Protein Kinase-Actuated Resonance Energy Transfer in Quantum Dot–Peptide Conjugates

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ABSTRACT Bioconjugates of quantum dot nanocrystals possess unique optical properties that allow them to serve as exceptional biological imaging and sensing reagents. Protein kinases are an important family of enzymes that phosphorylate serine, threonine, or tyrosine side chains and are critical in cell signaling and cancer biology, but despite their biomedical and pharmaceutical significance, their activity has been little explored using quantum dot technology. We demonstrate that self-assembled peptide–quantum dot conjugates can serve as surrogate substrates in a simple homogeneous assay for protein kinase activity. Enzymatic phosphorylation of the peptide-conjugates is detected by means of a complementary FRET-acceptor labeled antiphosphotyrosine antibody, with formation of the immunocomplex resulting in energy transfer between the quantum dot and FRET acceptor molecules. This approach should facilitate the development of new assays for protein kinases and other enzymes based on quantum dot FRET donors.

KEYWORDS: quantum dots · FRET · biosensors · bionanotechnology · protein kinases

Kinase-mediated protein phosphorylation plays a pivotal role in the regulation of numerous key cellular processes and their dysfunction is often manifested in the development of disease states, notably cancer.[1,2] As such, protein kinases are considered to be excellent targets for pharmaceutical intervention, and numerous radioactive and fluorometric assays to measure their activity have been developed.[3−5] For a number of enzymes, nanoparticle-specific phenomena, such as dispersion-dependent optical and magnetic properties, have been demonstrated to circumvent some of the associated caveats of traditional radioactive and fluorescence-based sensing approaches.[6−8] Photoluminescent semiconductor nanocrysals (quantum dots, QDs) are one such class of nanomaterial that has attracted much attention in biosensing. Notably, they are persuasive energy transfer donors in FRET-based applications owing to their exceptional brightness, broad excitation spectra, large effective Stokes' shifts, and narrow size-tunable emissions, providing the ability to maximize donor–acceptor spectral overlap.[9,10] Several QD energy transfer assays for the detection of small molecules, biological recognition events, and biocatalytic transformations have been reported, demonstrating the increased sensitivity of using quantum dots as energy transfer donors.[11−15] In light of these studies, we sought to design a new system to monitor protein kinase activity. For instance, the ability to selectively tune QD emission to red-shifted wavelengths provides a convenient means to minimize signal interference due to sample autofluorescence, frequently encountered in fluorometric kinase assays.[7,8] This is a novel demonstration of QD-based technology that can measure the activity of this crucial family of enzymes, in a simple and rapid assay that does not require separation or washing steps and can be carried out without specialized instrumentation.

We applied our system (Figure 1) to study the activity of the prototypal nonreceptor tyrosine kinases Abl and Src owing to their role in the progression of several cancers.[1] We first prepared water-soluble CdSe/ZnS QDs (605 nm emission maximum) by base-promoted ligand exchange of the native hydrophobic surfactant coating with mercaptopropionic acid (MPA).[16] MPA-capped QDs were then conjugated to peptide substrates for Abl and Src (H_2N-EAIYPFAEEH$_6$–$CONH_2$, Ac-IYGEFKKKH$_6$–$CONH_2$) by metal-affinity driven self-assembly *via* an appending

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Received for review June 5, 2010 and accepted July 04, 2010.

