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Energy Transfer-Based Multiplex Analysis Using Quantum Dots

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1. Introduction

Semiconductor nanocrystals, also known as quantum dots (QDs), have emerged as a significant new class of materials over the past decade. The capabilities of QDs – high quantum yield, improved sensitivity, high photostability, and size-tunable colors have paved the way for numerous studies including imaging, sensing and targeting biomolecules. Thus QDs are now rapidly replacing traditional fluorophores in almost all fluorescence-based applications. Unlike organic dyes and fluorescent proteins, QDs are size-tunable with non-overlapping emission band profiles due to their narrow and symmetric emission bands (full width at half maximum of 25–40 nm) that can span the light spectrum from the ultraviolet even to the infrared. As illustrated in **Figure 1**, this property enables the QDs to be useful for multiplexing assay in a single run (Chan et al., 2002; Jaiswal et al., 2003; Wu et al., 2003). Moreover, QDs typically have very broad absorption spectra with very large molar extinction coefficients ($0.5–5×10^6$ M^{-1} cm^{-1}) (Hawrylak et al., 2000; Moreels and Hens, 2008). This makes QDs absorb 10–50 times more photons than organic dyes at the same excitation photon flux, providing a sufficient brightness for the sensing system (Gao et al., 2004). Owing to high photostability, QD-based sensing and imaging are favorable for continuous tracking studies over a long period of time. Most importantly, when the QD is harnessed in fluorescence resonance energy transfer (FRET), several advantages over organic dye-based probes have been acquired. Multiple binding of an energy acceptor per a single QD is expected to increase the overall energy transfer efficiency (Clapp et al., 2004; Zhang et al., 2005). Additionally, large Stokes shift of the QD can avoid the crosstalk between the donor QD and the acceptor counterpart because its broadband absorption allows excitation at a short wavelength that does not directly excite the acceptor. The continuously tunable emissions that can be matched to any desired acceptor, makes it possible to use many fluorophores for multiplexed assay (Medintz et al., 2003). The quencher (orgarnic (Mauro et al., 2003) or metal (Kim et al., 2008a; Oh et al., 2005; Oh et al., 2006; Wargnier et al., 2004) substances) or emissive fluorescent molecules (fluorophores, proteins, or other QDs) (Wang et al., 2002) can be promising acceptors. While QDs are frequently used as donors in FRET, they may also play a critical role as energy acceptors either in bioluminescence resonance energy transfer (BRET)(Rao et al., 2006a; Rao et al., 2006b) or chemiluminescene resonance energy transfer (CRET)(Huang et al., 2006) as the energy donor (**Figure 2**). To this end, energy transfer system allows QDs to be suitable for many biological applications, such as the analyses of enzyme activity, protein-protein

interactions, and other environmental conditions (pH, ion concentration and so forth). To avoid redundancy in a myriad of applications of QDs, the energy transfer-based detection will be focused here between QDs and other binding partners. A short overview regarding QD-FRET, QD-BRET, and QD-CRET will be demonstrated.
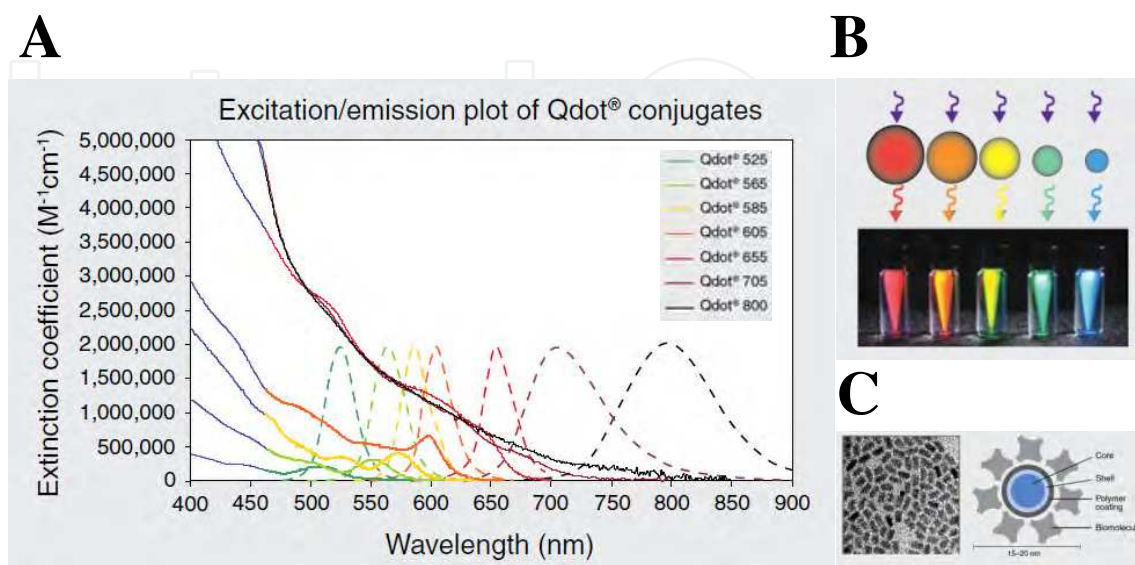

Fig. 1. Characters of Quantum Dot (QD). (A) Excitation (solid line) and emission spectra (dotted line) of CdSe quantum dots. (B) Size tuneability and emission color of five different QDs with the same long-wavelength UV lamp. (C) Structure of a QD nanocrystal: transmission electron microscope image of core shell QD at 200,000× magnification (left) and schematic of the overall structure (right). Figures are obtained from Invitrogen website (www.invitrogen.com).

2. Fluorescence resonance energy transfer (FRET) system using QDs

FRET is the most commonly utilized technique in these applications because of the high sensitivity, good reproducibility, and real-time monitoring capabilities. General configuration of FRET consists of chromophores with different combinations, such as auto-quenched probes (Kim et al., 2009; Weissleder et al., 1999), dual chromophore probes (Kircher et al., 2004), or multiphoton FRET-based probes (Stockholm et al., 2005). However, such organic fluorophores often have problems such as photobleaching, susceptibility to environment, difficulty in multiplexed analysis by specific paring between donor and acceptor. As aforementioned, these problems in FRET assays can be overcome when appropriate fluorophore or quencher is used in conjunction with quantum dots (QDs) (Medintz et al., 2006; Shi et al., 2006). In FRET, QDs are typically used as fluorescence donors while the fluorescent (or quenchable) acceptors are used as acceptors by appropriated labeling with biomolecules (DNA, aptamer, peptide and protein).

