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.

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 Ni2+ cations, the carboxylic acids on the QDs will bind the metal ions and form complexes with the 6 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 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-
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 Ni2+–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 Ni2+ 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 6His tag on the Luc8 and the carboxylate groups on the QDs. However, when Ni2+ (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 peak at 480 nm, became 1.94. This value is comparable to that of the conjugate prepared through covalent coupling between the QDs and Luc8, which suggests a short distance between the QDs and the Luc8 fusion protein in this Ni2+–His-tag-mediated complex and a high BRET efficiency.

Both the carboxylate groups on the QDs and the histidine tag on the Luc8 are important for the Ni2+–mediated BRET. Addition of an excess amount of ethylenediaminetetraacetic acid (EDTA; 200 μM), a strong chelator for Ni2+ ions, to a mixture containing QD-COOH, the fusion protein, and Ni2+ greatly decreased the BRET emission (Figure 3). EDTA competed with QD-COOH for the Ni2+ 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 Ni2+ ions and BRET (see the Supporting Information). When imidazole was added to a mixture containing QD-COOH, the Luc8 fusion protein, and Ni2+, 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 Ni2+ 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, Ni2+ ions induced the highest BRET signal. The presence of Ca2+, Mg2+, and Cu2+ 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 Ca2+ and Mg2+ ions were able to induce BRET efficiently. No emission from Luc8 was detected when Hg2+ ions (100 μM) were present, so no BRET was induced. Zn2+ ions could induce BRET but the overall activity of Luc8 decreased fivefold, and thus a lower BRET emission was observed than with Ni2+ ions.

We applied the Ni2+–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 6His-tagged complex and a high BRET efficiency.
The Luc8 fusion protein (16 nm) was preincubated with MMP-2 (10 μg 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 Ni²⁺ (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 μg mL⁻¹,[11] which is a much lower sensitivity than that reported here with the BRET-based 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),[29] 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; 

SDT) A solution of 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.
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.\[13\]

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 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 measurements were performed with a ChemBioChem (4349) instrument manufacturer was used for the correction of emission substrates. The approach offers great sensitivity and may 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.

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