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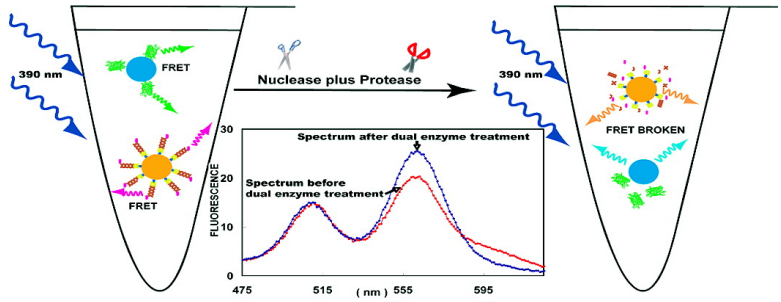

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Quantum Dot FRET Biosensors that Respond to pH, to Proteolytic or Nucleolytic Cleavage, to DNA Synthesis, or to a Multiplexing Combination

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Abstract: Fluorescent acceptors have been immobilized on nanoparticulate quantum dots (QDs), which serve in turn as their FRET donors. The broad excitation and narrow emission bands of QDs mark them as having excellent potential as donors for FRET and, in principle, differently colored QDs could be excited simultaneously. The present work describes the preparation and operation of FRET-based QD bioprobes individually able to detect the actions of protease, deoxyribonuclease, DNA polymerase, or changes in pH. In addition, two such QD-mounted biosensors were excited at a single wavelength, and shown to operate simultaneously and independently of each other in the same sample solution, allowing multiplex detection of the action of a protease, trypsin, in the presence of deoxyribonuclease.

1. Introduction

Quantum dots (QD), fluorescent colloidal semiconductor nanoparticles, have been developed to provide materials compatible in size with biomolecules for use as fluorescent biosensors as they are reasonably resistant to photobleaching, to denaturants of biomolecules, and to alterations in pH and temperature.[1–3] Proteins, nucleic acids and other biomolecules have been immobilized on QDs through combinations of organic fluorescent dyes or dye-labeled biopolymers, to enable detection of specific analytes (for example, antibody–antigen interaction, biotin–avidin biochemistry, maltose-binding protein (MBP) modifications with maltose derivatives, oligonucleotide-protein interaction and nucleotide hybridization).[4–15] FRET-based sensing has been used to probe many biological phenomena, including structural or binding changes and enzymatic reactions.[16–18] However, FRET pairs have rarely been organized to multiplex and respond to phenomena occurring for ensembles, or for a series of events such as on a signaling pathway. Although QDs have been applied to labeling specific proteins to reveal their localization or amount, there are few FRET-based QD biosensors for cellular events.[14] There is a report of a pH-sensitive QD prepared by conjugating a squaraine dye to a CdSe/ZnS nanocrystal.[19] QDs have also been described that show

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quenching of their fluorescence by an attached organic dye[6] or gold nanoparticles,[17,20] chemically linked through a protease-digestible peptide, with the degree of quenching changing on proteolysis (quantum dot multiplexing).

To probe signal transduction in a living cell, a number of FRET pairs will have to be used at once, each monitoring a different enzymatic or binding event. Using several FRET pairs would require complicated equipment for discrimination of respective emission profiles, appropriate excitation in terms of filters, lasers, mirrors etc. and also calculations to calibrate cross-excitation. However, FRET pairs composed of QDs would be expected to partly overcome such problems, as the ability to carry out simultaneous excitations for different colors of QD using a single excitation wavelength implies that QDs could be excellent donors for FRET-based molecules in multiplexing combinations for sensing biological phenomena. While there is a simple labeling case with different QD−antibody complexes on biomolecules *in vitro*,[7–9,21] we are not aware of signaling involving more than one biological process in mixtures of QD biosensors.

We recently established chimeric FRET-based biosensors based on conjugation of Green Fluorescent Protein (GFP) to fluorescent organic dyes[22] via a cleavable link, and showed their use to detect glutathione, borohydride ion[23] or specific protease actions both *in vitro* for trypsin[24] and in real time during apoptosis for caspase 3 in different cellular compartments *in vivo*.[25] FRET performance depended on the location of the dye on the GFP surface, permitting fine-tuning of the orientation and distance between the GFP chromophore and its appended chemical FRET partner.

