

Quantum Dot/Bioluminescence Resonance Energy Transfer Based Highly Sensitive Detection of Proteases**

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Semiconductor quantum dots (QDs) are bright fluorescence emitters with high quantum yields, high molar extinction coefficients, size-dependent tunable emission, and high photostability.^[1–6] These attractive fluorescence properties prompt a wide interest in developing QD-based sensors for biological detection and imaging.^[7–13] One often-used strategy towards the development of QD nanosensors is based on fluorescence resonance energy transfer (FRET) with the QDs as the FRET donor. There are numerous examples of FRET-based QD biosensors that include self-assembled nanocomplexes for detecting maltose, pH, 2,4,6-trinitrotoluene, thrombin, and enzyme activity.^[7–13] In these FRET-based QD nanosensors, multiple copies of the FRET acceptor were often present on one QD, which may result in self-quenching and lead to low emission from the FRET acceptor.^[14]

We have recently demonstrated that QDs can also serve as an energy acceptor for a light-emitting protein (for example, the bioluminescent protein *Renilla* luciferase) in bioluminescence resonance energy transfer (BRET).^[15,16] When the QD conjugates are exposed to the luciferase substrate, the energy released in the oxidation of the substrate is transferred to the QDs through BRET, thus generating light emission from the QDs. The QD BRET has been shown to have high sensitivity for in vivo imaging and detection. Herein, we report the design of a

BRET-based QD biosensor for detection of the activity of matrix metalloproteinases (MMPs).

MMPs are a family of zinc-dependent secreted endopeptidases that are crucial for the regulated degradation and processing of extracellular matrices, and are upregulated in almost every type of human cancer.^[17,18] The significant role of MMPs in promoting cancer progression makes them important targets for drug development and in vivo tumor detection. Fluorescence- and magnetic-resonance-based approaches have been used to detect the activity of MMPs.^[19–23] Here, we describe a BRET-based QD nanosensor for detecting the activity of gelatinase MMP-2 with high sensitivity.

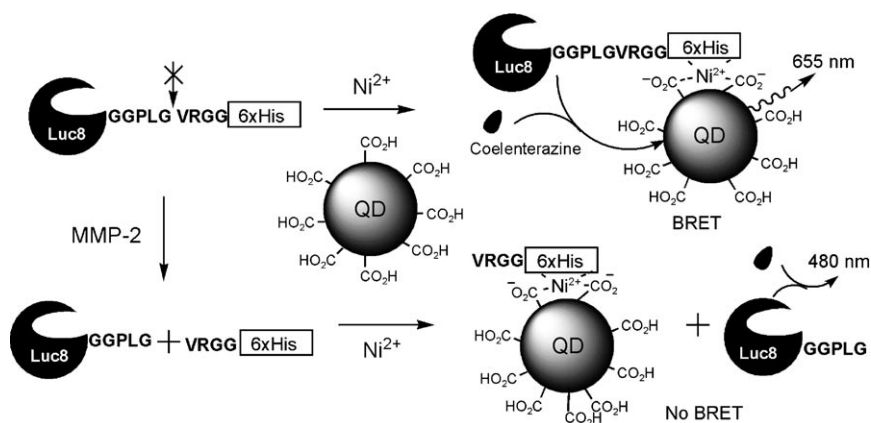


Figure 1. BRET-based detection of MMP-2 with assembled QD nanosensors.

The gelatinase MMP-2 has been identified as one of the key MMPs in degrading type IV collagen. MMP-2 hydrolyzes peptide substrates that contain an amino acid sequence of PLGVR.^[24] Our design of a BRET-based QD sensor for the detection of the MMP-2 activity is shown in Figure 1. A short 15 amino acid peptide (GGPLGVRGGHHHHHH), which contains the MMP-2 substrate and a six-histidine tag, is genetically fused to the C terminus of the BRET donor, a mutant of *Renilla* luciferase (Luc8). The Luc8 was chosen because of its high stability in biological media.^[25] In this study, we used commercially available QDs with carboxylic acids presented at the surface, QD-COOH. In the presence of Ni^{2+} cations, the carboxylic acids on the QDs will bind the metal ions and form complexes with the $6 \times \text{His}$ tag on the Luc8 fusion protein. BRET will take place and produce light emission from the QDs. The cleavage of the amide bond between Gly and Val by MMP-2 will release the $6 \times \text{His}$ tag from the fusion Luc8 and thus no BRET will occur (Figure 1).

