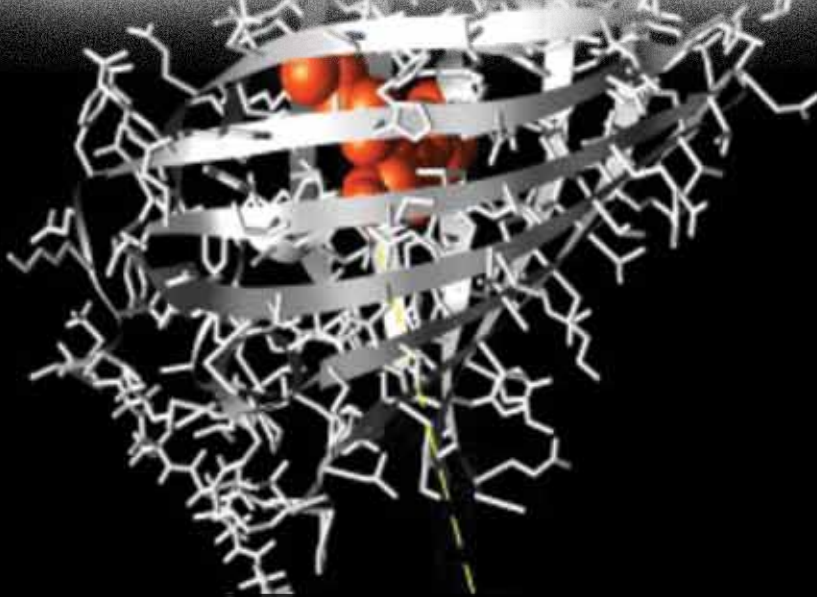
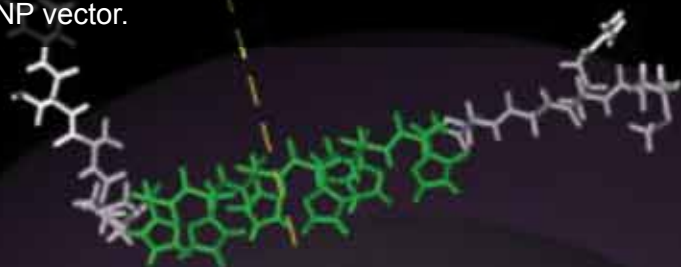




# It's Alive!...Sort of



**W**hat do you get when you combine the organic and the inorganic? Not Frankenstein's monster, the Six Million Dollar Man, or Robocop, but rather the most amazing new materials, if you think small (very, very small). The novel classes of nanomaterials that a team of NRL biomolecular and optical sciences researchers are creating and investigating are composed of biomolecules, such as proteins, peptides, and nucleic acids, integrated with inanimate nanoparticles (NPs) derived from metals, metal oxides, noble metals, and semiconductors. Due to their size, NPs exhibit unique quantum-confined photophysical, electronic, and chemical qualities not present in the parent bulk materials. These hybrid biological-NP functional composites have the best of both worlds, exhibiting properties and performing activities that neither component alone has or could achieve, and therefore show untold potential to both biotechnology and national defense. NRL scientists, in response to the President's National Nanotechnology Initiative, are developing the chemistry by which the biomolecules are assembled on the NPs and thus control their properties. In the work described in this article, a fluorescent protein, mCherry, was assembled on a central luminescent semiconductor nanocrystal or quantum dot (QD) to produce an optically active biosensor that can measure enzyme activity by exploiting resonance energy transfer within the bio-NP composite. Once perfected, these types of composite nanomaterials should prove useful in defense applications including the detection and remediation of explosives and chem/bio threats, improved battlefield diagnostics and therapeutics, and lightweight electronic and energy harvesting materials. Biotechnological applications include targeted drug delivery, improved pharmaceuticals, targeted diagnostics, improved imaging, sensing of molecular-scale events inside cells, or even the combination of multiple applications on a single NP vector.



## Monitoring Enzyme Activity with Hybrid Semiconductor Quantum Dot–Fluorescent Protein Assemblies

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Creating new functional nanomaterials by integrating biomolecules such as proteins with inorganic nanoparticles has been tasked to the Department of Defense as a critical long-term priority under the National Nanotechnology Initiative. NRL continues to be a leader in this field by developing chemistries that allow biomolecules to be assembled on nanoparticles with control over their properties. These abilities are highlighted by the approach described here to assemble a fluorescent protein–semiconductor quantum dot (QD) optical sensor that can measure enzymatic activity by exploiting resonance energy transfer within the complex. The fluorescent protein, mCherry, was grown in bacteria and engineered to express both a polyhistidine tag that facilitates high-affinity QD coordination and a sequence that can be cleaved by the protease, caspase 3. The latter is an enzyme involved in programmed cell death, which can be triggered by exposure to biothreat agents. Protein self-assembly onto the QDs completes the active sensor, which is capable of detecting caspase 3 activity with significantly better sensitivity than other available assays.