With a need for multiplexing biosensors that may become applicable to intracellular signaling problems such as signaling cascades, we now describe FRET systems with QD conjugates for distinctive targets, but which can function in a harmonized manner in multiplex situations. These were designed based on chimeric fluorescent QD bioconjugates operating by FRET between the QD as donor and an appended fluorophore as acceptor. Figure 1 shows a set of QD-based molecules designed to give FRET changes on cleavage (by protease or nuclease), on pH change, or on chain growth as a result of polymerase action. Their purpose is to demonstrate proof of principle of this concept and its potential for multiplexing. In the cases of protease and deoxyribonuclease (DNase), FRET would be destroyed on cleavage by the respective enzyme, as donor (QD) and acceptor (GFP or fluorophore-modified double-stranded DNA) become separated. For the case of DNA polymerase action, donor–acceptor interaction would occur as fluorescently labeled nucleotides become incorporated to extend the double-stranded DNA (dsDNA) attached to the surface of the QD. For the pH-switch construct, pH change would cause alteration of the fluorescent profile of the dye, through energy transfer from the QD to produce changes in FRET efficiency. We will also describe how more than one of these signaling systems can be used simultaneously in the same solution with a single excitation wavelength, i.e. multiplexing.

2. Experimental Procedures

2.1. Preparation of Functionalized QD Conjugates. 2.1.1. ds-DNA Immobilization onto QD and Examination of FRET Efficiencies. dsDNA was amplified by PCR with primer sets of 5′-biotinylated ACTCCAATTGGCGATGGCCCTG-3′ and unlabeled 5′-AGAACCACTCCCAGCAGCAGTTACAAACTC-3′ (0.5 μM each) and pUV5casS22tag[6] as template supplied with 0.2 mM of dATP, dCTP, and dGTP, 0.15 mM of dTTP, 0.045 mM of Alexa-labeled dUTP (AlexaFluor532-5-dUTP, Invitrogen Corporation) and 0.2 unit DNA polymerase (KOD Dash, TOYOBO Life Science) and purified using a QIAquick PCR Purification Kit (Qiagen). The PCR products (0.5, 2.5, and 10 μL) were immobilized on 0.2 μM QD (Qdots 525 ITK™ Streptavidin Conjugate, Invitrogen Corporation) by combining in the buffer provided by the supplier for dilution of the QD at 37 °C for 3 h: these products are indicated as ratios of DNA to QD, respectively, of 2, 0.4, 0.1, and ∞, the last indicating QD not modified with DNA, see Figure 2A) The QD−conjugate was isolated using a NICK column (GE Healthcare) equilibrated with phosphate-buffered saline (PBS) to eliminate free PCR products following dilution with PBS (400 μL). FRET spectra between the QD (excited at 330 nm) and labeled dsDNA were then determined.

2.1.2. DNase Sensor Type. dsDNA was amplified with the same primer sets, template and other components but using 5 times the quantity of KOD Dash with 0.2 mM of dATP, dCTP and dGTP, 0.15 mM of dTTP, 0.045 mM of Alexa-labeled dUTP (AlexaFluor532–5-dUTP or AlexaFluor568–5-dUTP, Invitrogen Corporation) and 1 unit DNA polymerase (KOD Dash, TOYOBO Life Science). The PCR products, purified with QIAquick PCR Purification Kit (Qiagen), were immobilized on 2.5 μL of 0.2 μM QD (Qdots 525 and 565 ITK™ Streptavidin Conjugate, Invitrogen Corporation), corresponding to 0.5 μL of 0.2 μM QD as described in the Immobilization Procedure above by combining at 4 °C overnight in the buffer provided by the supplier for dilution of QD. The QD conjugate was isolated using a NICK column to eliminate free PCR products in the case of Qdots 525, and a Microcon YM-50 membrane in the case of Qdots 565.

2.1.3. DNA Polymerization Sensor Type. Primer-hybridized ssDNA for fixation on QD (Qdots 525 ITK Streptavidin Conjugate, Invitrogen Corporation) was synthesized by asymmetric PCR using the same primers (5′-biotinylated ACTCCAATTGGCGATGGCCCTG-3′ and 5′-AGAACCACTCCCAGCAGCAGTTACAAACTC-3′) and template as described in the previous sections, but using one tenth the amount of unlabeled primer to biotinylated primer (0.05 μM and 0.5 μM of each). After using the purification steps described for dsDNA, the major PCR product, biotinylated ssDNA, was rehybridized with fresh, unlabeled primer by heating at 94 °C for 1 min, cooling on ice quickly and then mixing with QD (3 μL of 0.2 μM) and buffer (11 μL) for the polymerization procedure (10 mM Tris-Cl, 7 mM $MgCl_2$, 0.1 mM dithiothreitol pH 7.5) to bind at room temperature overnight.