We first examined whether MMP-2 could cleave the peptide substrate fused to Luc8. A NuPAGE assay (Invitro-

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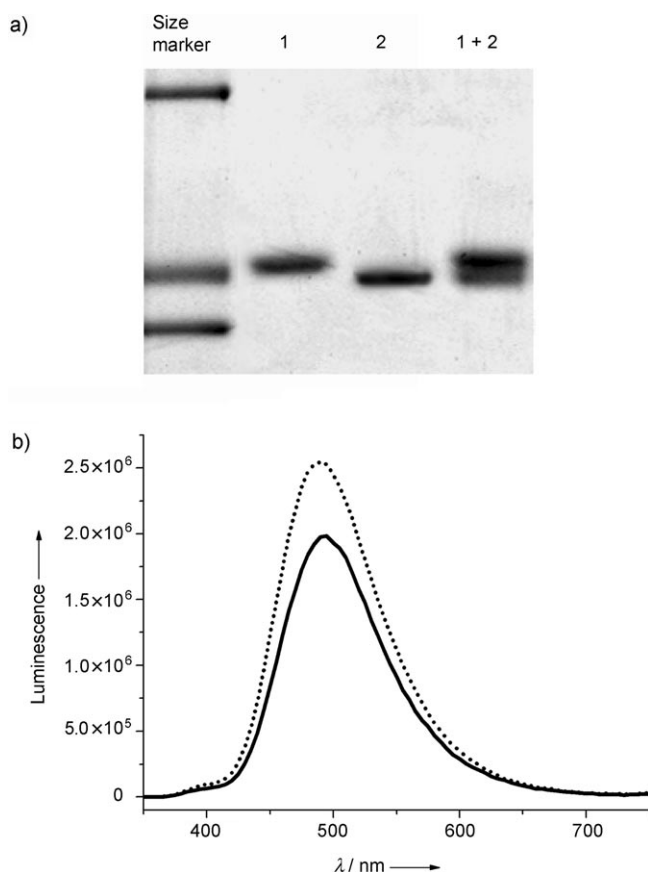


Figure 2. MMP-2 cleavage of the Luc8 fusion protein. a) NuPAGE assay. Lane 1: Luc8 fusion protein (size: 37.9 kD); lane 2: MMP-2-treated Luc8 fusion protein (size: 36.7 kD); lane 1 + 2: mixture of compounds in lanes 1 and 2. b) Bioluminescence emission of the Luc8 fusion protein before (—) and after (.....) MMP-2 cleavage.

gen Corp., USA) detected a protein product with a smaller size (36.7 kD), which confirmed that the fusion protein can be hydrolyzed by MMP-2 (Figure 2). The cleaved product displayed a slightly higher (18%) luminescent emission than the fusion protein itself.

We then evaluated the efficiency of the Ni^{2+} -induced BRET between the QDs and the Luc8 fusion protein. When QD655-COOH (50 nM) was mixed with the fusion protein (400 nM) in the absence of Ni^{2+} ions, upon addition of the substrate of Luc8, coelenterazine (1 μg), there was a large emission from Luc8 at 480 nm but only a small emission from the QDs at 655 nm (< 10% of the total emission from Luc8; Figure 3). This small peak at 655 nm most likely arose from an electrostatic interaction between the 6 $\times$ His tag on the Luc8 and the carboxylate groups on the QDs. However, when Ni^{2+} (100 μM) was added to the mixture, the emission at 480 nm largely decreased and that at 655 nm from the QDs increased significantly, which indicated that BRET occurred efficiently between the QDs and Luc8. The BRET ratio, defined as the ratio of the integration of the peak at 655 nm to that of the conjugate prepared through covalent coupling between the QDs and Luc8,^[15] which suggests a short distance between the QDs and the Luc8 fusion protein in this Ni^{2+} -His-tag-mediated complex and a high BRET efficiency.