Published online July 12, 2010. 10.1021/nn101293s

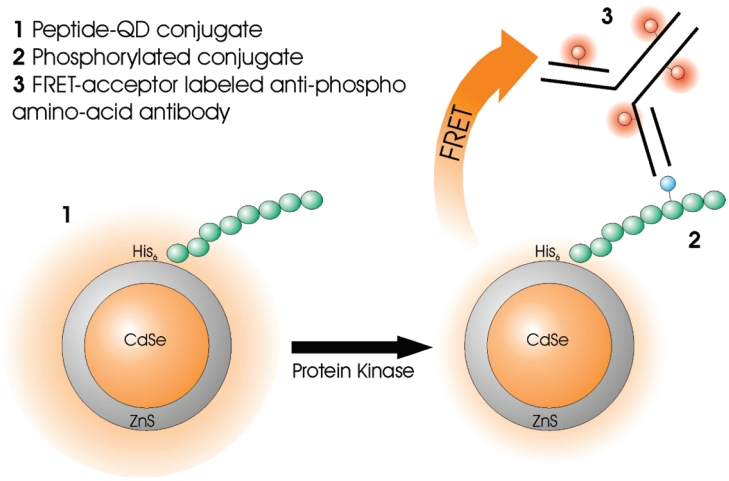

Figure 1. Schematic representation of kinase-mediated phosphorylation of peptide−QD conjugates, antibody recognition of phosphopeptide, and FRET detection.

hexahistidine motif.[17] Formation of the conjugates was confirmed by agarose gel electrophoresis (Figure 2), with the peptide-QD conjugates exhibiting altered electrophoretic mobility relative to unmodified QDs and having an approximate peptide/QD stoichiometry of 30:1. The peptide-modified QDs showed good colloidal stability in assay buffer and no signs of macroscopic aggregation for several weeks while stored at 4 °C.

The conjugates were then incubated with their respective tyrosine kinases in the presence of excess ATP for 1 h, after which the reaction was terminated with the addition of EDTA.[4] The phosphorylated reaction products were then detected by the addition of a phosphotyrosine-specific monoclonal antibody labeled AlexaFluor 647 (*ca.* 4 dyes/antibody, as measured by absorbance; see Methods). Owing to the appreciable overlap between the QD emission and dye absorption, the QD and fluorophore can participate in energy transfer.[18] Upon antibody recognition of the phosphorylated peptide−QDs, the fluorophores attached to the antibody are brought into the proximity of the nanocrystal surface, within a distance regime commensurate with efficient FRET (calculated Förster distance of 72 Å).[18]

Steady-state emission spectra revealed a concomitant decrease and increase in QD- and fluorophore-specific emission as a function of enzyme concentration, consistent with an enzyme-dependent FRET process for both Src and Abl (Figure 3), with maximum energy transfer efficiencies, *E*, of 75% and 45%, respectively. These experiments demonstrated variations in the extent of energy transfer in the two enzyme systems, most likely reflecting subtle differences in specific substrate preference and different assay buffer requirements for maximal activity.

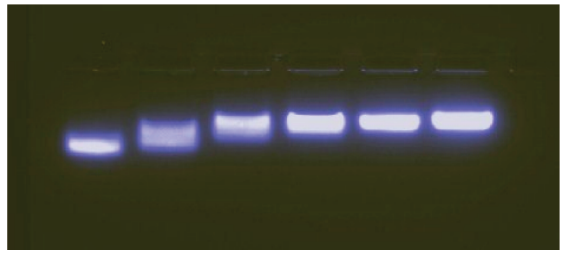

Figure 2. Gel mobility data of Src peptide incubated with 100 nM QDs. Lanes show increasing peptide/QD ratio, with (left to right): 0, 10, 20, 30, 40, 50:1 [peptide]:[QD].

To confirm that QD-fluorophore energy transfer was responsible for the observed intensity changes, time correlated single photon counting (TCSPC) was employed to examine changes in the QD exciton lifetime and revealed substantially diminished QD photoluminescence decay time in the presence of both enzyme and FRET acceptor-labeled antibody (Figure 4). Furthermore, control experiments in which ATP was omitted from the reaction mixture or with an inactive peptide substrate containing a Tyr to Phe mutation did not exhibit such behavior, providing strong support that the observed luminescence and lifetime changes were due to FRET originating from ATP-dependent tyrosine phosphorylation.