2.1.4. pH Sensor Type. Traut's reagent (1 μL of 14 μM, 2-iminothiolane·HCl, Pierce, Biotechnology Inc.) was mixed with QD (20 μL of 20 μM, Amine T2-MP EviTag (Lake-Placid Blue), Evident Technology Inc.) and incubated at room temperature overnight. Unreacted reagent was removed using a NICK column (GE Healthcare) equilibrated with PBS. The sulfhydryl group thus introduced onto the QD was allowed to react with fluorescent pH indicator (5 μL of 2 mg/mL of fluorescein-5-maleimide) at room temperature overnight. Excess fluorescent dye was removed from the mixture using a Microcon YM-10 membrane and the fluoresceinyl-modified QD recovered in PBS (30 μL).

2.1.5. Proteolysis Sensor Type. QD (20 μL of 20 μM, Amine T2-MP EviTag (Lake-Placid Blue), Evident Technology Inc.) was mixed with pH 9.0 sodium carbonate buffer (20 μL of 0.2 M) and

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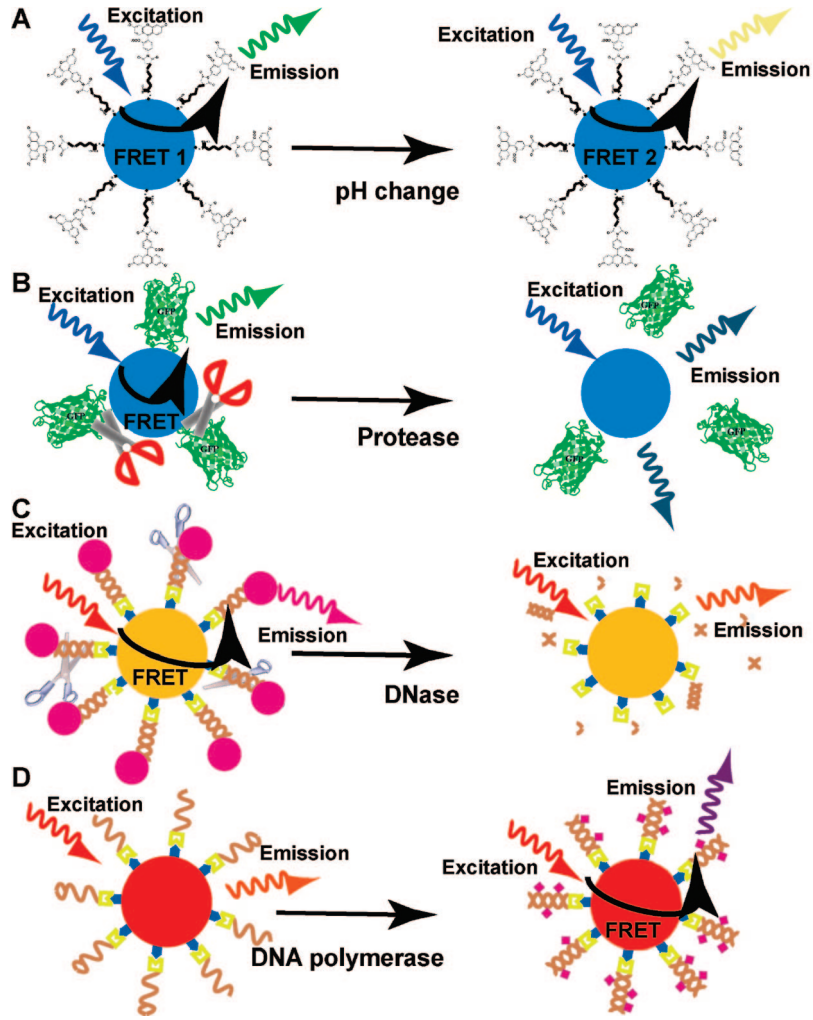

Figure 1. FRET-based QD bioprobes designed to give FRET changes on (from top to bottom): (A) pH change via pH-sensing dyes attached to a QD; (B) cleavage of a GFP variant with an inserted sequence recognized by a protease (e.g., trypsin) to release GFP from the QD surface; (C) digestion by DNase of dsDNA (labeled with fluorescent dUTP) bound to a QD; (D) incorporation of fluorescently labeled dUTPs into ssDNA on a QD by extension with DNA polymerase.

ethylene glycol bis-succinimidylsuccinate (EGS, 0.4 μL of 100 mM in dimethyl sulfoxide, Pierce Biotechnology Inc.) and the solution incubated at room temperature overnight. Excess EGS was removed with a Microcon YM-10 membrane. The recovered QD in pH 9.0 sodium carbonate buffer (25 μL of 0.2 M) was allowed to react with *N*-(5-amino-1-carboxypentyl)iminodiacetic acid at room temperature overnight. The resulting chemically modified QD was separated from unreacted *N*-(5-amino-1-carboxypentyl)iminodiacetic acid using a Microcon YM-10 membrane and reconstituted in PBS (15 μL). UV5trypS1tag GFP mutant[24] (22 μL of 15 μM in PBS) was conjugated to the QD derivative (2 μL) by mixing with 1 mM $NiSO_4$. Unbound GFP mutant was removed using a Microcon YM-50 membrane to obtain UV5trypS1tag immobilized on QD by coordination through its $(His)_6$ amino acid His-tag sequence.