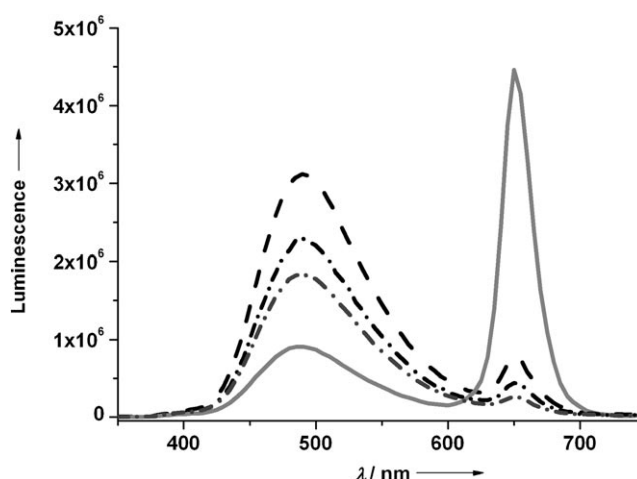


Figure 3. Luminescence spectra of mixtures of QD655-COOH (50 nM) and Luc8-6 $\times$ His fusion protein (400 nM) containing (from top to bottom at 480 nm): no Ni^{2+} , Ni^{2+} (100 μM) + EDTA (200 μM), Ni^{2+} (100 μM) + imidazole (300 mM), and Ni^{2+} (100 μM).

Both the carboxylate groups on the QDs and the histidine tag on the Luc8 are important for the Ni^{2+} -mediated BRET. Addition of an excess amount of ethylenediaminetetraacetic acid (EDTA; 200 μM), a strong chelator for Ni^{2+} ions, to a mixture containing QD-COOH, the fusion protein, and Ni^{2+} greatly decreased the BRET emission (Figure 3). EDTA competed with QD-COOH for the Ni^{2+} ions, which led to dissociation of the QD-COOH and Luc8 complex and, consequently, disruption of the BRET process. When QDs coated with amino groups (QD655-NH₂) replaced QD655-COOH, no BRET emission from the QDs was detected, further confirming that the carboxylate groups on the QDs are critical for the binding of Ni^{2+} ions and BRET (see the Supporting Information). When imidazole was added to a mixture containing QD-COOH, the Luc8 fusion protein, and Ni^{2+} , a large decrease in the BRET emission was observed; correspondingly, there was a large increase in the Luc8 emission at 480 nm (Figure 3). These results indicate that the interaction between Luc8 fusion protein and QD-COOH mediated by Ni^{2+} ions is specific and dependent on the histidine tag.

We examined whether other metal ions could induce BRET between the QDs and the Luc8 fusion protein. A series of divalent metal ions was tested at a concentration of 100 μM in a solution containing QD-COOH (50 nM) and Luc8 (400 nM). As shown in Figure 4, Ni^{2+} ions induced the highest BRET signal. The presence of Ca^{2+} , Mg^{2+} , and Cu^{2+} ions (100 μM) afforded a similar Luc8 emission as the control, and the BRET emission from the QDs was small. At a much higher concentration (a few millimolar), both Ca^{2+} and Mg^{2+} ions were able to induce BRET efficiently. No emission from Luc8 was detected when Hg^{2+} ions (100 μM) were present, so no BRET was induced. Zn^{2+} ions could induce BRET but the overall activity of Luc8 decreased fivefold, and thus a lower BRET emission was observed than with Ni^{2+} ions.

We applied the Ni^{2+} -His-tag-mediated BRET between the QDs and the Luc8 fusion protein to the detection of the protease MMP-2. Hydrolysis by MMP-2 releases the 6 $\times$ His

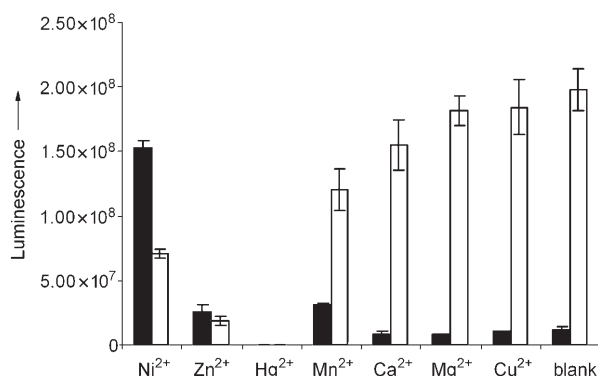


Figure 4. Bioluminescence emission from the QDs (50 nm) and Luc8 fusion protein (400 nm) in the presence of the indicated divalent ions (each at 100 μM) in borate buffer solution. Black columns: emissions from QDs; white columns: emissions from Luc8 proteins.

tag from the Luc8 fusion protein and thus decreases the BRET ratio. A solution of the Luc8 fusion protein (16 μM in borate buffer solution) was incubated with MMP-2 (10 $\mu\text{g mL}^{-1}$) for various periods of time at 22°C, and then the mixture was added to a solution containing QD-COOH (40 nm) and Ni^{2+} (100 μM) for the measurement of the BRET emission (Figure 5). Before cleavage of the MMP-2 ($t=0$), there was a strong emission from the QD655 at 655 nm, and the BRET ratio was 1.64. Within 30 min the BRET ratio dropped to 0.62, and the reaction was nearly complete within 2 h with a BRET ratio of 0.21.