FRET-based QD-DNA nanosensor allows for detecting low concentrations of DNA, where the target strand binds to a dye-labelled reporter strand thus forming a FRET between QD and dye (Zhang et al., 2005). The QD also functions as a concentrator that amplifies the target signal by confining several targets in a nanoscale domain. They applied the nanosensors in combination with the oligonucleotide ligation assay to the detections of Kras

point mutations (codon 12 GGT to GTT mutation) in clinical samples from patients with ovarian serous borderline tumours. Unbound nanosensors produce near-zero background fluorescence, but on binding to even a small amount of target DNA (~50 copies or less) they generate a very distinct FRET signal.
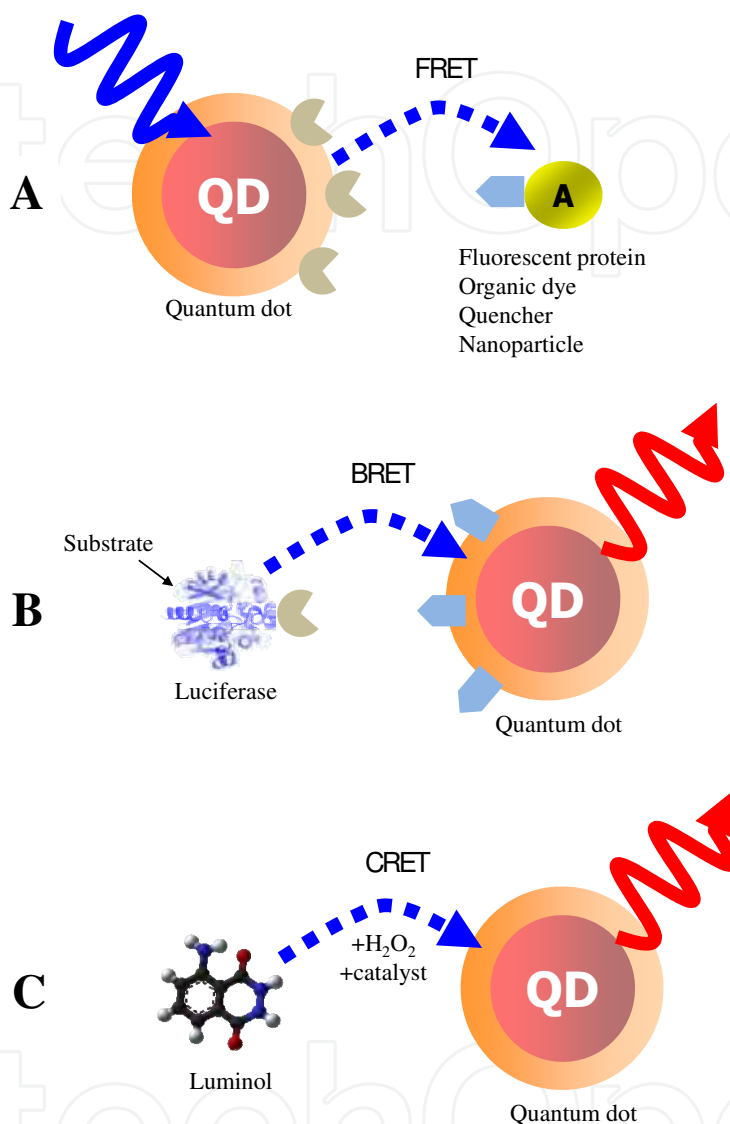

Fig. 2. Representative schematic of resonance energy transfer-based QD probes. QD-FRET (A), QD-BRET (B), and QD-CRET (C). A indicates energy acceptor.

QD-based FRET sensor has been increasingly combined with aptamers which take a great deal of advantages including high affinity, easy development through systematic evolution of ligands by exponential enrichment (SELEX), great stability and immuno-resistance. It has been reported that QD-aptamer (QD-Apt) conjugate can image and deliver anticancer drugs to prostate cancer (PCa) cells to the targeted tumor cells based on the mechanism of FRET(Bagalkot et al., 2007), in which RNA aptamers covalently attached to the surface of QD serves a dual function as targeting molecules and as drug carrying vehicles. When doxorubicin (Dox) intercalated within the A10 aptamer, a donor-acceptor quenching between QD and Dox was induced by the energy transfer mechanism. Specific uptake of QD-Apt(Dox) conjugates

into target cancer cell through PSMA mediate endocytosis evokes the release of Dox from the QD-Apt(Dox) conjugates, resulting in the recovery of fluorescence from both QD and Dox and the intracellular delivery of Dox inside cancer cells. Levy et al. demonstrated an aptamer beacon-based approach for detecting the protein thrombin with QD-based fluorescence readout, in which the detection limit was determined to be 1 μM (Levy et al., 2005). Most recently, Cheng et al. Once MUC1 peptide binds to the aptamer strand on the surface of QD, fluorescence intensity successively decreases through FRET effect, showing low detection limit for MUC1 (nanomolar level) and a linear response found in blood serum.