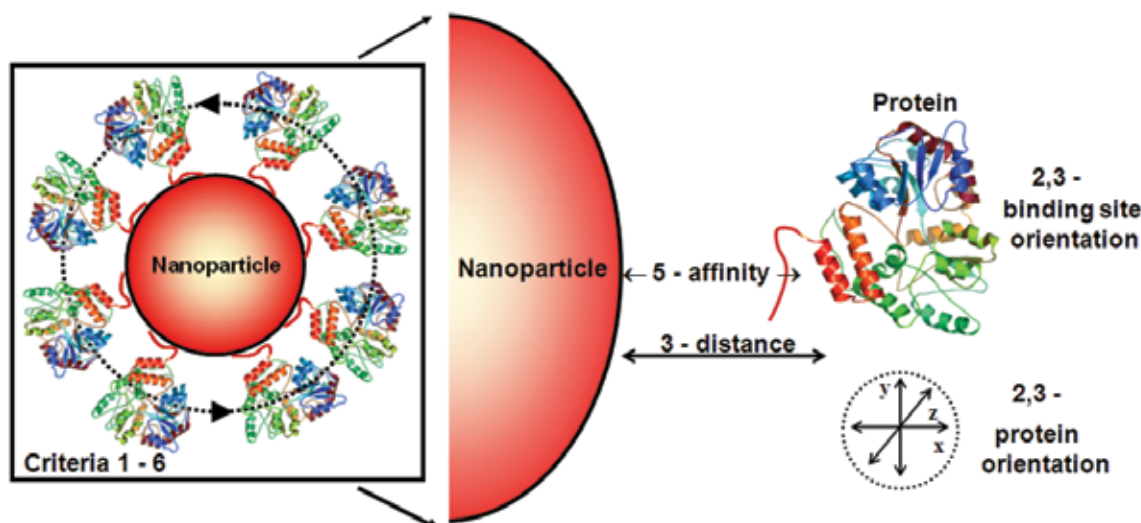
### INTRODUCTION

Nanotechnology offers the potential for developing new classes of materials that can significantly impact biotechnology and national defense. Nanoparticles (NPs) derived from metals, metal oxides, noble metals, and semiconductors display unique photophysical, electronic, and chemical properties that are not present in the parent bulk material. Biologicals, including proteins, peptides, nucleic acids, and the like are capable of synthesis, catalysis, scaffolding, self-assembly, biorecognition, and energy harvesting. Integrating both into hybrid biological–NP functional composites will provide new classes of materials capable of far more than each component alone. For biotechnology, combining NPs with biologicals provides opportunities for targeted drug delivery, improved pharmaceuticals, targeted diagnostics, improved imaging, sensing of molecular-scale events inside cells, or even the combination of multiple applications on a single NP vector. Defense applications include active sensors for the detection and remediation of explosives and chem/bio threats and improved battlefield diagnostics and therapeutics along with lightweight electronic and energy harvesting materials. Department of Defense (DoD) research objectives outlined in the President's National Nanoscience Initiative include exploiting interactions between NPs and naturally occurring biomolecules for next-generation defense-related applications along with

developing methods for the inexpensive and controlled assembly of such composites.<sup>1</sup> Here we describe the recent development of a novel, optically active biosensor capable of monitoring enzymatic activity, that consists of a central semiconductor nanocrystal serving as a scaffold for the controlled assembly of a fluorescent protein.

