Energy Transfer

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A Resonance Energy Transfer between Chemiluminescent Donors and Luminescent Quantum-Dots as Acceptors (CRET)**

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Fluorescence resonance energy transfer (FRET) is a powerful technique for probing very small changes in the distance between donor and acceptor fluorophores, and is ideal for the sensitive detection of molecular binding events and changes in protein conformation in response to interactions with a particular target molecule or to changes in the solution environment. In recent years, luminescent semiconductor nanocrystals (also called quantum dots, QDs) have been favorably adopted in the FRET-based studies, such as deriving QD–protein–conjugate configuration,[1] QD–protein sensing assemblies,[2,3] photochromic switching,[4] photodynamic medical therapy,[5] and probing DNA replication and telomerization.[6] This success is due to the unique size-dependent physical and chemical characteristics of QDs. QDs were applied to FRET as both donors[7–10] and acceptors,[11–13] and some of the advantages offered compared to conventional dyes include sharp and symmetrical emission spectra, high quantum yield (QY), good chemical and photo stability, and size-dependent emission-wavelength tunability. Recently, QDs as acceptors were successfully used in bioluminescence resonance energy transfer (BRET).[14]

As with any fluorescence technique, however, photobleaching and autofluorescence limit the usefulness of FRET. Herein we report a resonance energy transfer between chemiluminescent donors and QDs as acceptors, which is called chemiluminescence (CL) resonance energy transfer (CRET), and is similar to BRET.[14–19] CRET involves nonradiative (dipole–dipole) transfer of energy from a chemiluminescent donor to a suitable acceptor molecule. In contrast to FRET, CRET occurs by the oxidation of a luminescent substrate without an excitation source. In our system, we chose the luminol/hydrogen peroxide CL reaction catalyzed by horseradish peroxidase (HRP) because this is one of the most sensitive CL reactions. In capillary electrophoresis with CL detection, the detection limit of HRP was below 10^{-19} mol

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in the presence of *para*-iodophenol (*p*-IP) as an enhancer.[20] And more importantly, the CL spectrum (425–435 nm) of luminol overlaps well with the absorption of the QDs (Figure 1 A). The principle of CRET is illustrated in Scheme 1. In Scheme 1 A, the CL donor, luminol, is not directly linked with the QDs, and the catalyst, HRP, is conjugated to the QDs. HRP can continuously catalyze the luminol/hydrogen peroxide CL reaction. In this system, the QD–HRP conjugates can be used as probes in cell and tissue imaging similar to BRET.[14] In Scheme 1 B, QDs are linked with bovine serum albumin (BSA), and HRP is conjugated with the BSA antibody (anti-BSA). When the anti-BSA–HRP binds to the BSA–QDs, CRET can occur. This system has potential to be used in immunoassay in non-competition and competition modes (see Supporting Information).

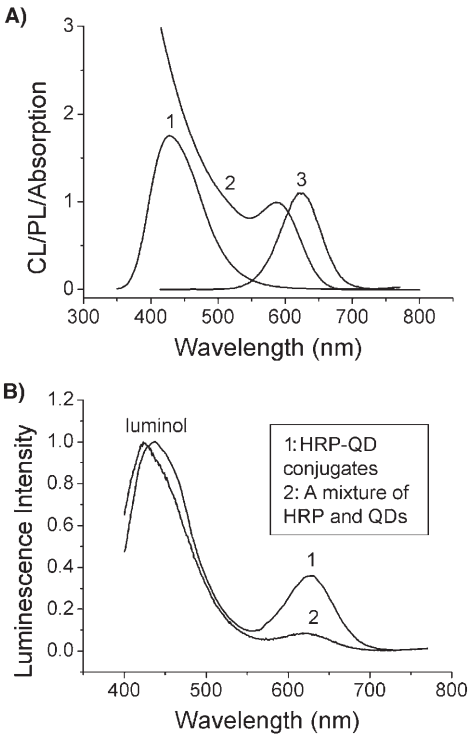
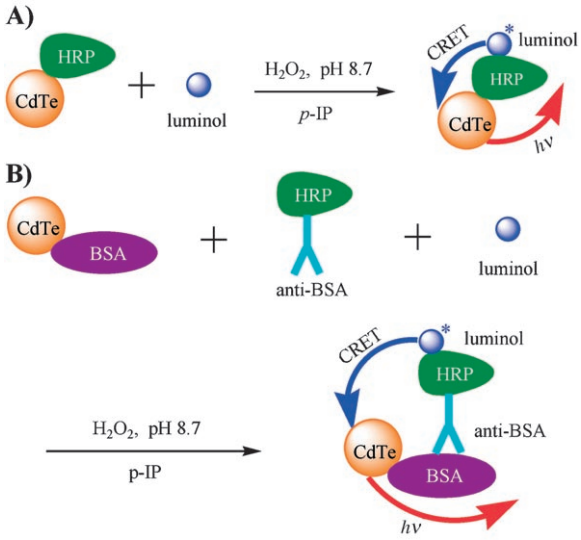