Much attention has also been paid to QD-FRET for *in vitro* and *in vivo* assay of proteases. The ability of enzymes to target the specific substrate in complex milieu is essential for understanding the fidelity of most biological functions. Among enzymes, proteases are particularly significant because proteolytic processing is a final step to establish the functional roles of many expressed proteins and most of proteases are involved in major human cancers. Thus, methods to assay proteases and their inhibitors using QD-FRET have been of great interest in diagnosis of protease-relevant diseases and development of potential drugs. Assaying proteolytic activity can be easily designed by combining such peptide substrates with QD-FRET. Upon cleavage of the substrates by the protease, the resulting signals are regenerated from quenched or energy-trapped QDs because of the distruption of the energy transfer. Although conventional methods to adapt this FRET-based principle for monitoring *in vivo* protease activity has been made by using genetically engineered fluorescence protein (typically CFP-YFP), the real analysis of energy transfer is often hindered by cross-talk and bleed-through between two fluorescent proteins due to the narrow Strokes shift. By conjugating the energy acceptor groups (organic fluorophore or quencher (Chang et al., 2005; Kim et al., 2008b; Medintz et al., 2006; Shi et al., 2006)) with a QD through a peptide sequence, this problem can be overcome. A recent study by Shi *et al.* has shown a ratiometric QD-FRET probe to measure protease activity *in vivo*, consisting of the donor QD and the acceptor rhodamine labeled peptide (Rosenzweig et al., 2007; Shi et al., 2006). Local excitation of the QDs is able to induce efficient energy transfer into the adjacent rhodamine dye. Approximately 48 numbers of a rhodamine dye labeled substrate (RGDC) for collagenase were conjugated on a single QD through a sulfhydryl group of cysteine residue. When the probes were first tested for trypsin (500 μg/mL for 15 min) in solution, they gave rise to 60% increase in the photoluminescence of the QDs and a corresponding decrease in the emission of the rhodamine molecules, based on FRET signal changes (Rosenzweig et al., 2007). Similarly, upon the cleavage by collagenase, fluorescence from QD at 545 nm was recovered up to 60% while that of rhodamine at 560 nm were diminished. Thus, ratiometric change in fluorescence emissions of QD and rhodamine allowed for the real-time detection and quantification of collagenase activity. The activity of collagenase, matrix metalloproteinases (MMPs) was monitored in normal (HTB 125) and cancerous breast cells (HTB 126) in which collagenase is negligible. This result clearly showed that QD-dye FRET sensors could be very useful to detect protease activity by measuring the ratiometric change. Mattoussi group has reported the similar approach to measure the activities of different proteases by tuning the appropriate peptide substrate (Medintz et al., 2006). Modular peptides were rationally designed to have four different parts; i) an N-terminal hexahistidine(His$_6$) domain for self-assembly with QD, (ii) a helix-linker spacer region, (iii) an exposed protease recognition/cleavage sequence, and (iv) a C-terminal site-specific location (cysteine thiol) for dye attachment. In particular, the artificial

residue alpha-amino isobutyric acid (Aib) and alanine were incorporated into the helix linker to provide rigidity, and an organic fluorophore or a quencher was attached to the C-term via thiol chemistry. For the detection of caspase-1 activity, dihydrolipoic acid (DHLA)-capped QD538 and the Cy3 was employed as the FRET pair, and QD-FRET probes for three other proteases (thrombin, collagenase and chymotrypsin) were also designed to have the quencher (QXL-520) instead of Cy3. They described that the relative FRET efficiency within each QD nano-assembly can be controlled through the number of peptides assembled per QD. Therefore, these assays provide quantitative data for protolytic activity by measuring enzymatic velocity, Michaelis–Menten kinetic parameters, and mechanisms of enzymatic inhibition. Unlike the conventional protease assay format in which substrate concentration has to be used in the micromolar range, they demonstrated that the developed format can be consistently used with lower substrate concentrations (200nM QD and peptide concentration of 0.2–1.0 µM) over a diverse selection of enzymes that manifest different specific activities. Moreover, it is likely that the substrates incorporated on the QD complex allow easy access to the desired protease and provide the high sensitivity and low background by being easily tuned. More recently, they utilized this system to detect caspase-3 activity, a key downstream effector of apoptosis. Along with that, the presence of calcium ions with the acceptor CaRbCl can increase the FRET efficienty, enabling calcium-sensitizing sensors (Medintz et al., 2010). Also they have reported this QD-dye FRET system to serve as a papain or proteinase K sensor, based on tunable coupler, 520QD and Cy3 (Clapp et al., 2008). In a similar way, Biswas *et al.* has showed that a genetically programmable protein module was designed to have a His$_6$, a cleavage site labeled with a Alexa dye via cysteine residue, an elastin-like peptide (ELP) domain for purification, and a flanking TAT peptide (Chen et al., 2011). This QD-dye FRET module was used for the detection of HIV-1 Pr activity *in vitro* and in cancer cells, which particularly takes responsibility for drug-resistance against rapidly mutating viruses such as HIV-1. Analysis of enzymatic inhibition was also performed in the presence of specific inhibitors. QD-FRET assay system to measure protease activity has been applied to chip-based format by Kim *et al* (Kim et al., 2007). While the photoluminescence (PL) of donor streptavidin-QD525 immobilized on a surface was quenched due to the presence of an energy acceptor (peptide substrates modified with TAMRA and biotin at N- and C-terminus, respectively) in close proximity, the protease activity caused modulation in the efficiency of the energy transfer between the acceptor and donor, thus enabling the highly sensitive detection of MMP-7 activity. In contrast to a solution-based analysis, the chip-based format allowed more reliable analysis, with no aggregation of QDs. Plus, this format required a much smaller reaction volume. This method is likely to have a potential to screen the activity of disease-associated proteases for the development of therapeutics and diagnostics in a high-throughput manner. In addition to organic fluorophores, a fluorescent protein was easily designed as an energy acceptor against QD donor. Boeneman *et al* (Medintz et al., 2009) has demonstrated that a red fluorescent protein (mCherry) expressing the caspase 3 cleavage site and a His$_6$ sequence were self-assembled to the surface of CdSe–ZnS DHLA QDs via metal affinity coordination, leading to FRET quenching of the QD and sensitized emission from the mCherry acceptor. Caspase-3 activity caused the FRET efficiency to be reduced. Owing to the favorable spectral overlap (Förster distance R_0=4.9 nm) between QD550 and mCherry, considerable loss in QD PL was observed along with an increase in sensitized mCherry emission. A FRET efficiency of approximately 50% was measured when the

number of mCherry per a single QD was six. Caspase 3-induced changes in FRET efficiency were comparable to those observed in fluorescent protein sensors. However, compared to two fluorescent proteins, some advantages of QD-fluoresecent protein encompass 5–10 times less substrate and ~3 orders of magnitude less enzyme in terms of quantity to be used. As a result, they were able to detect enzymatic activity for caspase 3 concentrations as low as 20 pM. This capability seems to be mainly due to multivalent effect of QDs.