The ability to screen potential small-molecule inhibitors of tyrosine kinase activity is of significant importance in drug discovery.[3−5] As a proof of concept, we further sought to investigate whether the system could be employed to quantitatively assess enzyme inhibitor potency, with Abl kinase reactions carried out in 384-well fluorescence microplates. The enzyme was preincubated with serial dilutions of staurosporine, a broad spectrum kinase inhibitor, prior to initiating the reaction with the addition of ATP. The relatively long wavelength emission of both QD and dye effectively prevented staurosporine autofluorescence from contributing to the measured signal. Following quenching and antibody addition, the emission intensities at 605 and 670 nm (QD and Alexa Fluor 647 emission maxima, respectively) were measured and converted into a ratio, 670 nm/605 nm, to generate a dose

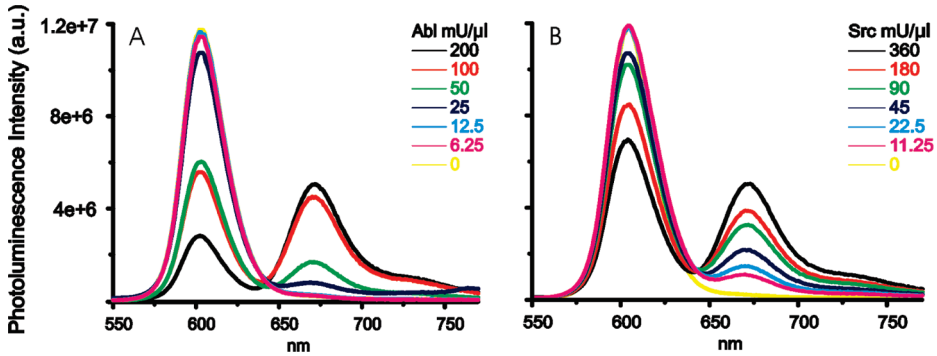

Figure 3. Steady-state emission spectra (λ_{ex} = 400 nm) of Abl (A) and Src (B) kinase reactions using peptide−QD conjugate substrates following 1 h enzyme reactions and addition of FRET-acceptor labeled antibody.

response curve which provided an IC_{50} value of 100 nM, in good accordance with previously reported literature values.[19] To determine the detection limits of this assay, we incubated the peptide−QD conjugates with serial dilutions of enzyme (Figure 5). Subnanomolar concentrations of the tyrosine kinases are detectable, corresponding to low mU/µL activity levels and comparable to current state-of-the-art techniques.[20]

In summary, we have demonstrated a new rapid, homogeneous, and generic assay for protein kinase activity based on QD-fluorophore energy transfer. This approach exhibits several advantages compared to the most sensitive kinase assays: it does not require radioisotope tracers specialized time-gated fluorescence detection procedures or additional coupled enzyme reactions for signal development affording a greater degree of simplicity in operation.[21,22] Furthermore, the use of color-tunable QDs and fluorophores here provides a convenient means to operate within a spectral window in which cellular autofluorescence, a common source of signal interference encountered by standard fluorometric approaches is at a minimum.[23] In addition, given the amenability of QDs to multiplexed biosensing, through judicious choice of QD/fluorophore pairs, enzyme substrates and specific antibodies, it should be possible to measure the activity of multiple kinases within a single reaction mixture. Finally, given the wide variety of post translational modifications and complementary antibodies available, our approach should open the door for the development of a new generation of enzyme assays based on QD FRET.