Figure 1. A) The CL of the luminol (1) spectrally overlaps with the absorption of the QDs (2). The diameter of CdTe QDs used is approximately 4 nm, and their emission wavelength is around 622 nm (3); CL = chemiluminescence, and PL = photoluminescence. Luminescence spectra of the QD–HRP-luminol system using luminol as donor and QDs as accepters. B) Comparison of CL spectra of HRP-QDs conjugates and a mixture of HRP and QDs using the QD–HRP-luminol system. CL reaction buffer was 0.05 M sodium borate solution (pH 10.0) containing of 1.0×10^{-4} M luminol, 5.0×10^{-4} M H_2O_2 and 5.0×10^{-4} M *para*-iodophenol. The concentrations of the QD–HRP conjugates are 1×10^{-6} M (HRP). All spectra are normalized.



Scheme 1. A) Schematic illustration of CRET based on luminol donors and HRP-labeled CdTe QD accepters, B) Schematic illustration of CRET for luminol donors and QD accepters based on the immuno-reaction of QD–BSA and anti-BSA–HRP. See text for details.

In our study, different sized water-soluble CdTe QDs were synthesized in the aqueous phase using the reaction between Cd^{2+} and NaHTe solution in the presence of mercaptopropyl acid (MPA) as a stabilizer[21] and their QYs were measured to be 40–50 %. The MPA-coated CdTe QDs were conveniently conjugated to certain proteins (such as HRP and BSA) using EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) as a coupling reagent. The mixtures were purified using ultra-filtration membrane. The QD bioconjugates were characterized by capillary electrophoresis with laser-induced fluorescent detector. Our results showed that ultra-filtration was an effective and simple approach to purify QD–HRP conjugates and the conjugates were stable after being kept for one week (see Supporting Information). The diameter of HRP (M_W = 40 kDa) was estimated to be 35 Å, by using spherical approximation.[22] The hydrodynamic diameter of 622 QD–HRP conjugates and 622 QD (that is, QDs with an emission wave length of 622 nm), measured by fluorescence correlation spectroscopy (FCS),[23] were approximately 8 nm and 4 nm, respectively (see Supporting Information). Each 622 QD–HRP conjugate was estimated to contain one or two copies of immobilized HRP.

We investigated the CRET between luminol and QD bioconjugates in the QD–HRP system and the results obtained are shown in Figure 2. In Figure 2 A, four different sized QDs (with emissions at 557, 587, 622, and 657 nm) were used as acceptors. An efficient CRET between luminol and QD bioconjugate was observed, which looked like FRET. The CRET ratios, determined by dividing the acceptor emission by the donor emission, ranged from 0.22 to 0.32. The small differences of the CRET efficiency among the QDs are attributed to their different donor–accepter distances and quantum yields.

A comparison of CL spectra between HRP–QDs conjugates and a mixture of HRP and QDs (Figure 1 B) shows that the CL intensity ratio of QDs:luminol from the mixture of QDs and HRP was very low (Figure 1 B (2)), which was attributed to the low adsorption of QDs onto HRP. The electropherograms (see Supporting Information) of a mixture of HRP and QDs confirmed that a little of HRP was adsorbed onto QDs in the buffer solution (PBS, 0.01 M) by electrostatic interaction. There is a slight red-shift in luminol emission in