The sensitivity of this assay was measured by incubating various concentrations of MMP-2 with the Luc8 fusion protein (5 μM) for 24 h at 22°C (see the Supporting Information). The BRET ratio for the control without MMP-2 remained unchanged at 1.55. However, the BRET ratio for a concentration of MMP-2 of 2 ng mL^{-1} (≈ 30 pM) decreased by about 10% from 1.55 to 1.40, and by 15% to 1.31 for a concentration of MMP-2 of 5 ng mL^{-1} (≈ 75 pM). Previously reported FRET-based QD sensors detected MMPs at a concentration of 0.5 $\mu\text{g mL}^{-1}$,^[11] which is a much lower sensitivity than that reported here with the BRET-based

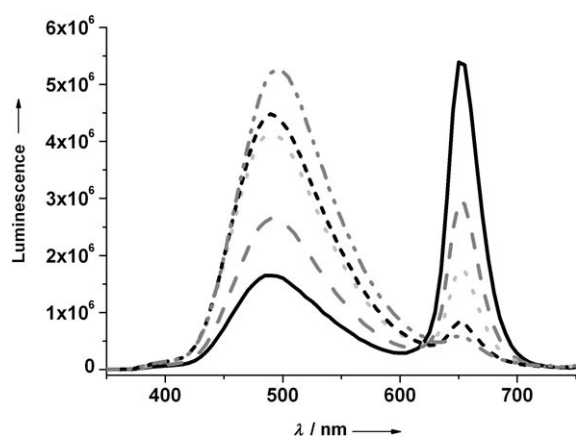


Figure 5. Detection of MMP-2 with the BRET-based QD nanosensor. The Luc8 fusion protein (16 μM) was preincubated with MMP-2 (10 $\mu\text{g mL}^{-1}$) for 0, 0.5, 1, 2, and 16 h (from top to bottom at 655 nm) before addition to a solution containing QD655-COOH (40 nm) and NiCl_2 (100 μM) for BRET measurements.

QD sensor. The detection limit is also better than that for the magnetic sensor, which was reported to be 19 ng mL^{-1} .^[22]

Finally, we evaluated the specificity of the QD sensor for MMP-2 by testing its activity against two other proteases: another member of the MMP family, MMP-7 (matrilysin that cleaves a peptide substrate RPLALWRS efficiently between Ala and Leu),^[26] and the tobacco etch virus (TEV) protease (Figure 6). A solution of the Luc8 fusion protein (5 μM) was incubated with AcTEV protease (100 Unit mL^{-1} ; an

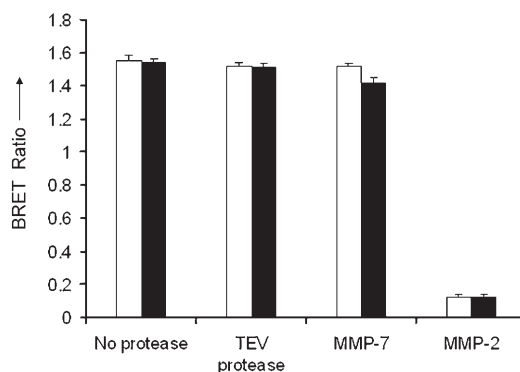


Figure 6. BRET ratio of the assembled QD nanosensor complex containing QD655-COOH, NiCl_2 , and the Luc8 fusion protein. Luc8 fusion protein (5 μM) was preincubated with AcTEV protease (100 Unit mL^{-1}), MMP-2 (15 nM), or MMP-7 (15 nM), or without any proteases, for 2 h (white columns) and 6 h (black columns).

enhanced TEV protease that recognizes the seven amino acid sequence ENLYFQG and cleaves between Gln and Gly with high specificity),^[27] MMP-7 (15 nM), or MMP-2 (15 nM) in borate buffer solution for 2 h at room temperature, and QD-COOH (40 nm) and Ni^{2+} (100 μM) were added immediately for the BRET measurements. The BRET ratio for MMP-2 decreased to 0.12, whereas it remained unchanged at 1.55 for both MMP-7 and AcTEV protease, which was the same as that of the control without any protease. After 6 h of incubation, the BRET ratio for the AcTEV protease did not change, and that for MMP-7 dropped slightly to 1.44. These results demonstrate that this QD-BRET sensor system is specific to the MMP-2 activity.