2.2. Biosensing Using QD Biosensors. 2.2.1. DNA Digestion Sensing. The DNase-activated QD−DNA conjugate FRET probe was reconstituted in the buffer supplied for RQ-1 RNase-free DNase (Promega Corporation). DNase (2 units) was added to the QD−DNA conjugate solution (one-eighth of the preparation of PCR products immobilized onto QD as described in the DNase Sensor Type section) to initiate digestion. Reaction was stopped after 1 h incubation at 37 °C by application to a Nick column (GE Healthcare) equilibrated with PBS. As a negative control, the QD−DNA conjugate solution was treated similarly in the absence of DNase. Emission spectra of both reaction mixtures were recorded, with excitation at 370 nm.

2.2.2. DNA Polymerization Sensing. To half of the ligation-based QD-labeled nucleotide FRET probe, prepared above, in the buffer supplied for the Klenow fragment (Takara Bio Ltd.,) was added 0.16 mM dATP, dGTP, dCTP, 0.11 mM dTTP and 0.04 mM of Alexa-labeled dUTP (Alexa Fluor532–5-dUTP, Invitrogen Corporation), and DNA elongation polymerization initiated by adding Klenow fragment (8 units). After 2 h at 37 °C, reaction was stopped by applying mixture onto a NICK column to exchange buffer with PBS for measurement of the emission spectra with excitation at 390 nm, compared to a control with no added Klenow fragment.

2.2.3. pH Variation Sensing. An aliquot of the fluoresceinyl−QD FRET probe (4 μL of the 30 μL recovered, described in the Preparation section) was diluted with phosphate buffer (450 uL) of the required pH, and the fluorescence emission spectra were measured, with excitation at 330 nm.

2.2.4. Proteolysis Sensing. Half of the proteolysis-activated QD−GFP FRET probe preparation described in the Proteolysis Sensor Type section was incubated in the presence or absence of 0.006% trypsin for 90 min at 37 °C, and the fluorescence spectra of the resulting solutions were measured with excitation at 390 nm.

2.2.5. Simultaneous Nucleolysis and Proteolysis Sensing. Dual enzymatic reaction monitoring was carried out by combining aliquots of proteolysis-activated QD−GFP FRET probe (one-third the amount used in the Proteolysis Sensing experiments) and nuclease-activated QD−DNA conjugate FRET probe (0.8 of the