In order to construct QD-FRET probe, quenching groups (organic quenchers or metal nanoparticles) (Chang et al., 2005; Kim et al., 2008b; Medintz et al., 2006; Shi et al., 2006) can be bound to the surface of a QD through a peptide sequence. Unlike QD-dye FRET system based on the ratiometry of dual emission, this close proximity causes only quenching of the QD emission via the resonance energy transfer, while subsequent cleavage of the peptide sequence by the corresponding protease led to a recovery of the QD fluorescence. The quenching ability of the gold nanoparticle (AuNP) has been known to be much higher than that of organic quenchers as described elsewhere (Oh et al., 2006). As such, the use of AuNPs as energy acceptors enables the energy transfer to be valid even in the excess distance of the traditional FRET. One of the feasible mechanisms might be associated with the property of metal surface; it was reported that the metal surface extended the effective energy transfer distance up to 22 nm, resulting in a high energy transfer efficiency (Dulkeith et al., 2002; Jennings et al., 2006a; Jennings et al., 2006b; Pons et al., 2007; Yun et al., 2005). The initial report of protease detection with a QD-AuNP system was made by Chang *et al* (Chang et al., 2005). A peptide substrate, GGLGPAGGCG, was employed to measure the activity of collagenase. The N-terminal amines of the peptides were coupled to the carboxylic acids on the QDs by EDC, and the cysteine was conjugated to the maleimide functionalized gold nanoparticles. When the AuNP level was six per QD, a quenching efficiency of 71% was observed. To be expected, the excess peptides on the QD possibly decrease the probe sensitivity to protease hydrolysis even if they cause the quenching efficiency to increase. By the release of the gold quencher in the presence of collagenase (0.2 mg mL^{-1}), QD fluorescence was recovered up to 51%, meaning that a number of enzyme molecules are not enough to be fully accessible between two large nanoparticles.

It is demonstrated by Suzuki et al (Suzuki et al., 2008a) that QD-based nanoprobes were used to detect multiple cellular signaling events including the activities of protease (trypsin), deoxyribonuclease, DNA polymerase, as well as the change in pH. This system was designed based on the FRET between the QD as donor and an appended fluorophore as acceptor; protease and deoxyribonuclease (DNase) induces the change in FRET efficiency between donor (QD) and acceptor (GFP or fluorophore-modified double-stranded DNA), whereas DNA polymerase action leads to the close proximity of fluorescently labeled nucleotides to the surface of the QD, and pH-sensitive fluorophore conjugated on the surface of QD produces changes in FRET efficiency by pH change (**Figure 3**). This mixture of modified QDs showed distinct changes in emission peaks before and after enzyme treatment by simultaneous-wavelength excitation in the same tube.

A multiplexed system to detect the activity and inhibitory effects of several proteases (MMPs, thrombin, and caspase-3) has been proposed by Kim *et al*, which is utilized by the principle of energy transfer between the AuNP and respective QDs on a glass slide (**Figure 4**) (Kim et al., 2008a). For construction of nanoprobes, the AuNP acceptors conjugated with a peptide substrate including cysteine and biotin were associated with streptavidin (SA)-

conjugated QDs (SA-QDs, energy donor) deposited on a glass slide, thus quenching the PL of the QD by the energy transfer. Upon addition of a protease to cleave the peptide substrate
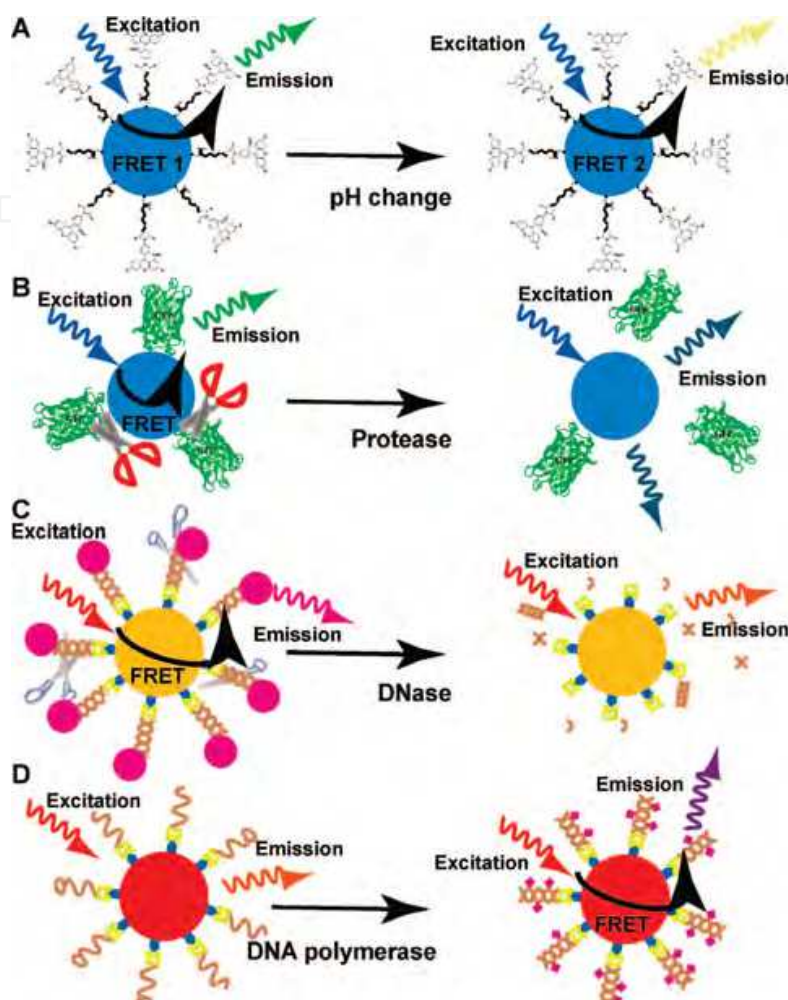

