

Sensing Caspase 3 Activity with Quantum Dot–Fluorescent Protein Assemblies

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The creation of hybrid biological–inorganic nanomaterials capable of enhanced sensing, catalysis, or actuation is a major goal of nanotechnology.¹ Sensors consisting of nanoparticle bioconjugates in particular are predicted to find utility in medicine, bioresearch, security, and defense applications. Among the challenges in creating these materials is efficiently interfacing the biological elements (proteins, peptides, DNA) with the nanoparticle surface. Chemistries for accomplishing this should be facile, allow both participants to function in concert, and be amenable to the creation of a wide variety of other functional nanomaterials.^{1–3} We have shown that polyhistidine-appended proteins, peptides, and even DNA can self-assemble to CdSe–ZnS core–shell semiconductor quantum dots (QDs) via metal-affinity coordination.² This rapid, high-affinity interaction allows control over the number of biological moieties attached per QD and can even allow for control over protein orientation.² Bioconjugation using this strategy allows utilization of the QD as both a central nanoscaffold and an exciton donor for self-assembly of a variety of QD–protein, –peptidyl, and –DNA nanoconjugates capable of sensing nutrients, explosives, DNA, and enzymatic activity via fluorescence resonance energy transfer (FRET).^{1–3}

Use of QDs as FRET donors provides several inherent photophysical benefits cumulatively unavailable to organic dyes, including the ability to optimize spectral overlap by size-tuning the QD photoluminescence (PL), control over intra-assembly FRET by arraying multiple acceptors around the QD, reduced direct excitation of the acceptor, and access to multiplex FRET configurations.^{2c} These properties have led a growing number of groups to adopt QD–FRET as the signal transduction modality for sensors targeting pH changes, HIV-related peptides, nucleic acids, sugars, β -lactamase activity, and antibiotics.^{2,3} Here we demonstrate that the fluorescent protein mCherry modified to express a caspase 3 cleavage site can be ratiometrically self-assembled onto QDs to create a sensitive and specific FRET-based protease sensor (Figure 1A). Caspase 3 or apopain is an important downstream protease in apoptosis. Once activated by upstream initiator caspases, this cysteine protease specifically cleaves substrate proteins as part of the apoptotic cascade.⁴ Caspase 3 is of particular interest in cancer research, as it is down-regulated in different types of tumors and decreased activity is a prognostic indicator of chemosensitivity in breast and ovarian tumors.⁵ Treatments targeting caspase 3 inhibitors are being sought to restore chemosensitivity and improve clinical outcomes.⁵ Clearly, sensitive detection of caspase 3 activity is important for monitoring and evaluating treatments, and initial FRET-based protein sensors have been reported.^{5c}

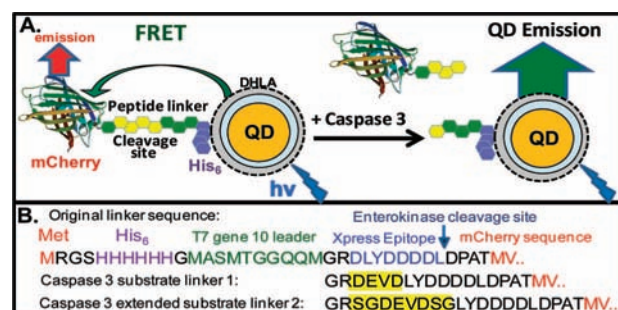


Figure 1. (A) Schematic of the QD–fluorescent protein sensor. mCherry with an N-terminal linker expressing the caspase 3 cleavage site and a His₆ sequence were self-assembled to the surface of CdSe–ZnS DHLA QDs, resulting in FRET quenching of the QD and sensitized emission from the mCherry acceptor (mCherry PDB structure 2H5Q). Caspase 3 cleaves the linker, reducing the FRET efficiency. (B) Linker sequences. The original 35 residue N-terminal linker is shown with colors highlighting functionalities, including the start methionine (Met), the His₆ NTA-purification/QD-assembly sequence, several other functionalities, and the first residues of the mCherry protein. Caspase 3 cleavage site insertions into the linkers are highlighted in yellow.