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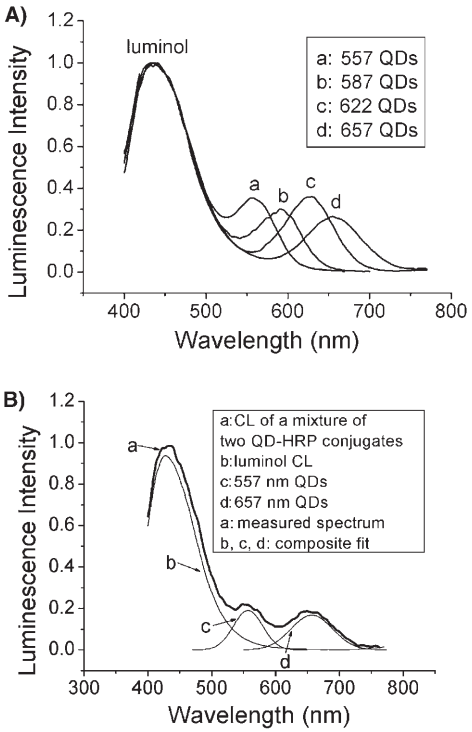

Figure 2. A) CL spectra of the QD–HRP–luminol system using luminol as donor and different sized QDs as accepters. B) CL spectra of the QD–HRP–luminol system using two QD accepters simultaneously. Other conditions are the same as in Figure 1. All spectra are normalized.

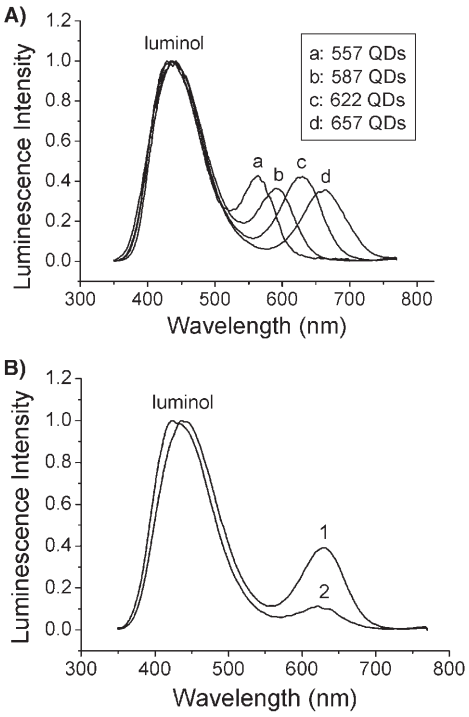

Figure 3. A) CL spectra for the QD–BSA/anti-BSA–HRP/luminol system using multiple QDs accepters interacting with luminol donors, respectively. B) Comparison of CL spectra of the QDs-BSA/anti-BSA–HPR immuno-complex (1) and a mixture of QD–BSA, anti-BSA, and HPR (2). CL reagents are delivered by a syringe to the quartz cuvette which containing QD–BSA/anti-BSA–HRP immuno-complex. Other conditions are the same as in Figure 1. All spectra are normalized.

the CRET experiment (Figure 1 B (1)). This phenomenon is unclear and may be due to the decrease of confinement in one dimension.[24,25]

Furthermore, we also observed the CRET of the luminol donor with two different size QD accepters (557 nm and 657 nm) simultaneously, and the CL spectra are shown in Figure 2 B. This result demonstrated that the multiplexed CRET could be realized similar to the multiplexed FRET.[3]

On the basis of the above results, we attempted to apply CRET to immunoassay based on antigen(BSA)–antibody(anti-BSA IgG) interactions. Immunoglobulin antigen (BSA) was first conjugated to CdTe QDs by electrostatic interaction, while HRP was covalently conjugated to the corresponding antibody (anti-BSA IgG). The mixtures were purified using an ultra-filtration membrane. The purified mixtures were mixed and incubated at 37 °C for 2–4 h. The CL reaction of luminol and hydrogen peroxide was used in the CRET process.

The CL spectra using different sized QDs as accepters are shown in Figure 3 A based on the immuno-interaction. These data illustrate that an efficient CRET between luminol and QD bioconjugates is generated when the anti-BSA–HRP binds to the BSA–QDs. In this system, the CRET ratios ranged from 0.27 to 0.36, which are approximately the same CRET ratios as those of the directly coupled conjugates. The distance between the donor and acceptor of the antibody-sandwiched conjugates is not the simple addition of the sizes of BSA, anti-BSA, and HRP since proteins are folded. However, the distance in the antibody-sandwiched conjugates is still expected to be longer than in the directly coupled conjugates, this longer distance should result in a lower CRET ratio. We attribute the results to a little uncoupled horseradish peroxidase in the purified QD–HRP solution, which thus led to the higher CL intensity of luminol and a lower CRET ratio for the directly coupled conjugate.