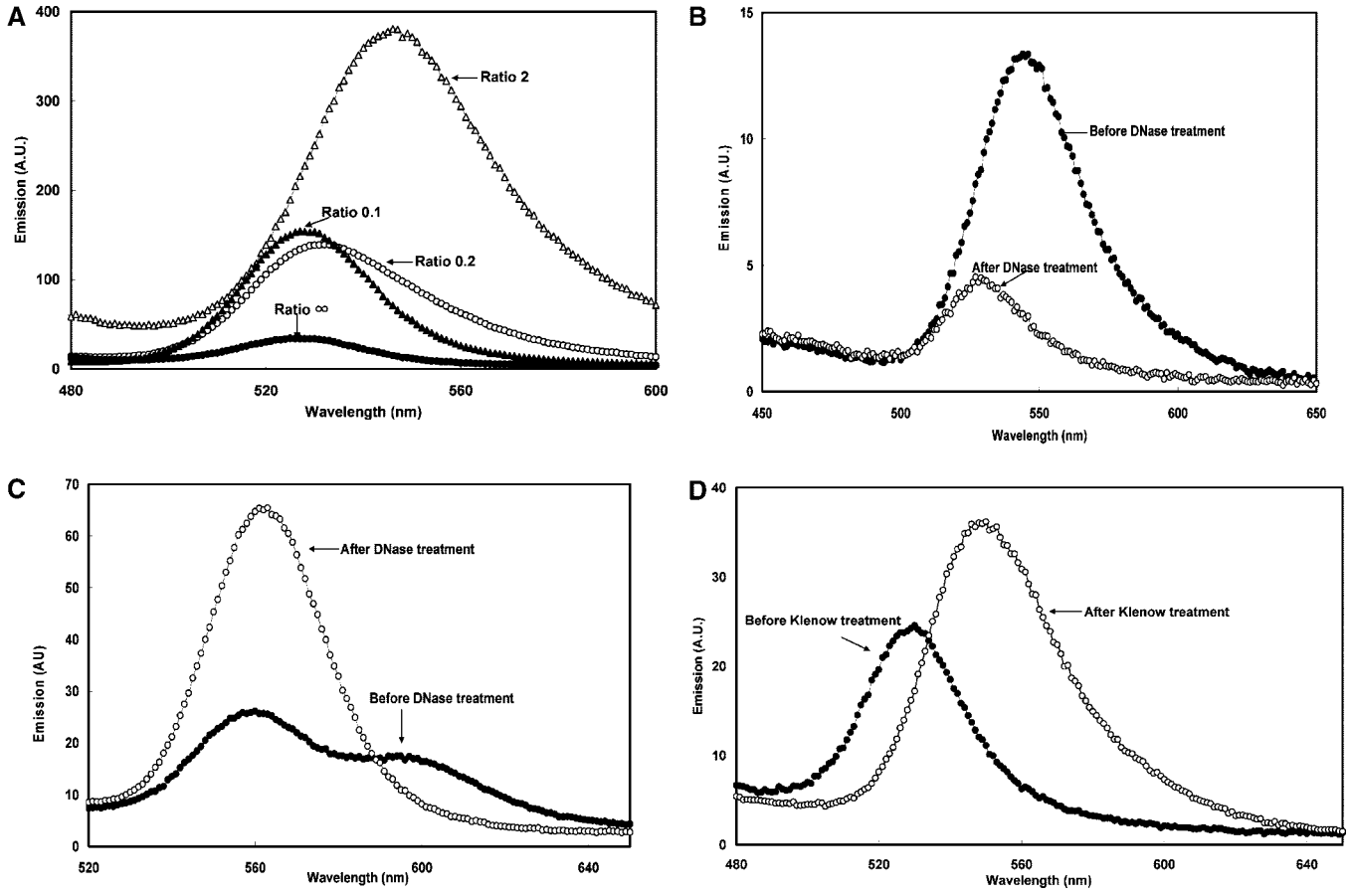

Figure 2. Emission spectral changes of DNA attached to Quantum Dots.

amount used in the DNA Digestion Sensing experiments) in the buffer used for RQ-1 RNase-free DNase. The combination of QD conjugates was exposed for 1.5 h at 37 °C to enzyme mixtures containing: (i) both trypsin (0.006%) and DNase (2 units), (ii) trypsin (0.006%), but no DNase, (iii) DNase (2 units), but no trypsin, and (iv) neither trypsin nor DNase. The fluorescence spectra of the resulting mixtures were measured with excitation at 390 nm.

2.3. Fluorescence–Excitation and Emission Spectra. Fluorescence–excitation and emission spectra were recorded at ambient temperature on a Shimadzu RF-5300 spectrofluorophotometer using 1 mL, 4-sided, quartz cuvettes. In most cases slit widths were set from 3 to 5 nm for excitation and emission spectra, depending on the intensity of emission. All experiments were carried out at least twice and all data reported were reproducible (representative spectra are shown rather than averaging spectral data).

3. Results and Discussion

3.1. Emission Profiles of QD−DNA Conjugate FRET Probes. Since we could only obtain information on the average quantity of streptavidin attached to the QD from the supplier, samples were prepared with four different amounts of DNA bound to the QD−streptavidin conjugate described in the Experimental Procedures to examine FRET efficiencies between dye-labeled DNA and QD conjugate. As shown in Figure 2A, a strong emission originating from acceptor dye (∼550 nm) excited at 330 nm was observed when the dsDNA/QD ratio was highest, marked as ratio 2. It is necessary to mix QD and dsDNA in such a ratio to obtain occupancy of the QD's binding sites satisfactory to show good FRET efficiency. We then used this ratio to construct a QD−DNA conjugate FRET probe for nuclease sensing. Emission profiles were not different for the QD−dsDNA conjugates when they were excited at 370 nm (data not shown).