The parent mCherry gene we utilized was encoded in the multicloning site of plasmid pRSetB (Invitrogen) and expressed a 35 residue linker upstream of the mCherry protein that included a His₆ tag and a T7 transcript stabilizing sequence among other functional sequences (see Figure 1B). The linker was analyzed for native structure to evaluate caspase 3 steric accessibility when the His₆ sequence is assembled onto the QD. A comparison of more than 25 crystallographic protein sequences in the Protein Data Bank (PDB, www.rcsb.org) containing this N-terminal linker found no structure for the 35 residues, strongly suggesting that the linker adopts a flexible conformation. A sequence requiring the least amount of modification near the enterokinase site was chosen for insertion of the cleavage sites. Stratagene’s QuikChange site-directed mutagenesis kit was used to introduce the caspase 3-recognized cleavage sequence DEVLD (substrate 1) and an extended serine–glycine-flanked sequence SGDEVDSG (substrate 2) previously shown to increase activity in a fluorescent-protein FRET sensor (Figure 1B).⁶ DNA sequencing confirmed the plasmid insertions. Substrate plasmids along with the unmodified parent were transformed into *Escherichia coli* Rosetta 2 (DE3) cells and expressed overnight, and mature mCherry was purified over Ni-NTA media and quantitated using chromophore absorbance ($72\,000\text{ M}^{-1}\text{ cm}^{-1}$ at 587 nm) as described elsewhere.⁷ Dihydropolipoic acid (DHLA)-functionalized QDs emitting at 550 nm were selected for assembly with mCherry because of the favorable spectral overlap (Förster distance $R_0 = 4.9\text{ nm}$).⁸ Agarose gel electrophoresis of the QD–mCherry conjugates confirmed ratiometric self-assembly (data not shown). Figure 2A shows spectra for 550 nm QDs self-assembled with increasing ratios of mCherry substrate. Figure 2B

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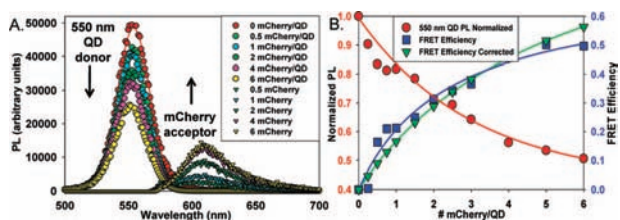


Figure 2. (A) Deconvoluted spectra of 550 nm QDs (quantum yield 0.2) self-assembled with an increasing ratio of mCherry substrate 1. Data are corrected for mCherry direct excitation. (B) Plots vs mCherry/QD ratio of (red) normalized QD PL, (blue) FRET efficiency, and (green) efficiency corrected for heterogeneity using a Poisson distribution function.⁸

shows the normalized QD PL and the FRET efficiency E determined from QD PL loss as functions of the mCherry/QD ratio n . The data show a progressive loss in QD PL along with an increase in sensitized mCherry emission. An intra-assembly FRET efficiency of $\sim 50\%$ was measured at $n = 6$. The FRET was analyzed using eq 1, which has been derived for centrosymmetric QD conjugates:^{2,8,9}

$$E = nR_0^6 / (nR_0^6 + r^6) \quad (1)$$

where n is the acceptor/QD ratio, R_0 is the Förster distance, and r is the QD–acceptor separation distance. An average r value of 5.6 nm was found for the unmodified parent mCherry. This value increased to 6.5 nm upon cleavage-site insertion for the longer substrate 2 sequence, indicating that insertion of the additional residues into the linker slightly increases the separation distance without affecting the conjugate structure.

