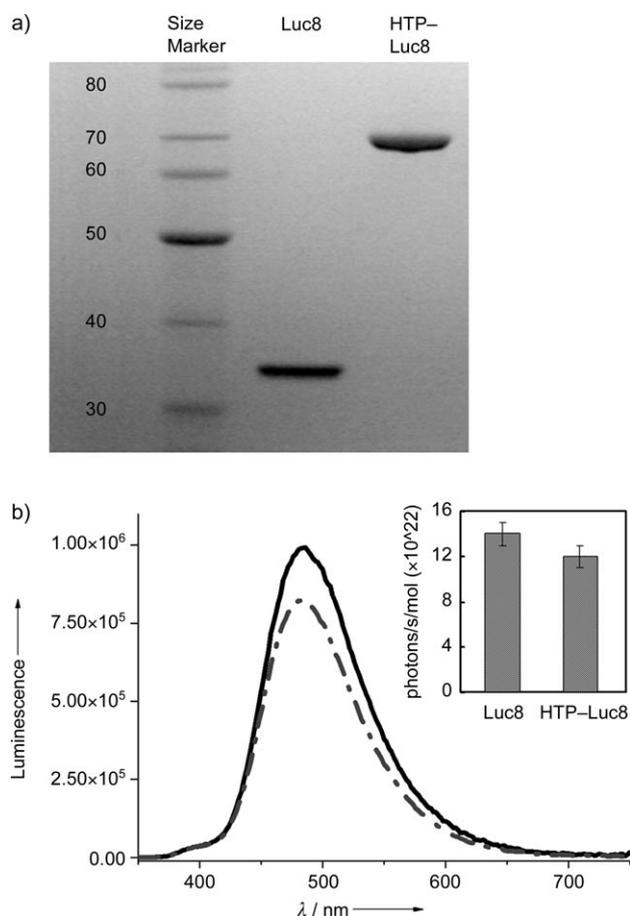


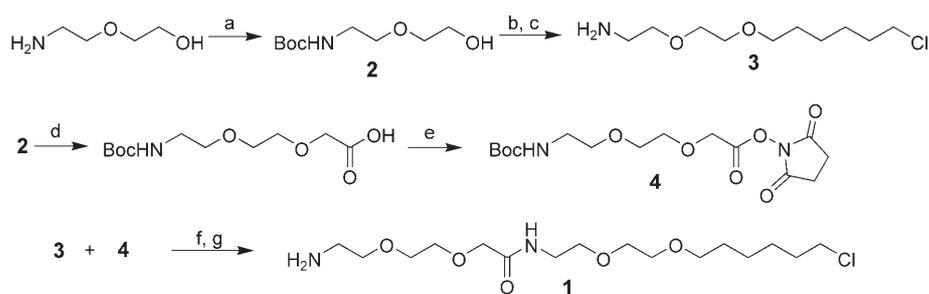
Figure 1. Characterization of the size and function of the fusion protein. a) Gel electrophoresis analysis confirmed the size of HTP-Luc8. Both proteins (0.5 μ g each) were run on a 4–12% Bis-Tris (bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methane) gradient denaturing gel and stained with Coomassie Blue. The expected sizes for Luc8 and HTP-Luc8 were 37.1 and 70.3 kDa, respectively. b) Bioluminescence emission spectra of Luc8 (solid line) and HTP-Luc8 (dashed line). The inset shows the total photon production of Luc8 and HTP-Luc8.

10^{23} photons s^{-1} mol $^{-1}$, which is approximately 86% of Luc8 (Figure 1b).

To minimize potential steric hindrance between the quantum dots and HaloTag proteins during conjugation, we designed a HaloTag ligand containing an amino ethylene glycol group that would help orient the ligand away from the quantum-dot surface (Scheme 1). The HaloTag ligand **1** was prepared from 6-chloro-1-iodohexane and 2-(2-aminoethoxy)ethanol by the synthetic route outlined in Scheme 2, and was then immobilized through its amino group to the carboxylate-presenting quantum dots (QD@COOH). The resulting quantum dots coated with the HaloTag ligand **1** (QD@1) showed good solubility in neutral pH buffer solution. The conjugation of the fusion protein HTP-Luc8 to QD@1 was carried out by a simple mixing of both at 37°C, resulting in the formation of an irreversible covalent bond between ligand **1** on quantum dots and HTP-Luc8.