Fig. 3. FRET-based QD bioprobes designed to give FRET changes on (from top to bottom): (A) pH change via pH-sensing dyes attached to a QD; (B) cleavage of a GFP variant with an inserted sequence recognized by a protease (e.g., trypsin) to release GFP from the QD surface; (C) digestion by DNase of dsDNA (labeled with fluorescent dUTP) bound to a QD; (D) incorporation of fluorescently labeled dUTPs into ssDNA on a QD by extension with DNA polymerase.

on the AuNP–QD conjugates, there was a significant regeneration of the photoluminescence emission of the QDs. Protease inhibitors also prevented any recovery of the photoluminescence of QDs by inhibiting the protease activity. When three types of SA-QD (SA-QD525, SAQD605, and SA-QD655) were independently complexed with the AuNPs with different peptide substrates, a specific reaction of the protease induced strong photoluminescence intensity from each spot, at a specified wavelength. Marginal cross-reactional images of the protease against other peptide substrates were observed, thus confirming the multiplexed capability of this assay system (**Figure 4A**). Since the AuNPs can be employed as a common energy acceptor, a variety of QDs with different colors could be used as the energy donor (**Figure 4B and 4C**), thus enabling a multiplexed assay. Moreover, high quenching efficiency of AuNPs allows application of the assay system to an extended

separation distance between a donor and an acceptor. This developed system also overcome some drawbacks resulting from a solution-based format, including the aggregation of nanoparticles and the fluctuation in photoluminescence, and the consumption of large amounts of reagents. This multiplexed assay using a QD-FRET nanosensor has also been applied to different types of enzymes other than proteases. Suzuki *et al* (Suzuki et al., 2008b). designed QD-based nanoprobes on a FRET with QD as donor and an appended fluorophore as acceptor in order to detect multiple cellular signaling events, including the activities of protease (trypsin), deoxyribonuclease, and DNA polymerase, as well as changes in the pH. Notably, this mixture of modified QDs showed distinct changes in emission peaks before and after enzyme treatment by simultaneous-wavelength excitation in the same tube.
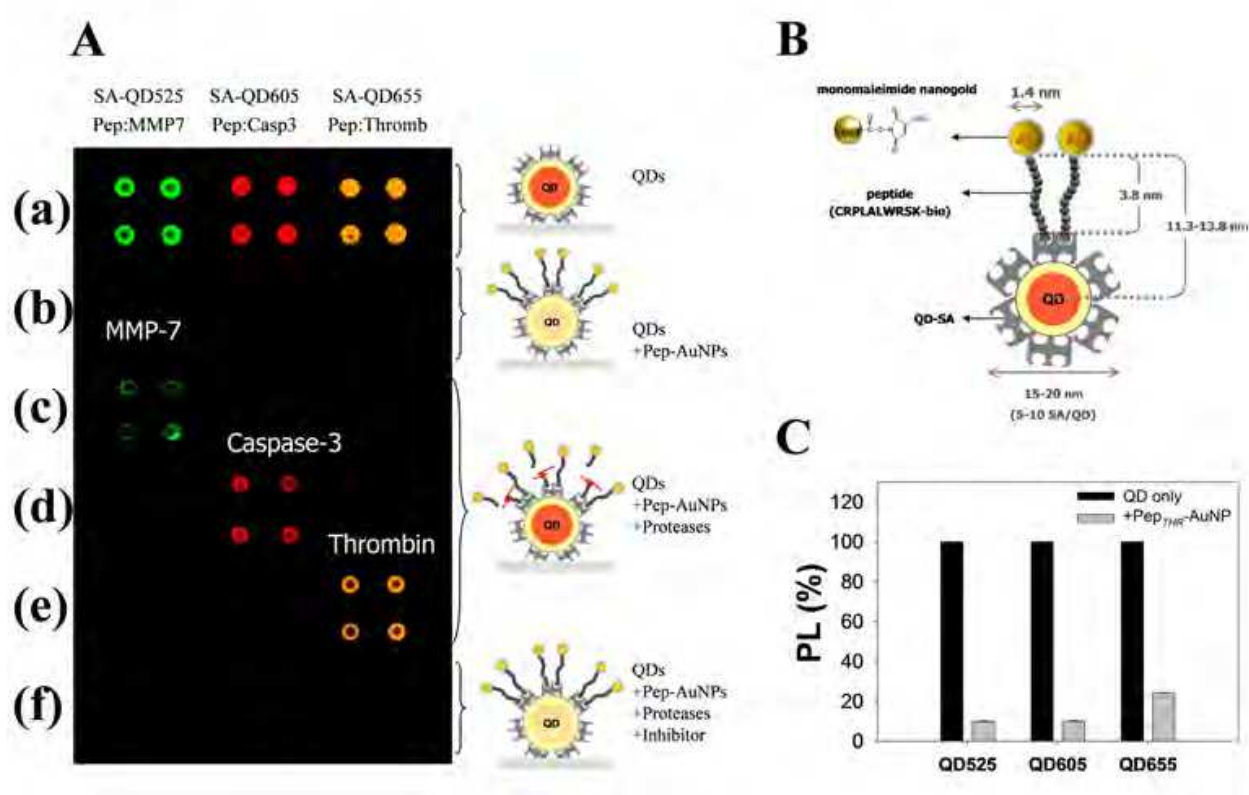

Fig. 4. Development of multiplexed system to detect protease activity with QDs. (A) Multiplexed assay of proteases by using QDs with different colors. SA-QD525, SA-QD605, and SA-QD655 were used (from left to right). Biotinylated peptide substrates for MMP-7, caspase-3, and thrombin were conjugated to the AuNPs, and then the resulting Pep-AuNPs were associated with SA-QD525, SA-QD605, and SA-QD655, respectively. (a) SA-QDs only. (b) SA-QDs + respective Pep-AuNPs. (c) SA-QDs + Pep-AuNPs + MMP-7. (d) SA-QDs + Pep-AuNPs + caspase-3, (e) SA-QDs + Pep-AuNPs + thrombin, (f) QDs + Pep-AuNPs + mixture of the respective protease and its inhibitor. (B) Configuration of QD-AuNP nanosensor to detect the activity of MMP-7 (SA: streptavidin). (C) Changes in the : photoluminescence (PL) intensities of QDs having different colors (SA-QD525, SA-QD605, and SA-QD655) in the presences of biotinylated Pep$_{THR}$-AuNPs in solution. The molar ratios of Pep-AuNPs to respective QDs were equally maintained at 50.