3.2. Nuclease-Activated QD−DNA Conjugate FRET Probe. The QD-labeled dsDNA conjugate developed to detect DNase activity was prepared as described in the Experimental Procedures using streptavidin-coated QD (λ_{em} 525 nm) and Alexa Fluor 532-5-dUTP (λ_{em} 550 nm; λ_{ex} 528 nm) for donor and acceptor molecules. Emission profiles were compared between systems undergoing nuclease treatment and control with no nuclease treatment. As shown in Figure 2B, their patterns reverted to being similar to those in Figure 2A for ratios 0 and 2, respectively, suggesting that FRET was destroyed on digestion of labeled dsDNA bound to the QD. A similar combination of donor and acceptor molecules was also tested with Alexa Fluor 568-5-dUTP (λ_{em} 600 nm; λ_{ex} 575 nm) incorporated into the dsDNA bound to a streptavidin-coated QD (λ_{em} 565 nm). This QD conjugate showed two emission peaks (560 and 595 nm) when excited at 370 nm (Figure 2C), the peak at 560 nm arising from residual unquenched QD fluorescence and the 595 nm peak from the Alexa dye as a result of FRET. Thus, less efficient FRET quenching occurred even though the conjugation process was almost same as for QD (λ_{em} 525 nm) linked to dsDNS at ratio 2, which gave the efficient FRET achieved in Figure 2A. It was also distinctive from the pattern from the QD (λ_{em} 525 nm)−dsDNA conjugate at ratio 0.2, which demonstrated much lower FRET efficiency than ratio 2. The final shape of the emission curve for such conjugates depends on the precise natures and fluorescence properties of the various donors and

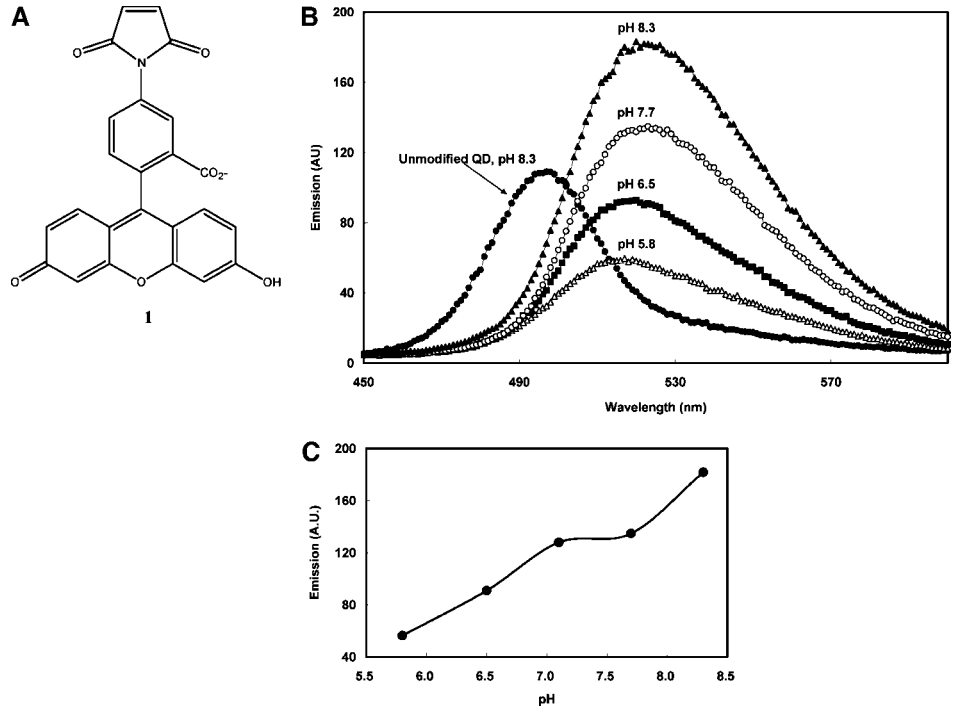

Figure 3. (A) Structure of fluorescein-5-maleimide. (B) pH-Dependence of emission spectra (λ_{ex} 330 nm) of fluorescein-modified quantum dots. (C) Relative fluorescence intensity at 517 nm as a function of pH.

acceptors. The peak at 595 nm in Figure 2C, however, entirely disappeared on treatment with DNase, which cleaved nucleotide-bound dyes from the QD to disrupt the FRET. Thus, it is possible to obtain two distinct FRET pattern changes (Figure 2B and 2C) depending on how the QD conjugate FRET system is constructed. Such different QD conjugations can be exploited for different tasks, for example in multiplexing.

3.3. Ligation-Based QD-Labeled Nucleotide FRET Probe. A QD biosensor was constructed to register DNA elongation by DNA polymerase using asymmetric PCR products for organization of ssDNA on the QD. This procedure gave a construct with a fluorescence peak at 530 nm on excitation at 390 nm before Klenow treatment (Figure 2D). Extension by the Klenow fragment with reaction mixtures that included fluorescently labeled dUTP (λ_{em} 550 nm; λ_{ex} 528 nm) led to a QD conjugate emitting at longer wavelength (λ_{em} 550 nm excited at 390 nm) than the starting QD conjugate as a result of FRET between the fluorescently labeled nucleotide and the QD (Figure 2D).