The QD BRET in this sensing system is mediated by the interaction between Ni^{2+} ions and the His tag. Other conjugation systems, such as biotin and streptavidin, and halotag protein and halotag ligand,^[28] may also be exploited for the design of similar QD-BRET assays. When a different peptide substrate is used, the system can be extended to other hydrolytic enzymes. For example, when an AcTEV protease substrate (ENLYFQG) replaced the MMP-2 substrate sequence in the Luc8 fusion protein, the nanosensor specifically detected the TEV protease activity.

In comparison to FRET-based QD sensors, BRET-based QD biosensors offer several attractive features. The spectral separation between the BRET donor and acceptor emissions is large; for instance, the difference of the maximal emission wavelength in this example is more than 175 nm, which makes it easy to detect both emissions for ratiometric measurements. The sensitivity is high with low background emission. As a result of the wide absorption spectra of the QDs, one

bioluminescent protein, such as *Renilla* luciferase, can efficiently excite multiple QDs with different emissions.^[15] For the FRET-based QD sensors, different FRET acceptors are required for multiple QDs with different emissions.^[12] Furthermore, the presence of multiple copies of the BRET donors on the QD does not decrease but increases the BRET emission.^[15]

In summary, the BRET-based QD biosensing system described here utilizes commercially available carboxylate QDs and a bioluminescent fusion protein containing a histidine tag and MMP-2 substrate to detect the activity of protease MMP-2 with high sensitivity. This system is simple to use with no need for QD modifications, and can be extended to other hydrolytic enzymes by simply choosing appropriate substrates. The approach offers great sensitivity and may serve as a general strategy to design QD nanosensors for multiplex detection of biological analytes.

Experimental Section

QDs were from Invitrogen and coelenterazine was from Prolume. The vector to make the Luc8 fusion protein was from Promega. Fluorescence and bioluminescence spectra were collected with a Fluoro Max-3 spectrometer (Jobin Yvon Inc.). Bioluminescence spectra were acquired with the excitation light blocked. MMP-2 and MMP-7 were obtained from Calbiochem, and AcTEV protease was from Invitrogen.

Preparation of the Luc8 fusion protein: The plasmid Luc8-MMP-2-pRSF-Duet was constructed from plasmid pBAD-RLuc8 by PCR and ligation. *Escherichia coli* BL21 cells were transformed with this plasmid and induced with isopropyl β -D-1-thiogalactopyranoside (0.5 mM) for 3 h until the optical density at a wavelength of 600 nm reached 0.5. Cells were lysed by thawing in wash buffer (WB) solution (NaCl (300 mM), 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES; 20 mM), pH 8.0) containing lysozyme (1 mg mL⁻¹), RNase A (10 μ g mL⁻¹), and DNase I (5 μ 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 8.0) and further purified by anion-exchange chromatography (Source 15Q resin, GE/Amersham) followed by gel-filtration chromatography with borate buffer solution.

MMP-2 assay: Typically, a solution of the Luc8 fusion protein (5 μ M) in borate buffer solution (10 mM CaCl₂, pH 7.5) was incubated with various amounts of active human recombinant MMP-2 for a period of time at room temperature. A small volume of the reaction solution (6 μ L) was withdrawn and mixed with QD655-COOH (15 μ L, 160 nM) and NiCl₂ (15 μ L, 400 μ M) in borate buffer solution (23 μ L, pH 7.5) for the BRET measurements. The Luc8 substrate coelenterazine (1 μ g in 1 μ L) was added immediately before the acquisition of bioluminescence emission spectra, and the scan was typically finished within 20 s. The emission-correction file provided by the instrument manufacturer was used for the correction of emission spectra. Gel electrophoresis analysis confirmed the cleavage of the fusion protein by MMP-2. Proteins were run on a 4–12 % Bis-Tris gradient denaturing gel and stained with Coomassie Blue.

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