3. Bioluminescence resonance energy transfer (BRET) system using QDs

In FRET, QDs are not able to function as effective acceptors (Nabiev et al., 2004) because a direct excitation of donor fluorophores has to accompany by unavoidable excitation of the QD with a broad absorption. However, when QDs can serve as an energy acceptor with a light-emitting protein, luciferase, this problem can be overcome; since the bioluminescence energy of a luciferase-catalyzed reaction occurs only in its active site and cannot excite the acceptor QD, it can be successfully transferred to the QDs to produce quantum dot light emission. Upon addition of the luciferase substrate (coelenterazine) to QD-luciferase conjugate, a short blue light emission at 475 nm is transferred the QDs. Main advantage of QD-luciferase system is to eliminate the need for excitation light which causes inevitable background autofluorescence. In the case of QD-FRET that has been widely employed as activity-based probes, the excited illumination can partly increase the background noise level especially in serum sample, thus diminishing the value of acquired information as a result of the false-positive signal. Moreover, a common luciferase protein serves as the BRET donor for several QDs with different colors because QDs have similar absorption spectra and absorb blue light efficiently. Especially in contrast to QD-FRET system where the FRET efficiency improves as the number of FRET acceptors per QD increases, the BRET ratio with varying numbers of luciferase on the surface of single QD were quite similar although the intensity of both luciferase and QD emissions varied by approximately100-fold. This means that the QD-BRET system is more dependent on the donor-to-acceptor distance rather than varying donor number, thus being applied to detect the distance-dependent assay with high fidelity. Rao group has initially demonstrated the feasibility of using QDs as the acceptor in a bioluminescence resonance energy transfer (BRET) system (So et al., 2006). This conjugate was known as "self-illuminating quantum dots." in the sense that no external illumination light source is needed for the QDs to fluoresce (**Figure 5**). The self-illuminating QDs are advantageous over conventional QDs for the *in vivo* animal imaging purposes, due to extremely high sensitivity (an *in vivo* signal-to-background ratio of >1000 for 5 pmole of conjugates subcutaneously injected) and capability to look into deep tissue without considering external light delivery.

This QD-BRET based probe can be applied to the detection of protease activity. Yao *et al.* has focused on the detection of the activity of MMPs that is a promising cancer biomarker enzyme. Since the secretion level of MMPs in human serum is of very interest as a promising prognostic marker (Lein et al., 1997; Nikkola et al., 2005) due to the up-regulation of MMPs in almost all human cancerous cells or in malignant tissues, the designed probe to measure MMP activity and related inhibitory effect is valuable for discovering drug candidates for anticancer therapeutics (Coussens et al., 2002; Denis and Verweij, 1997; Hidalgo and Eckhardt, 2001). For the construction of QD-BRET energy donor, a bioluminescent protein fused to the MMP-2 substrate (GGPLGVR) and a hexahistidine tag at its C terminus was genetically expressed in *E. coli*. The bioluminescent protein is a *Renilla reniformis* luciferase mutant dubbed Luc8, which has eight mutations and shows higher stability and improved catalytic efficiency than the wild-type luciferase (Loening et al., 2006). Since the simultaneous coordination is generated between carboxyl groups on the QD surface and the Luc8 His tag in the presence of Ni^{2+}, a strong BRET signal was observed just immediately after luciferase-GGPLGVRGGH$_6$ is mixed with carboxyl QD655 and Ni^{2+}. Upon cleavage of the flanking peptide region by MMP-2, the His tag was released from the fusion Luc8 and the BRET signal decreased. In comparison to FRET-based QD sensors,
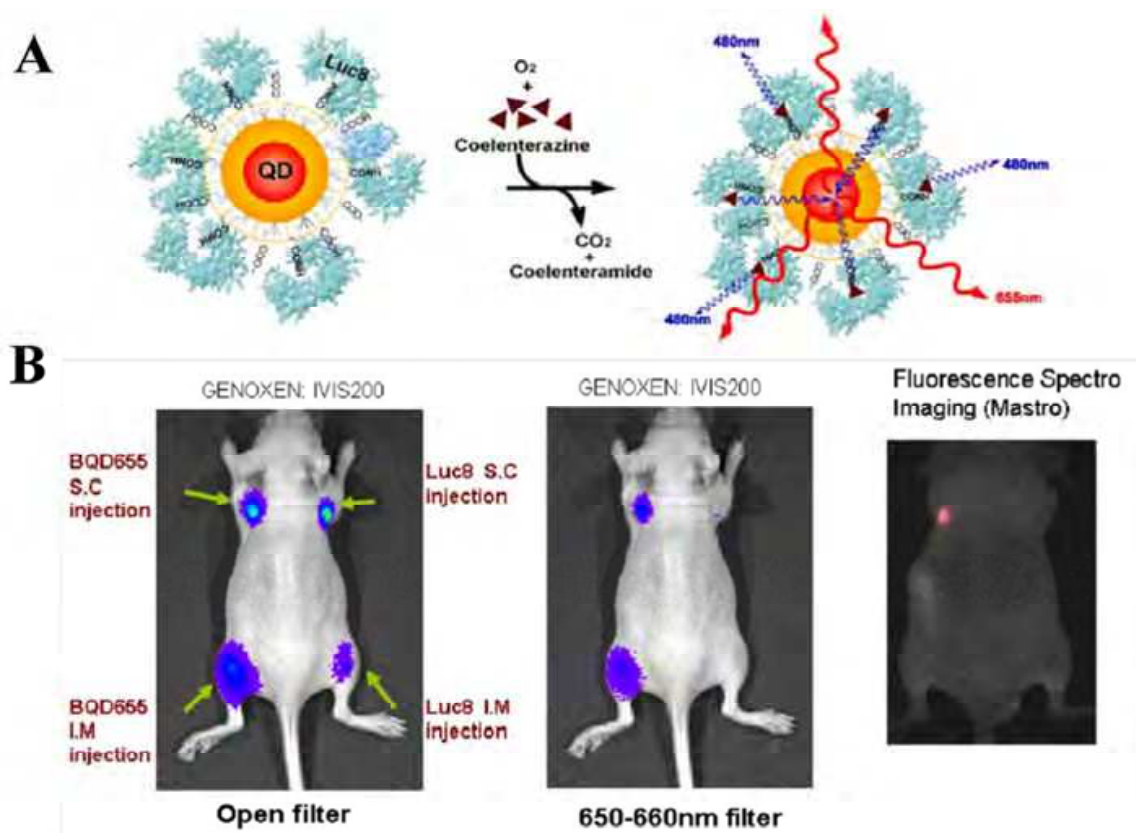
Fig. 5. QD-BRET based in vivo animal imaging. (A) Schematic showing bioluminescence resonance energy transfer between *Renilla* luciferase mutant, Luc8 protein (donor) and QD (acceptor) The bioluminescence energy of Luc8-catalyzed oxidation of coelenterazine is transferred to QDs in close proximity, resulting in the QD emission (655 nm); (B) In vivo imaging using QD-BRET conjugates. Luc8-QD bioconjugates were injected into nude mice either intramuscularly or subcutaneously and imaged bioluminescently (Xenogen, open filter (left image) and QD filter (middle image)) or fluorescently (Mastro, right image, QD signals shown in bright white). Luc8 alone were also injected into the same animal for comparison.