### INTERFACING BIOLOGICALS WITH NANOMATERIALS

NPs are similar in size as midsize to large proteins and can therefore serve as stable scaffolds for the central attachment and display of multiple copies of the same or even different biomolecules. It is helpful to envision the ultimate criteria desired from chemistries used in forming these types of composite materials as they directly impact subsequent activity (see Fig. 1). Ideally, the chemistry would attach a biomolecule to a NP (1) in a homogenous manner, (2) with control over its orientation, (3) with control over its distance from the NP, and (4) with control over the number of biomolecules per NP. The goal is to uniformly display the biomolecules on the NP surface with their active regions all clearly available for subsequent interactions. (5) Controlling the affinity for the NP surface is becoming increasingly important as it can also allow attachment and release of biologicals from the NP in response to external stimuli. It would be ex-



**FIGURE 1**

Schematic of a nanoparticle–protein bioconjugate and the criteria desired for optimized bioconjugation. Figure adapted from Ref. 2.

tremely beneficial if these chemistries would be generally applicable to (6) allow any biomolecules to be attached to any NP while still maintaining the above-described attributes.<sup>2</sup> There are relatively few available bioconjugation methods that satisfy even some of these criteria, and research at NRL is focused on addressing this specific need.

## MATERIALS AND TECHNIQUES

### Quantum Dots

Nanocrystalline semiconductor quantum dots (QDs) have remarkable photoluminescent (PL) properties that make them especially attractive for use in biological sensing and imaging applications.<sup>3</sup> These include excellent photo- and chemical stability, high quantum yields, and large absorption cross-sections, which are coupled to size-tunable narrow emission spectra spanning from the UV to near-IR depending upon the constituent semiconductor materials used. The latter properties are especially useful in biology as they allow multiple differentially emissive QD populations to be effectively excited using a single wavelength, far removed from their respective emissions. This enables simultaneous monitoring of different QD emissions and allows tracking and potential correlation of complex spatiotemporal cellular events. High-quality CdSe/ZnS core/shell QDs emitting in the visible range are reproducibly prepared via thermal decomposition of organometallic precursors in organic solvents. Post-synthetic chemical modification of the nanocrystal surface is required to render the QD suspensions stable in aqueous media for biological applications and is usually accomplished by one of two principal routes:

(1) ligand-exchange with multifunctional hydrophilic surface capping ligands, or (2) use of amphiphilic polymers that interdigitate with the native organic surface.<sup>3</sup> The Optical Sciences Division at NRL has long been a leader in developing ligands based on the first route for providing biologically compatible QDs. They have pioneered the combination of appending multidentate thiols, which provide high-affinity QD surface interactions, with either small charged groups such as in the dihydrolipoic acid (DHLA) utilized here or short polyethylene glycol (PEG) segments, both of which mediate solubility and provide compact, pH-stable QDs.<sup>4</sup>

### Fluorescent Proteins and Self-Assembly to Quantum Dots

Numerous organisms express naturally fluorescent proteins. The first to be extensively characterized was green fluorescent protein (GFP) isolated from the luminescent jellyfish *Aequorea victoria*. Mutational engineering of this, and similar proteins, has resulted in variants with numerous fluorescent emissions and enhanced stability, which have established them as powerful tools in bioimaging and sensing applications. Here we create a self-assembled QD hybrid with a fluorescent protein termed mCherry that originated in Roger Tsien's laboratory (2008 Nobel Prize laureate in medicine for work on GFP). mCherry was first cloned from coral as a tetramer and then extensively engineered into the current, stabilized monomeric version. To conjugate proteins to QDs, we exploit metal-affinity coordination between polyhistidine residues and the ZnS shell of the QD.<sup>5</sup> This requires that the proteins be recombinantly engineered to express a polyhistidine sequence at one of their termini. The same sequence allows the proteins



to also be easily purified using commercial Ni-chelate media. We have extensively characterized self-assembly between QDs and both polyhistidine-appended proteins and peptides and have found the interaction to be rapid and stable (equilibrium binding constant  $K_d \sim 1 \times 10^9 \text{ M}^{-1}$ ) across a wide range of pHs.<sup>5</sup> More importantly, the ratio of proteins assembled per QD is easily controlled through the molar amounts added together.

### Förster Resonance Energy Transfer (FRET)

FRET is a process in which an excited state donor fluorophore transfers energy nonradiatively to an acceptor fluorophore. FRET requires that the donor emission wavelengths overlap with the acceptor absorption spectrum; that is, that they share spectral overlap, and the two molecules must be relatively close to each other, usually within 100 Å. FRET is characterized by a detectable loss in donor fluorescence intensity, which is sometimes coupled to an increase in the acceptor's emission intensity. Measuring these changes provides the FRET efficiency and allows estimation of the nanoscale distance separating the two fluorophores; this is why FRET is sometimes referred to as a molecular-scale spectroscopic ruler. QDs make superior FRET donors as their unique photophysical properties provide numerous advantages that are cumulatively unavailable to organic dyes functioning in the same role. When a QD donor interacts with dye acceptor(s), FRET efficiency,  $E$ , is calculated using the relationship