As successful immobilization of HTP-Luc8 to quantum dots should allow BRET to occur, we measured the BRET emission from the quantum dots to follow the conjugation reaction. Addition of coelenterazine, the substrate for *Renilla* luciferase, to the purified conjugate QD@1-HTP-Luc8 resulted in a dual-peak bioluminescence emission spectrum (Figure 2). In addition to the Luc8 peak at 480 nm, there was an emission maximum at 655 nm that overlapped well with the fluorescence emission of the quantum-dot conjugates excited at 480 nm.

To evaluate whether the observed BRET emission was owing to specific conjugation between the quantum dots and fusion proteins, we examined the dependence of the BRET emission on the HaloTag ligand **1** that was used in the conjugation. QD@COOH was first functionalized with various concentrations of **1**. These modified quantum dots were subsequently conjugated with 20 equivalents of HTP-Luc8. Figure 3 shows that with increasing amounts of ligand **1** used in the conjugation, the bioluminescence emissions from both the immobilized HTP-Luc8 and the quantum dots through



Scheme 2. Synthesis of HaloTag ligand **1**. Reagent and conditions: a) $\text{Boc}_2\text{O}/\text{EtOH}$, 0°C , 2 h; b) $\text{NaH}/\text{DMF-THF}$ and 6-chloro-1-iodohexane; c) $\text{TFA}/\text{anisole}$ in DCM ; $\text{K}_2\text{CO}_3/\text{MeOH}$; d) $\text{NaH}/\text{DMF-THF}$ and iodoacetic acid sodium salt; e) N -Hydroxysuccinimide and DCC in DCM ; f) DIPEA/THF ; g) $\text{TFA}/\text{anisole}$ in DCM ; $\text{K}_2\text{CO}_3/\text{MeOH}$. Boc = *tert*-butoxycarbonyl, DMF = N,N -dimethylformamide, TFA = trifluoroacetic acid, DCM = dichloromethane, DCC = 1,3-dicyclohexylcarbodiimide, DIPEA = diisopropylethylamine.

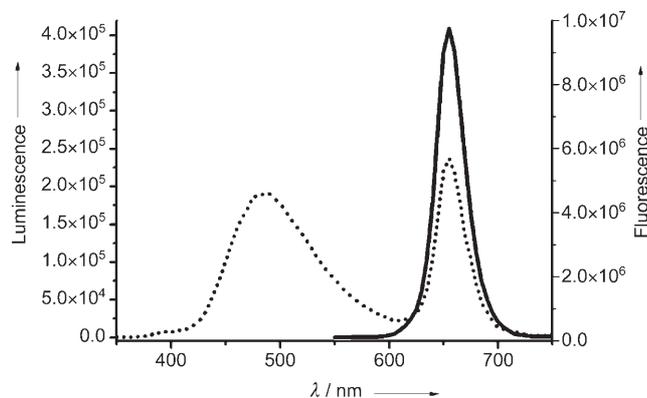


Figure 2. Bioluminescence (dashed line) and fluorescence (black solid line) spectra of conjugate $\text{QD}@1\text{-HTP-Luc8}$ in borate buffer solution. The fluorescence emission was collected with excitation at 480 nm.

BRET increased. When quantum dots (without a HaloTag ligand **1** attached) were similarly mixed with the fusion protein, there was only a small emission from HTP-Luc8 at 480 nm and a small BRET emission from the quantum dots. The small BRET emission probably arises from an electrostatic interaction between the $6 \times \text{His}$ tag on HTP-Luc8 and the negative carboxylate groups on the quantum dots. These results confirm that the BRET emission reflects specific conjugation occurring between the quantum dots and the fusion protein HTP-Luc8, and that the fusion protein HTP-Luc8 retains its enzymatic activity after conjugation.

The efficiency of the resonance energy transfer process can be quantitatively estimated from the BRET ratio. The BRET ratio is defined by the acceptor emission relative to the donor emission.^[23,24] In the quantum dot and HTP-Luc8 conjugate, the donor is Luc8 and the acceptor is the quantum dot. We calculated the BRET ratio by dividing the total emission from quantum dots by the total emission from HTP-Luc8, shown in Figure 3b. With the increase in the number of HaloTag ligand **1** and in turn the increase in immobilized fusion protein, the BRET ratio decreased gradually from 0.6 to 0.4. In principle, the BRET ratio should not depend on the

number of immobilized proteins if the BRET distance remains unchanged.^[23] This small decrease in the BRET ratio may be due to a shift in the orientation of the conjugated fusion proteins on the quantum dot surface.^[25]