BRET based QD biosensors have several attractive features. Most significantly, the large spectral separation between the BRET donor and acceptor emissions makes it easy to detect both emissions. In this case, a ratiometric measurement is far more accurate and reliable than that of QD-FRET because the integrated intensities from two separable spectra was favorable to reflect the energy-transferred efficiency (Rao et al., 2006a). In addition, considering that the BRET ratio for a concentration of MMP-2 of 2 ng mL^{-1} (~30 pM) decreased by about 10%, and by 15% for a concentration of MMP-2 of 5 ng mL^{-1} (~75 pM), the sensitivity is high due to a low background emission. By taking advantage of effective ratiometry where the spectral separation between the BRET donor and acceptor emissions is large, a QD-BRET nanosensor with a stable covalent linkage has been developed that allows the detection of protease activity in mouse sera and tumor lysates(Xia et al., 2008). MMP-2, MMP-7, and urokinase-type plasminogen activator (uPA). Most importantly, the nanosensors were not only capable of detecting these proteases in complex biological media (e.g., mouse serum and tumor lysates) with sensitivity down to 1 ng mL^{-1}, but could also detect multiple proteases present in one sample. To accomplish the site-specific conjugation,

the carboxylated QDs had been functionalized with adipic dihydrazide and subsequently treated with the luciferase–protease substrate recombinant protein with an additional intein segment. Since hydrazides are excellent nucleophiles to attack the thioester intermediate of inteins that are natural protein ligation mediators. The reaction proceeded rapidly when the two components were mixed together, and resulted in cleavage of the intein and ligation of the C terminus of the recombinant protein to the QDs.

It has been demonstrated that luciferase enzyme can be directly used as the template to generate new near-infrared (NIR) QD-BRET nanosensor via a biominealization process (Ma et al., 2010). The synthesis was accomplished by incubating luciferase with lead acetate (Pb(Ac)$_2$) at ambient conditions to allow the binding of Pb^{2+} to Luc8 and facilitate the heterogeneous nucleation. Then a sodium sulfide (Na$_2$S) was added into the Luc8-Pb^{2+} to produce a Luc-PbS hybrid nanostructure with a mean hydrodynamic diameter of 19.9 nm. Despite the very low quantum yield (~3.6%) of the Luc-PbS complex, the BRET signal was comparable to other NIR QDs. The small size of the complex is expected to be more advantageous for *in vivo* imaging and other sensing applications than that (30–40 nm in diameter) of the conjugates between luciferase and as-prepared QDs. However, the long-term stability in luminescence intensity, which might be due to the QD surface oxidation, should be overcome for more practical applications of this protein-nanomateral hybrid.

4. Chemiluminescence resonance energy transfer (CRET) system using QDs

Similar to bioluminescence, chemiluminescence offers many advantages such as high detection sensitivity, a wide linear range for quantification, and no requirement for a light source. Typically, chemiluminescence can be produced when luminol is mixed with oxidizing agents (e.g. hydrogen peroxide) in an alkaline solution in the presence of metal catalysts (iron or copper), leading to a strong blue emission at around 425 nm. A horseradish peroxidase enzyme (HRP) has been also used for catalyzing the oxidation of luminol in the presence of hydrogen peroxide via several intermediates, and the light emitted can be enhanced up to 1000-fold with enhancers such as *p*-iodophenol. This process is known to be the enhanced chemiluminescence. Based on this principle, nonradiative energy transfer can be accomplished between a chemiluminescent (CL) donor and a fluorophore acceptor (or QD), which is called chemiluminescence RET (CRET).

Ren and co-workers investigated the chemiluminescence resonance energy transfer (CRET) by using luminol–H$_2$O$_2$ system as energy donor and HRP-conjugated CdTe QD as acceptor (Huang et al., 2006). By the CL generation among the luminol, H$_2$O$_2$, and HRP, QD could absorb part of the excited-state luminol energy and re-emit it at longer wavelengths without external light source (**Figure 6**). A comparison of CL spectra between HRP–QDs conjugates and a mixture of HRP and QDs showed that the CL intensity ratio of QDs:luminol from the mixture of QDs and HRP was very low, which was attributed to the low adsorption of QDs onto HRP and to the resultant longer distance. The multiplexed CRET was able to be realized similar to the multiplexed BRET and a proof-of-concept experiment for multiplexed immunoassay was also explored using different QD-antibody-HRR complex. Although the QD-CRET efficiency is dependent on several factors, it is worth noting that the quantum yield of the acceptor QD in different status is the crucial factor to the CRET efficiency; that is, high quantum yield of QDs brings in a high CRET efficiency (Wang et al., 2008).