To extract kinetic data from changes in FRET efficiency, we utilized the ratios of mCherry to QD emissions as a calibration curve (see the Supporting Information). Such ratiometric data are less sensitive to changes in reagent concentration and allowed us (1) to select an mCherry/QD substrate ratio for which a large change in FRET efficiency following enzymatic cleavage can be measured and (2) to transform the proteolysis-induced FRET recovery data into quantitative velocity values, as demonstrated for similar QD–peptide conjugates.⁹ Figure 3 shows represen-

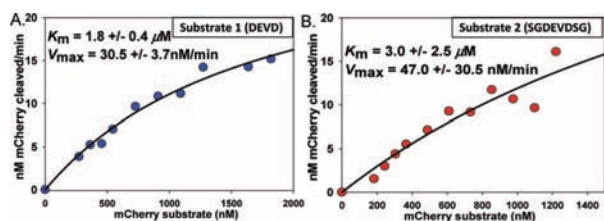


Figure 3. Proteolytic velocity vs substrate concentration. Changes in FRET efficiency were converted to enzymatic velocity (nM mCherry cleaved/min) as described elsewhere.⁹ Representative data are shown for (A) linker 1 and (B) extended linker 2 substrates (using mCherry/QD ratios of 3.5 and 1.5, respectively) along with estimated K_M and V_{max} values (means of three experiments). Data were fit with eq 2.

tative plots of velocity derived from monitoring changes in the mCherry/QD ratios for increasing concentrations of the two substrate conjugates (~ 200 nM to $2 \mu\text{M}$) exposed to 65 units (400 pM) of recombinant human caspase 3 enzyme (EC#3.4.22.56, activity $\sim 5.3 \times 10^6$ units/mg). Initial velocities were measured in 30 min reactions at 30°C .¹⁰ Corresponding Michaelis constants K_M and maximal velocities V_{max} were estimated using the Michaelis–Menten expression for excess substrate:^{9,10}

$$V = \frac{d[P]}{dt} = \frac{V_{max}[S]}{K_M + [S]} \quad (2)$$

where $[S]$ and $[P]$ are the substrate and product (cleaved peptide) concentrations and t is time. Similar K_M values were extracted for mCherry substrates 1 and 2: 1.8 ± 0.4 and $3.0 \pm 2.5 \mu\text{M}$, respectively. These are

slightly lower than the $\sim 11 \mu\text{M}$ value reported for a similar peptidyl substrate.¹⁰ Values of k_{cat} ($V_{max}/[Enz]$) of 1.3 and 2 s^{-1} for substrates 1 and 2 are comparable to the reported value of 2.4 s^{-1} , although the turnover numbers (k_{cat}/K_M) of $\sim 7.2 \times 10^7$ and $6.7 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ are slightly larger than the reported value of $2.2 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$.¹⁰ However, a direct benefit of our assay is that we utilize 5–10 times less substrate and ~ 3 orders of magnitude less enzyme than the latter format. Furthermore, with our assemblies, we were able to detect enzymatic activity for caspase 3 concentrations as low as 20 pM (~ 3.3 units, data not shown). Assays carried out with mCherry lacking the DEVD sites showed no activity, confirming that cleavage is substrate-specific.

Although qualitative (i.e., yes/no) QD–protease sensors have been reported,^{11a,b} we have demonstrated that QDs assembled with fluorescent proteins engineered as peptide substrates allow sensitive, specific quantitative monitoring of proteolysis. Our results showed that even though the substrate sequence was “sandwiched” between the QD and mCherry in the conjugate structure, it was still accessible to the enzyme. Caspase 3-induced changes in FRET efficiency were comparable to those observed in fluorescent protein sensors.^{5c} Our results build on and extend recent studies focusing on: understanding the interactions between QDs and fluorescent proteins via FRET interactions,⁷ energy transfer in multichromophore QD–DNA–fluorescent protein assemblies,^{11c} and endocytic delivery and intracellular imaging of QD–peptide–fluorescent protein conjugates.^{11d} Advantages of this sensing approach include a choice in pairing QD emission to a fluorescent protein acceptor, bacterial expression of a protease substrate in a fluorescent form, facile self-assembly to QDs, reduced use of enzyme/substrate, the possibility of in vivo utilization, and the ability to recombinantly modify substrates to target other proteases.

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Supporting Information Available: Representative assay data and experimental procedures. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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