Finally, we examined the dependence of the conjugation reaction on the amount of fusion-protein present. Quantum dots were reacted with 1000 equivalents of HaloTag ligand **1**, and then reacted with increasing concentrations of HTP-Luc8. As expected, the resulting conjugates showed increasing bioluminescence emissions both from HTP-Luc8 and from the quantum dots (Figure 4). As a control, Luc8 (without HTP fusion) was incubated with the $\text{QD}@1$. The control reaction showed no biolu-

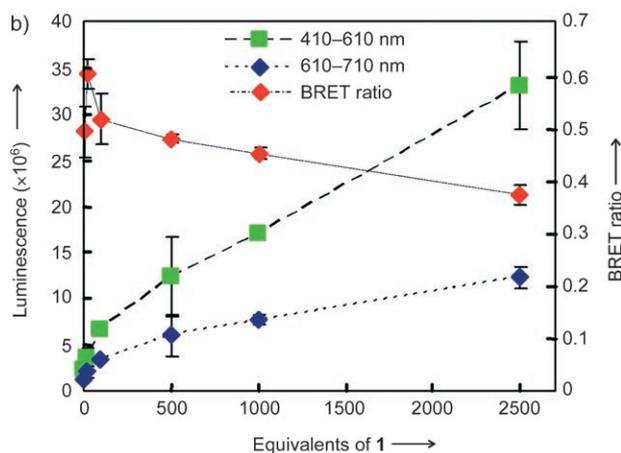
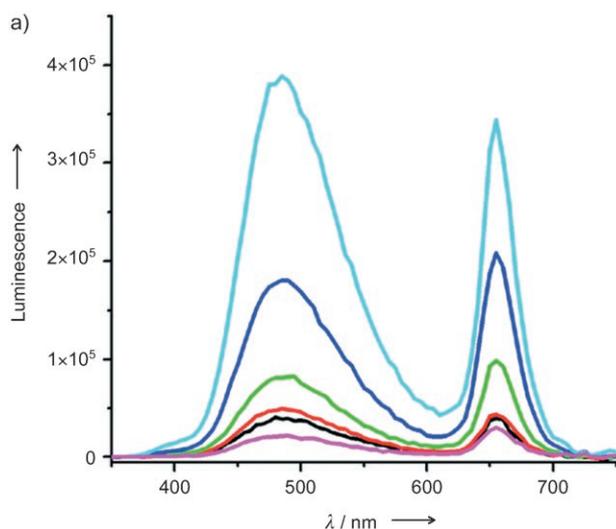


Figure 3. Dependence of the conjugation on the ligand **1**. a) Representative bioluminescence emission spectra of the conjugates synthesized at different concentrations of **1** (from bottom to top: 0, 20, 100, 500, 1000, and 2500 equivalents). b) Total bioluminescence emissions from HTP-Luc8 and from quantum dots, and the calculated BRET ratios of conjugates prepared in (a); in duplicate).

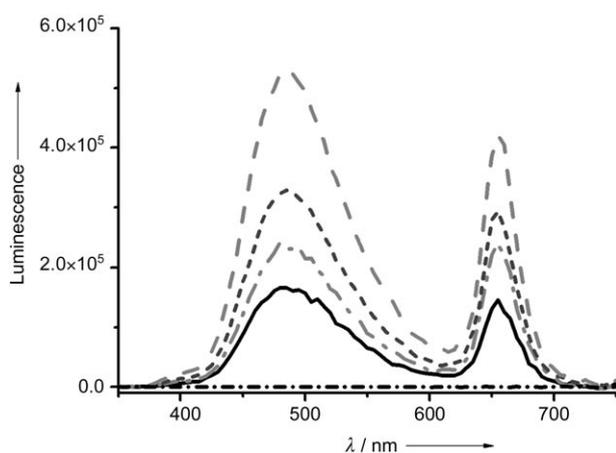


Figure 4. Bioluminescence emission spectra of quantum dots conjugated with HTP-Luc8 (going down from the top line: 100, 50, 20, and 10 equivalents) or 20 equivalents of Luc8 (bottom dash-dot line). The quantum dots were reacted with 1000 equivalents of HaloTag ligand **1** before the conjugation with HTP-Luc8. Unconjugated proteins were removed by filtration before measurement.

minescence emission, therefore indicating no immobilization of Luc8 on the quantum dots and further confirming that the conjugation between quantum dots and HTP-Luc8 was specific.