To avoid the requirement of labeling the energy acceptor with HRP and H_2O_2, which may limit the application of this CRET system, enzyme-free QD-CRET system was developed using a new oxidizing reagent, NaBrO (Zhao et al., 2010). The CRET ratio calculated by dividing the acceptor emission by the donor emission was comparable to that observed from the luminol–H_2O_2–HRP–conjugated QD system (~30%). A feasible explanation for this mechanism remains unclear, but an intermediate complex might be formed between BrO- anions and CdTe QDs, which bring the energy donor (luminol molecules) and the acceptor (QDs) very close to each other at the time of oxidation to produce chemiluminescence emission. Main advantage of this system is that highly stable QD-CRET sensor can be achieved because QD emission in HRP-based QD-CRET system is critically affected by free H_2O_2, leading to the instability of QD sensor. Therefore, this new luminol–NaBrO–QD CRET system is expected to have a great potential for simultaneous prognosis and diagnosis.
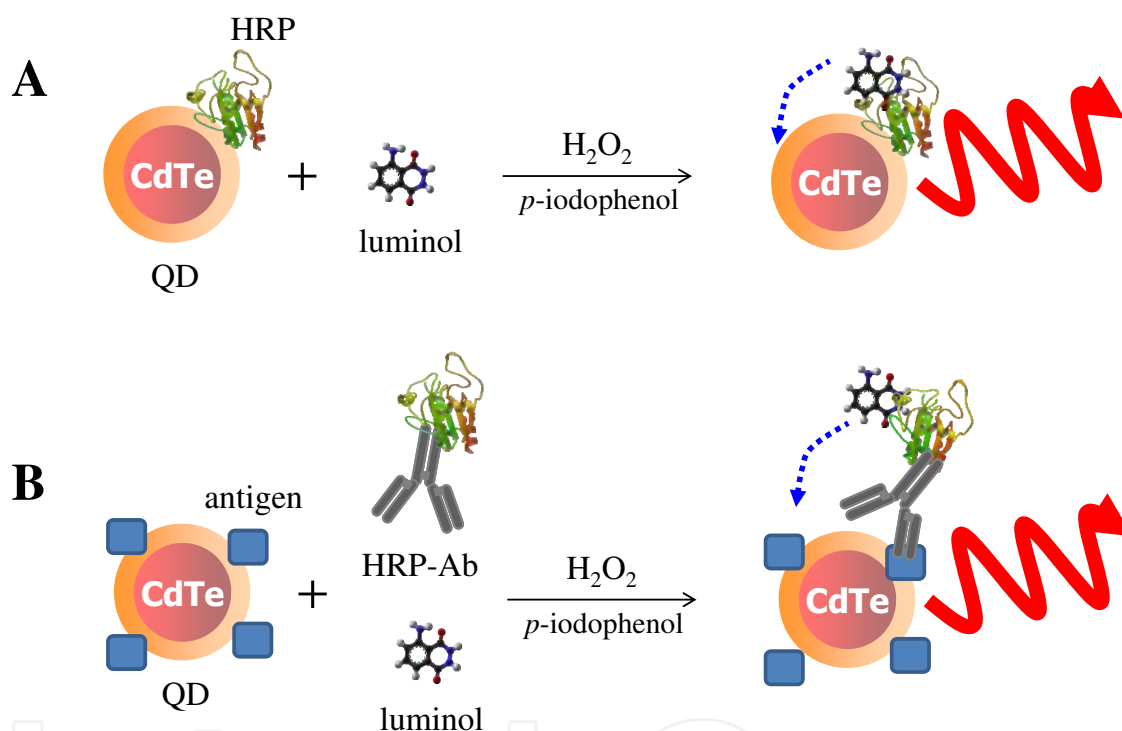

Fig. 6. (A) Schematic of QD-CRET based on luminol donors and HRP-labeled CdTe QD accepters, (B) Schematic of QD-CRET for luminol donors and QD accepters based on the immuno-reaction of QD–antigen and antibody–HRP.

5. Conclusion

The intrinsic properties of QDs have encouraged scientists to further develop this material for many biological applications. One of the most emerging uses of QDs lies in QD-based energy transfer systems, primarily consisting of QD-FRET, QD-BRET and QD-CRET. Many imaging and biosensing applications of QD-RET are now becoming very popular as a powerful tool for dissecting complex biomolecular detection and trafficking. Moreover, the ability to create different colors with QDs will certainly allow for multiplexed analysis both *in vitro* and *in vivo* applications. Given the rapid growth in new QD based materials, the improved interdisciplinary achievement will certainly be bright, but a lot of techniques

about QDs are still required to be improved with the development of new QDs in the near future as follows; (i) introduction of multiple functional groups to a single QDs for multimodal monitoring, (ii) improved properties of QD: reduced size and removing blinking phenomenon, (iii) need of toxicity and kinetics study in human before extensive application for clinical diagnosis and therapy, and (iv) development of near infrared QDs for in *vivo* imaging.

6. Acknowledgement

7. References

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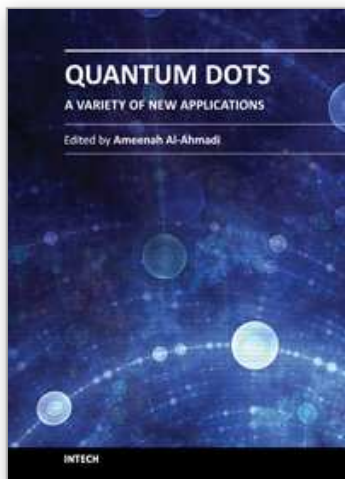
Quantum Dots - A Variety of New Applications

Edited by Dr. Ameenah Al-Ahmadi

The book "Quantum dots: A variety of a new applications" provides some collections of practical applications of quantum dots. This book is divided into four sections. In section 1 a review of the thermo-optical characterization of CdSe/ZnS core-shell nanocrystal solutions was performed. The Thermal Lens (TL) technique was used, and the thermal self-phase Modulation (TSPM) technique was adopted as the simplest alternative method. Section 2 includes five chapters where novel optical and lasing application are discussed. In section 3 four examples of quantum dot system for different applications in electronics are given. Section 4 provides three examples of using quantum dot system for biological applications. This is a collaborative book sharing and providing fundamental research such as the one conducted in Physics, Chemistry, Biology, Material Science, Medicine with a base text that could serve as a reference in research by presenting up-to-date research work on the field of quantum dot systems.

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