3.4. A pH-Switched Fluoresceinyl-QD FRET Probe. A pH-indicator dye (Figure 3A, fluorescein-5-maleimide) was attached to amine-modified QD (λ_{em} 490 nm) using Traut's reagent[26] to convert the $-NH_2$ group to an $-SH$ function and allow subsequent reaction through the maleimide. Dye attachment provided the QD with pH responsiveness with the fluorescence properties of the QD being maintained. Figure 3B shows that the emission peak at 495 nm of the unmodified QD is quenched by conjugation of pH-indicator dye, a new fluoresceinyl FRET-derived peak from QD excitation at 330 nm appearing in the region of 520 nm: its intensity and emission λ_{max} varies with pH, allowing this construct to behave as a pH sensor. Furthermore, the QD with the dye attached showed a fluorescent response (but not simple-sigmoidal, see Figure 3C) over an extended pH range (pH ~5.5 to ~8.5, except for the region pH 7.0−7.5 where the intensity was pH independent) relative to the free dye. The fluorescence of free pH-indicator dye is almost pH insensitive around pH 7.0[27–30] (data not shown): possibly the excited-state pK_{app} of the fluorophore is altered when bound to the QD. Such a QD-based pH sensor complex could also address problems caused by rapid leakage of cell-permeant fluorescein-based pH indicators from cells.[31]

3.5. Proteolysis-Activated QD−GFP FRET Probe. To build a prototype for QD-fluorescent protein systems we used a GFP mutant (UV5trypS2tag)[24] with a trypsin substrate-recognition sequence insertion and a six-histidine repeat near the C-terminus. This was conjugated to a Ni^{2+}-NTA-derived QD (λ_{em} 495 nm) with *N*-(5-amino-1-carboxypentyl) iminodiacetic acid through coordination with the $(His)_6$ tag. The emission maximum of this QD conjugate shifted (Figure 4A) due to FRET from 495 nm to the ~510 nm emission band of the GFP on excitation of the QD component (at 390 nm). An alternative manner of linking the QD and GFP mutant by making a covalent link between the cysteine residue[22] located close to the $(His)_6$ site of UV5trypS2tag using thiol-amine hetero-cross-linkers gave a comparable peak shift (data not shown). Thus, the FRET does not necessarily depend critically on the detailed manner of linkage between the QD and protein. Trypsinolysis of the UV5trypS2tag-QD conjugate shifted the peak at ~510 nm back toward the QD emission peak, Figure 4A.

3.6. Multiplexing: Dual Monitoring System. On the basis of the above results, two sets of QD biosensors were selected to set up a dual-monitoring system. This was to provide proof of

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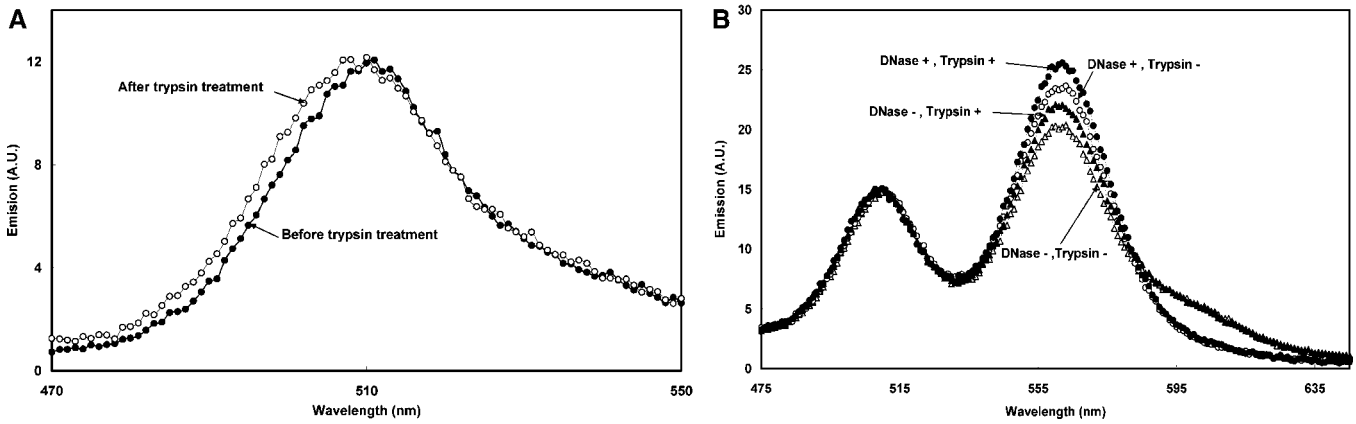