In our previous demonstration of self-illuminating quantum dots for *in vivo* imaging, the quantum dots were conjugated with Luc8 *in vitro* before their introduction into living cells and animals. An important further step will be to specifically conjugate luciferases with quantum dots for functional imaging *in vivo*. The mild conjugation conditions used to immobilize proteins to quantum dots, mediated by the HaloTag protein and its ligand, may allow this method to be applied to specific labeling of target proteins with quantum dots *in vivo*. This method also offers an advantage in comparison to a widely used conjugation method based on biotin and streptavidin in that the HaloTag protein is monomeric and relatively small.

In summary, this communication reports a new method, based on the specific interaction between the HaloTag protein and its ligand, to functionalize quantum dots for biological imaging. By using this method, we successfully conjugated a bioluminescent protein to quantum dots and produced self-illuminating quantum dot conjugates. This specific conjugation under mild physiological conditions offers promises for specific *in vivo* labeling of proteins or cells with quantum dots for imaging.

Experimental Section

Chemicals for HaloTag ligand synthesis were from Sigma-Aldrich. The coupling reagent 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) was from Fluka. Quantum dots were from Invitrogen and have typical CdSe/ZnS core-shell structures with the quantum yield (determined in 50 mM pH 9 borate buffer solution) of 83%. Coelenterazine was from Prolume. The plasmid pHT2 (HaloTag) was from Promega. NanoSep 100 K filters for quantum dot purification were from Pall, Life Science.

Synthesis of 1: the compound was synthesized from 6-chloro-1-iodohexane and 2-(2-aminoethoxy)ethanol according to Scheme 2. $^1\text{H NMR}$ (400 MHz, CDCl_3): $\delta = 3.92$ (s, 2H), 3.70–3.20 (m, 18H), 2.09 (m, 2H), 2.64 (m, 2H), 2.50 (m, 2H), 1.40–1.20 ppm (s, 4H); LC-MS: m/z 369.2 [$M+1$] $^+$; calcd M^+ : 368.2.

Conjugation of 1 to quantum dots: Quantum dots, HaloTag ligand **1**, and EDC (400 equiv) were mixed together in borate buffer solution (10 mM, pH 7.4) and incubated at room temperature for 1 h. QD@**1** was separated from free HaloTag ligand and excess EDC by filtration through a 100 K NanoSep filter. The quantum dot conjugates were washed three times with pH 8.5 borate buffer solution for 1 h before being recovered with pH 7.4 borate buffer solution. The concentration of QD@**1** was determined from the fluorescence intensity.

Preparation and purification of HTP-Luc8: the plasmid pBAD-Luc8-HaloTag encoded for the fusion protein was constructed from plasmid pBAD-RLuc8 and plasmid pHT2 by PCR and ligation. *E. coli* LMG194 cells transformed with this plasmid were induced with 0.2% arabinose and grown at 32°C to an optical density at a wavelength of 600 nm (OD_{600}) of 0.7. Cells were lysed by thawing in wash buffer solution (WB; NaCl (300 mM), 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES; 20 mM), imidazole (20 mM), pH 8) containing lysozyme (1 mg mL $^{-1}$), RNase A (10 $\mu\text{g mL}^{-1}$), and DNase I (5 $\mu\text{g mL}^{-1}$). 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, the protein was eluted with elution buffer solution (NaCl (300 mM), HEPES (20 mM), imidazole (250 mM), pH 8) and further purified by anion-exchange chromatography (Source 15Q resin, GE/Amersham) followed by gel-filtration chromatography with borate buffer solution.

Conjugation of QD@1 with HTP-Luc8: Typically QD@**1** (5 pmol) was incubated with of HTP-Luc8 (20 equiv) in borate buffer solution (10 mM, pH 7.4) at 37°C for 30 min. Free HTP-Luc8 was removed from the incubation mixture by filtration through a 100 K NanoSep filter at 4°C. The filtered conjugates were washed efficiently with pH 7.4 borate buffer solution at 4°C. The final quantum dot conjugates were recovered with ice-cold pH 7.4 borate buffer solution.

Fluorescence and bioluminescence spectra were collected with a Fluoro Max-3 (Jobin Yvon Inc.). Bioluminescence spectra were acquired with the excitation light blocked.

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