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

where  $R_0$  is the donor-acceptor separation distance corresponding to 50% energy transfer,  $r$  is the actual donor-acceptor separation distance, and  $n$  is the number of acceptors centrosymmetrically arranged around a QD donor.<sup>6</sup>

### Proteases

Proteases are enzymes that cleave specific peptidyl sequences and are involved in almost all cellular processes. Pathogens, such as the *Plasmodium* malaria parasite and *Yersenia pestis*, the cause of pneumonic plague, express specific proteases to aid in entry into host cells, to degrade host proteins for nutritional demands or to counter immune defenses, while *Clostridium botulinum* and influenza A use host proteases to activate essential virulence factors. More than 500 human proteases function as housekeeping proteins to degrade unneeded substrates during developmental processes or, in the specific case of caspase 3, to initiate apoptosis or programmed cell death, which sometimes occurs in response to pathogenic activity. When inhibited, the absence of protease activity can result in diseases and cancer.

## SENSOR DESIGN, ENGINEERING, AND CASPASE 3 ASSAYS

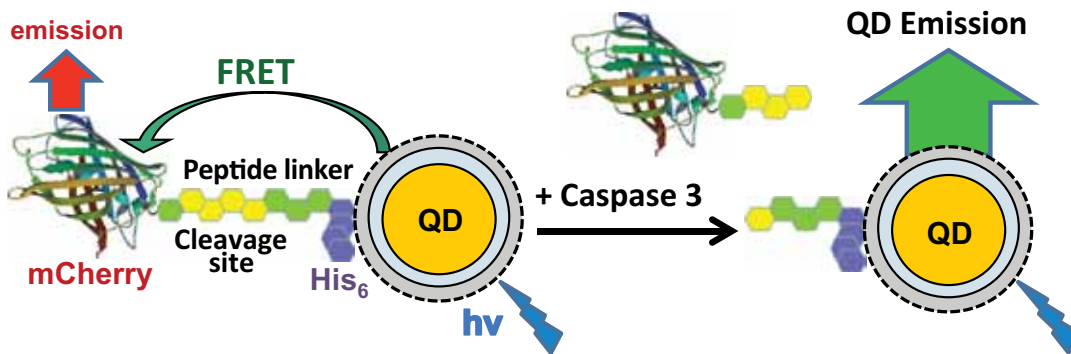
Sensor structure and assay function are schematically depicted in Fig. 2. mCherry expressing a terminal His<sub>6</sub> sequence self-assembles to the surface of QDs, resulting in FRET quenching of the QD and sensitized emission from the mCherry-acceptor. A cleavage site engineered in the linker sequence separating the QD from the mCherry is recognized and cleaved by added caspase 3, which reduces FRET efficiency and provides signal transduction. Experimental work proceeded in three phases: (1) modeling and protein engineering, (2) characterization of QD-mCherry self-assembly, and (3) caspase 3 assays.<sup>7</sup>