Figure 4. (A) FRET pattern change on trypsinolysis of a QD−GFP mutant conjugate. Emission spectra are shown with or without tryptic digestion (λ_{ex} 390 nm). (B) FRET pattern changes on simultaneous excitation of QD sensing trypsin and deoxyribonuclease action. A mixture of the two QD biosensors indicated in Figures 2C and 4A was simultaneously excited at 390 nm to test simultaneous, but independent, detection through changes in fluorescence spectra in response to individual enzymatic reaction. The mixture of QD conjugates was exposed for 2 h at 37 °C to (i) both trypsin and DNase, (ii) trypsin, but no DNase, (iii) DNase, but no trypsin, and (iv) neither trypsin nor DNase. The amounts of trypsin and DNase used were 0.006% and 2 units respectively. Relative fluorescence intensities for spectra with or without DNase were normalized to the same value at 510 nm, originating from the GFP choromophore, to demonstrate the pattern difference more quantitatively.

principle for constructing multiplex probes to monitor cellular events, or for assessments in drug screening or diagnosis. Simultaneous observation of protease and DNase reactions was selected as a test case. The two appropriate QD biosensors were thus mixed (Figure 4B) and their fluorescence spectra measured with or without trypsin- and/or DNase-treatment (λ_{ex} 390 nm). This mixture of modified QDs showed emission peaks at ∼510 and 560 nm and a shoulder at ∼600 nm before enzyme treatment (Figure 4B). On treatment with DNase, the shoulder at 600 nm was diminished in intensity and the 560 nm peak enhanced, similar to the change found (Figure 2C) for the action of DNase on the isolated QD−DNase sensor. On trypsinolysis of this dual QD sample, peak shifts took place to regenerate the emission spectrum of non-GFP-modified QD, as for the isolated system (Figure 4A and 4B). The fluorescence emission spectra show that the two QD biosensors on simultaneous-wavelength excitation in the same tube behave independently in mixtures. Data such as those in Figure 4B (and also parts A−D of Figure 2, and Figure 4A) are in principle amenable to quantitative extraction of the individual trypsin (in the region of 510 nm) and DNase activities (at >∼550nm) by deconvolution based on a sum of standard enzymatic curves, constructed along the lines of Figures 2C and 4A. Thus, it is feasible to combine nonoverlapped emission QD biosensors with tunable sensitivities, which is a prerequisite for the use of multiplexing monitoring systems in living cell situations.

4. Conclusions

The construction has been demonstrated of various FRET-based QD biosensors that take advantage of fluorescent properties of QD, and which are appropriate for sensing diverse biological phenomena. There are several requirements for multiplexing biosensors. One is to make a variety of sensor molecules, and the present contribution exhibits a range of functions already possible, *viz*., changes of ionization status, proteolysis or nucleolysis, and protein/chemical modification and processing such as DNA polymerization. Issues remain for such multiplexing to become useful *in vitro* and *in vivo*. For example, in the course of our (unpublished data) studies, it was apparent that QDs can sometimes affect properties of the bound protein, resulting in function depletion. In addition, the QD excitation profile can be influenced sometimes by attached proteins, and in practice it can be difficult to carry out a single-wavelength excitation for various QDs. However, this study has, to our knowledge, shown the first example of dual monitoring in such QD systems; now the goal is to use higher numbers of QD systems and work *in vivo*. (A) FRET pattern change upon fabrication of a QD (λ_{em} 525nm) with dsDNA incorporated fluorescent dUTP emitting 550 nm at several ratios. (B) FRET pattern change resulting from nuclease action on a QD−dsDNA conjugate (QD; λ_{em} 525 nm and dsDNA labeled with Alexa Fluor 532-5-dUTP). Emission spectra are shown before and after 1 h incubation (λ_{ex} 390 nm). (C) FRET pattern change on nuclease action using another (QD; λ_{em} 565 nm and dsDNA labeled with Alexa Fluor 568-5-dUTP) pair of QD (λ_{em} 550 nm) and fluorescent dsDNA emitting at 600 nm. Emission spectra are shown before and after 1 h incubation (λ_{ex} 390 nm). (D) FRET pattern change on incorporation of fluorescent dUTP emitting at 550 nm into ssDNA bound to a QD (QD; λ_{em} 525 nm) during DNA polymerase (Klenow fragment) action.

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