Comparisons of CL spectra of QD–BSA/anti-BSA–HRP immuno-complex and the mixture of QD–BSA conjugates and anti-BSA, and HRP are shown in Figure 3 B. The low CL intensity ratio of QDs:luminol from the mixture Figure 3 B (2) was also attributed to the weak adsorption of QDs onto HRP.

In summary, we demonstrated an efficient CRET between luminol and QDs based on HRP–QD conjugates and the immuno-interaction of the QD–BSA and anti-BSA–HRP in the luminol/hydrogen peroxide CL reaction. Further, we showed the potential for the application of multiple QDs acceptors with different emission wavelengths to multiplex analysis. Compared to FRET, CRET experiments are very simple and do not need an exciting light source. Additionally, in our system, the catalyst, HRP (not the CL donor, luminol), was directly linked with the QDs, and can continuously catalyze the CL reaction, which is probably the main reason for an efficient CRET. We anticipate that the CRET will become an attractive alterative method for quantitative bioanalysis, cell, and tissue imaging. CRET has its own limitations, however, and requires an oxidant, H_2O_2. High

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concentration H_2O_2 will quench the fluorescence of QDs. The future work is to develop the special detection system of CRET to enhance the detection sensitivity since the CL collection system of CRET used in this study is inefficient.

Experimental Section

CdTe QDs were prepared using the reaction between Cd^{2+} and NaHTe solution in the presence of MPA as a stabilizer according to the approach described in the ref. [21] Absorption spectra were collected using a Lambda 20 UV/Vis spectrophotometer (Perkin-Elmer). Emission spectra were measured with a Varian Cary spectrometer. The QYs of CdTe QDs are shown in the Supporting Information.

CdTe QDs were conjugated to proteins using EDC as a coupling reagent. First, a reaction mixture containing CdTe QDs (0.1–0.3 mg mL^{-1}), HRP (0.1 mg mL^{-1}), and EDC (0.5 mg mL^{-1}) in pH 7.0 PBS (0.01 M) was prepared and kept at room temperature for 2–4 h. Then the mixture was purified using an ultra-filtration membrane (Micoron YM-50–50 000 NMWL, Millipore, USA) according to the instructions from the manufacture. Finally the purified products were stored in refrigerator at 4 °C.

BSA can facilely conjugate to CdTe QDs by electrostatic attraction. A mixture (a) of QDs (0.1–0.2 mg mL^{-1}) and BSA (0.3 mg mL^{-1}; containing 0.01 M PBS, pH 7.0) was left for 0.5 h at the room temperature. Anti-BSA was conjugated to HRP using EDC as a coupling reagent: A mixture (b) of anti-BSA (0.3–0.5 mg mL^{-1}), HRP (0.1 mg mL^{-1}), and EDC (1.0 mg mL^{-1}; containing 0.01 M PBS, pH 7.0) was left for 2–4 h at room temperature. The mixtures (a) and (b) were purified using ultra-filtration membrane (Micoron YM-100–100 000 NMWL, Millipore, USA). Then, the purified mixtures (a) and (b) were mixed together and incubated at 37 °C for 2–4 h. Finally, the reaction solution was stored in refrigerator at 4 °C.

Fluorescence correlation spectroscopy (FCS)[23] was used to measure the hydrodynamic diameter of QD–HRP conjugates and QDs. A P/ACE MDQ capillary electrophoresis (CE) system (Beckman Coulter Inc., Fullerton, CA, USA) was applied to characterize the QDs conjugates. $Na_2B_4O_7$ solution (0.025 M, pH 8.0) was used as running buffer.

CL spectra were measured with a Varian Cary spectrometer using a 3 mL quartz cuvette (1 cm optical path). Herein, the luminol/hydrogen peroxide CL reaction catalyzed by HRP is adopted, and *para*-iodophenol is acted as an enhancer. The CL reaction buffer consists of sodium borate solution (pH 10.0; 50 mmol L^{-1}; containing 1.0×10^{-4} M luminol, 5.0×10^{-4} M H_2O_2, 5.0×10^{-4} M *para*-iodophenol). 0.5 mL the CL reaction buffer was added to the quartz cuvette containing a given HRP–QDs conjugate or QD–BSA/anti-BSA–HRP immuno-complex (containing 0.01 M PBS, pH 7.0), the pH value of the mixed CL reaction solution was 8.7. CL reactions were initiated, and CL spectra were measured with a Varian Cary spectrometer.

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