### Modeling and Protein Engineering

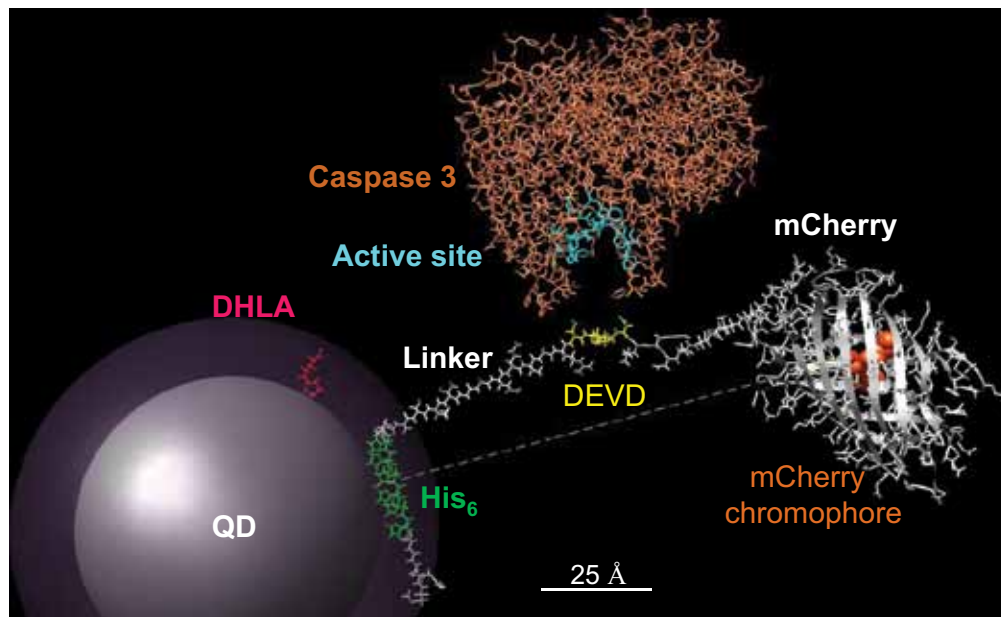
The mCherry gene is encoded in a commercial expression plasmid appended to a 35-residue linker that includes a His<sub>6</sub> tag and several other functional sequences. Prior to recombinant modification and protein expression, we modeled the putative QD-mCherry complex to ascertain if the assay as envisioned would function *in silico* (see Fig. 3). We apply this process to many of our QD-bioconjugate designs to optimize their function and it also represents one of the unique interdisciplinary capabilities available to NRL. The linker was first analyzed for native structure to evaluate caspase 3 accessibility when the His<sub>6</sub> sequence was 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 implying the sequence is present in a random-coil conformation. An area requiring the least modification in the linker was chosen for insertion of the caspase 3 cleavage site, which consists of the sequence DEVD. Constraints were applied to produce an extended linker conformation with the His<sub>6</sub> region in contact with the QD surface and the mCherry placed at the linker's other terminus. Placing the structure of caspase 3 near the DEVD portion of the linker and evaluating the possible binding interactions confirms that there is nothing intrinsically disfavored about the composite structure that should prevent caspase 3 binding or proteolysis. Site-directed mutagenesis was used to introduce DEVD (substrate 1) and an extended serine-glycine flanked SGDEVDSG sequence (substrate 2) into the linker. The proteins were expressed in bacteria and purified using standard techniques.

### Characterization of Quantum Dot-mCherry Self-Assembly

Following protein expression, we verified that mCherry-His<sub>6</sub> does indeed efficiently self-assemble to



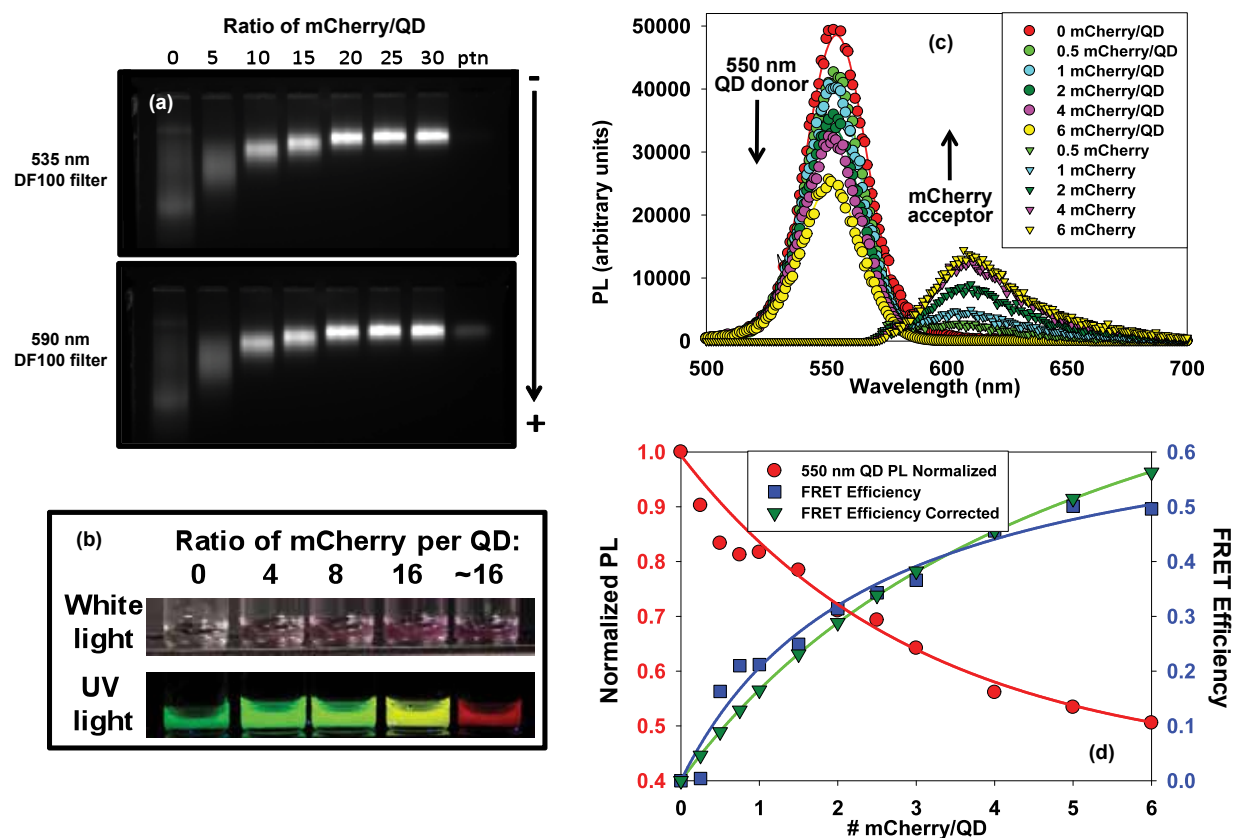
**FIGURE 2**  
Schematic of the QD–mCherry FRET biosensor for monitoring caspase 3 activity. mCherry appended with an N-terminal linker expressing the caspase 3 DEVD cleavage site and a His<sub>6</sub> sequence self-assemble to the QDs, resulting in FRET quenching of the QD and sensitized emission from the mCherry-acceptor. Caspase 3 recognizes and cleaves the linker, reducing FRET efficiency and providing signal transduction. Figure adapted from Ref. 7.



**FIGURE 3**  
Model of mCherry self-assembled to a QD. The QD is simulated by a sphere of 29 Å radius, while the dihydroliopic acid (DHLA) surface functionalizing ligand is represented in an energy minimized conformation by an outer sphere extending a further 11 Å. Three-dimensional coordinates for mCherry (2H5Q) and caspase 3 (3EDQ) were obtained from the Protein Data Bank. mCherry with its chromophore highlighted is appended to the extended linker and coordinated to the QD via the His<sub>6</sub> sequence. The caspase 3 DEVD cleavage site is shown in yellow. Caspase 3 (pale orange) and specifically its active site (cyan) are afforded unhindered access to the cleavage site.

QDs and engage in FRET. Figure 4(a) shows results in which the indicated ratios of mCherry were assembled to 550-nm emitting QDs, chosen due to favorable spectral overlap, and then separated in an agarose gel. The conjugates were visualized with different filters to isolate the FRET-sensitized emission. A clear loss in QD mobility is noted as the assembled mCherry ratio per QD increases along with corresponding increases in mCherry sensitization, especially as compared to directly excited mCherry alone. These data serve to confirm both self-assembly and FRET interactions. Figure

4(b) presents images of QDs assembled with increasing mCherry under both white light and UV excitation. With the latter, QD PL loss along with FRET excitation of mCherry manifest as an increasingly yellow composite emission rather than just the original QD (green) or mCherry (red) fluorescence. Deconvoluted and background-subtracted spectra tracking progressive QD donor PL loss and corresponding mCherry acceptor sensitization are shown in Fig. 4(c) along with plots of the data as a function of mCherry valence in Fig. 4(d). These plots also serve as calibration curves for

**FIGURE 4**

(a) Agarose gels of QDs self-assembled with 5 to 30 equivalents of mCherry. Lane '0' contains unmodified QD, while 'ptn' contains 30 equivalents of mCherry alone. Images captured with 535 nm and 590 nm filters to highlight QD and mCherry emission, respectively. (b) Images of QD/mCherry self-assembly reactions under visible and UV illumination. (c) Deconvoluted spectra from 550 nm QDs self-assembled with an increasing mCherry substrate 1. Data are corrected for mCherry direct excitation. (d) Plots of normalized QD PL vs mCherry valence  $n$  (red), FRET efficiency (blue), efficiency corrected for heterogeneity using a Poisson distribution function (green) as obtained with Eq. (1). Figure adapted from Ref. 7.

the subsequent caspase 3 assays. Average QD–mCherry separation values of 5.6 nm were derived within the self-assembled complex using Eq. (1) for the unmodified parent mCherry and separation distance increased to 6.5 nm with extended substrate 2 protein.<sup>7</sup>

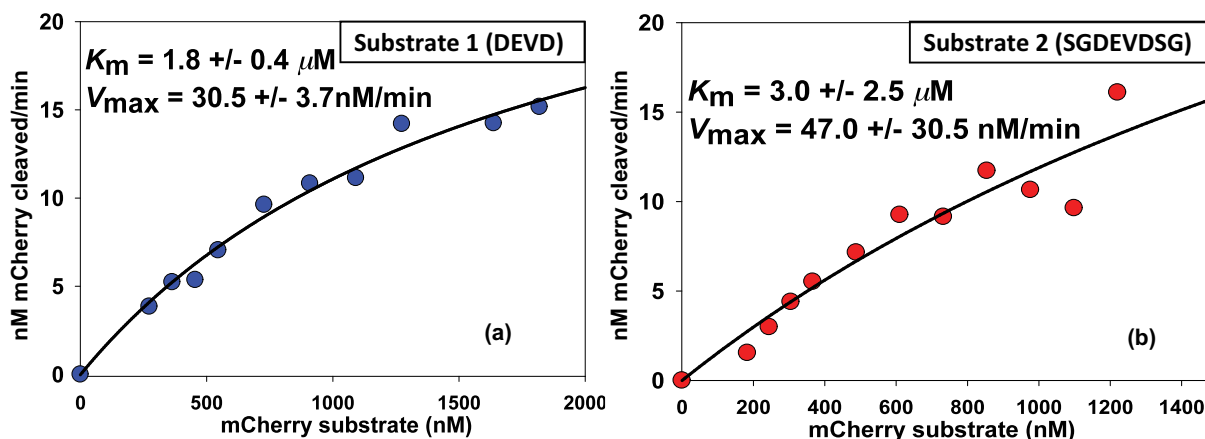
### Caspase 3 Assays

Confident in mCherry's ability to self-assemble to QDs and engage in FRET, we evaluated their efficacy in targeted proteolytic assays. Figure 5 highlights representative plots of enzymatic velocity derived from monitoring changes in FRET when increasing concentrations of QD–mCherry conjugates were exposed to recombinant human caspase 3 enzyme. To extract kinetic data from the FRET changes observed during the assay, we use the previously determined ratios of mCherry to QD emissions as a calibration curve (above). Use of this ratiometric data is less sensitive to changes in reagent concentration and also allows us to transform proteolysis-induced FRET recovery data into quantitative values. Corresponding Michaelis constants

$K_M$  and maximal velocity  $V_{max}$  were estimated using the Michaelis–Menten formula and were found to be comparable to other caspase 3 assay formats. Importantly, this reflects that there are no significant changes in enzyme activity when interacting with a nanocrystal–protein complex. However, the QD assay used 5 to 10 times less substrate and three orders of magnitude less enzyme than comparable formats and was capable of detecting enzyme levels down to the picomolar range.<sup>7</sup>

### SUMMARY AND CONCLUSIONS

Here we describe the development of a biosensor that is a self-assembled hybrid of a naturally occurring protein with an inorganic semiconductor nanoparticle. This optically active biosensor provides specific and sensitive quantitative monitoring of enzymatic proteolysis. As demonstrated here, this approach has multiple advantages: a choice in pairing QD emission to a fluorescent protein acceptor, bacterial expression of the protease substrate in a fluorescent form, facile self-

**FIGURE 5**

Caspase 3 assay results for QD–mCherry substrate 1 (a) and extended substrate 2 (b) along with corresponding kinetic values. Figure adapted from Ref. 7.

assembly to form the final active sensor, reduced use of enzyme/substrate, and the possibility of *in vivo* utilization. Additionally, the linker's cleavage sequence can easily be modified to be recognized by other proteases, thus allowing the sensor to monitor many different enzyme targets. Importantly, this hybrid sensor demonstrates that many of the DoD nanotechnology research goals are indeed feasible, and significant progress is being made toward them at NRL.

#### ACKNOWLEDGMENTS

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[Sponsored by DTRA and ARO]

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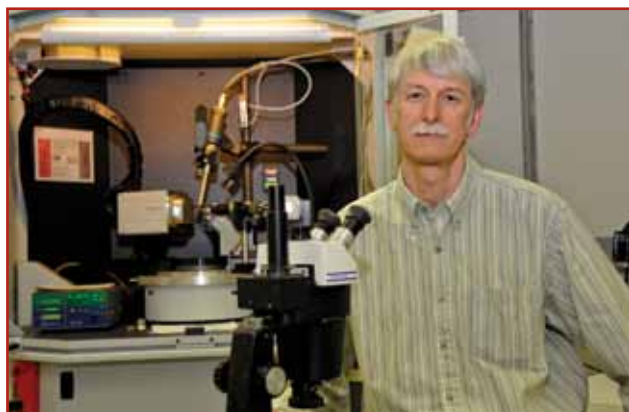
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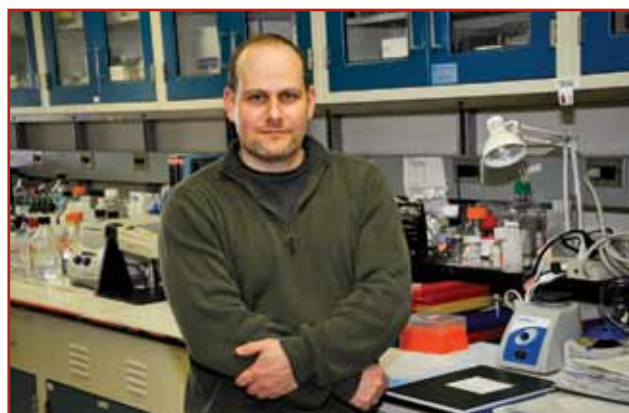
## THE AUTHORS



**KELLY BOENEMAN** received her B.S. in biology from Alma College in 2001 and her Ph.D. in tumor biology from Georgetown University in 2007, where she used confocal microscopy to study regulation of DNA replication mechanisms in bacterial model systems. She joined NRL's Center for Bio/Molecular Science and Engineering as an ASEE postdoctoral fellow in 2008 and has remained as a research biologist. Her primary focus has been on bioconjugation of semiconductor quantum dot nanocrystals and their potential applications as biosensors and molecular imaging agents. She won a Young Investigator of the Year Award for her presentation of this work at the 2009 Biophotonics West, International Society for Optics and Photonics (SPIE) Annual Meeting held in San Jose, CA. She is also currently working on projects involving nanoparticle delivery and biosensing in live cells, as well as improvement of nanoparticles/biomolecule conjugation techniques and photophysical analysis of bioconjugated materials.



**JEFFREY R. DESCHAMPS** has worked at the Naval Research Laboratory since 1985 on structural studies, structure function relationships of biological molecules, and biosensors. Prior to his position at NRL, Dr. Deschamps was a postdoctoral fellow in the Department of Pharmacology at the Johns Hopkins School of Medicine. He is the author of over 190 publications in diverse areas such as energetic materials, peptide and protein structure, and biosensor design and evaluation. His recent work with others at NRL on nanomaterials has resulted in new methods for characterizing these complex inorganic/biomaterial assemblies and is widely recognized as evidenced by the numerous citations.



**IGOR L. MEDINTZ** received a B.S. degree in forensic science from John Jay College of Criminal Justice, City University of New York in 1990. After working as an analytical chemist for 3 years, he received a Ph.D. in molecular, cellular, and developmental biology from the Graduate School University Center of the City University of New York in 1998. From 1998 to 2001, he was a National Cancer Institute postdoctoral fellow at the College of Chemistry, University of California, Berkeley. He joined NRL as a National Research Council fellow working on biosensing in 2002 and was hired in 2004. He is currently a research biologist in the Center for Bio/Molecular Science and Engineering and conducts research on biofunctionalizing nanoparticles and developing chemistries for attaching biomolecules to inorganic materials in a controlled manner. He has more than 100 publications and is on the editorial advisory board of *Bioconjugate Chemistry* and several other journals. He was awarded a Top Navy Scientists and Engineers of the Year Emerging Investigator Award in 2007.



**MICHAEL H. STEWART** is a materials chemist with over 8 years of synthetic experience, with expertise in organometallic chemistry. After receiving his B.S. degree in chemistry from Wittenberg University in 2002, he began training as an organometallic chemist as an NSF IGERT fellow at the University of Michigan, where he received his Ph.D. in 2007. As a graduate student, he developed a new synthetic route to rare terminal carbide transition metal complexes by chalcogen-atom transfer and studied their subsequent reactivity. Dr. Stewart is also trained in the application of scanning tunneling microscopy to study surfaces and molecular organization at solid-liquid interfaces. He became interested in semiconductor quantum dots (QDs) after spending a summer synthesizing PbSe QDs as an intern at Los Alamos National Lab. He further pursued QD research as an NRC postdoctoral fellow with the Optical Sciences Division at NRL, where he became a staff member in 2009. His research activities include developing multifunctional, biocompatible QDs as biological sensors and labels, and investigating QDs for renewable energy applications.