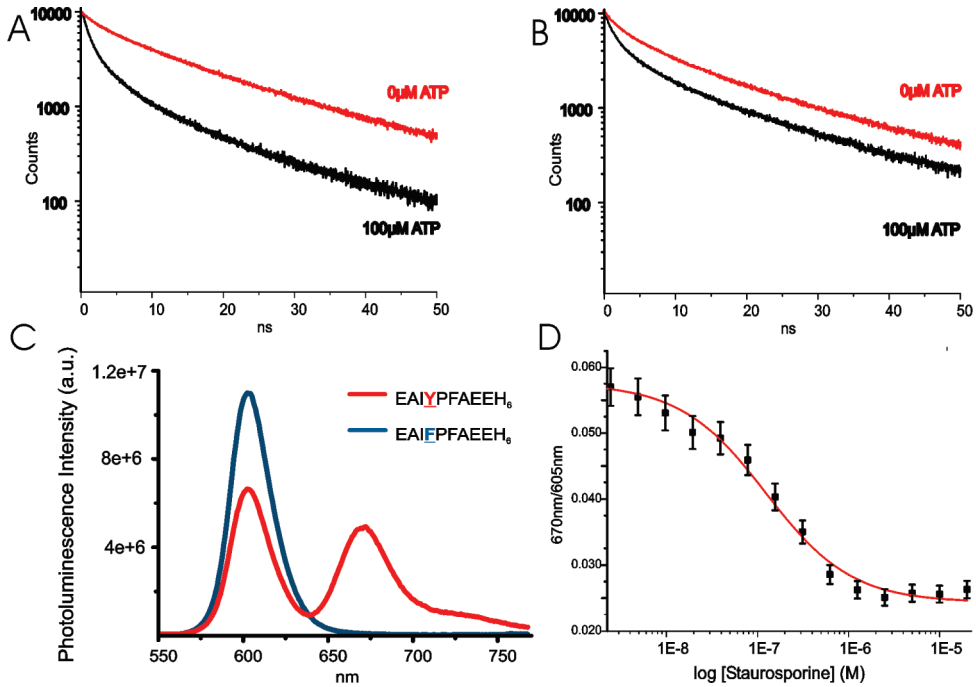

Figure 4. Transient PL emission detected by TCSPC, following 440-nm excitation of Abl- (A) and Src- (B) substrate-conjugated QDs, following kinase phosphorylation and acceptor-labeled antibody addition in the presence or absence of ATP; (C) effect of a Tyr to Phe mutation on steady state photoluminescence (Abl); (D) dose response curve of staurosporine inhibition of Abl activity.

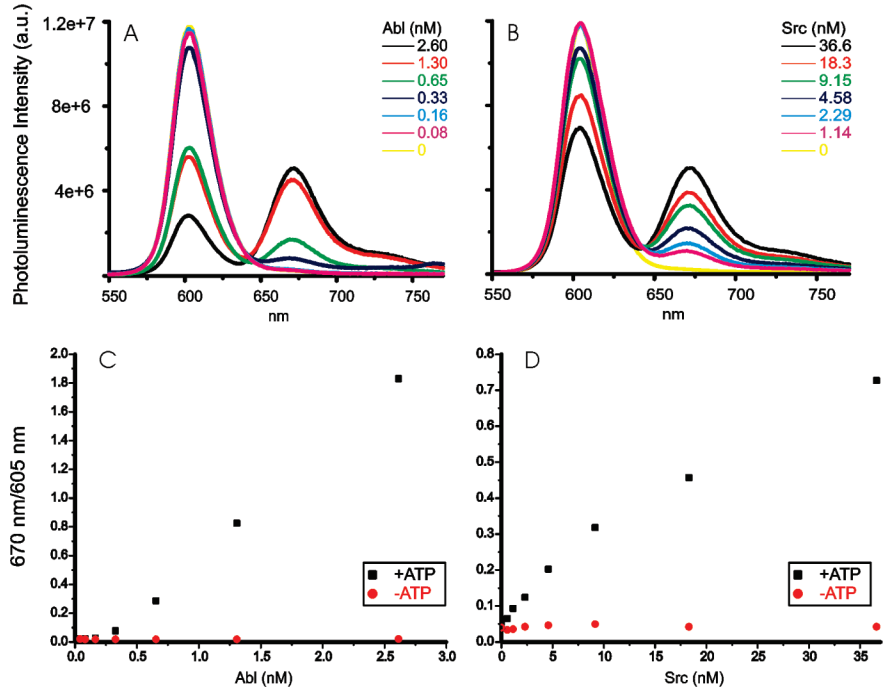

Figure 5. Photoluminescence FRET response as function of enzyme concentration for Abl (A) and Src (B) and plot of respective 670 nm/605 nm emission intensity ratios (C, D), demonstrating detection of subnanomolar concentrations of kinases.

METHODS

Quantum Dot-Peptide Conjugates. Peptide substrates and their respective Y/F substitution for v-Src and v-Abl (Ac-IYGEFKKKHHHHHH−$CONH_2$, Ac-IFGEFKKKHHHHHH−$CONH_2$, EAIYPFAEEHHHHHH−$CONH_2$, EAIFPFAEEHHHHHH−$CONH_2$[3]) were synthesized by standard automated Fmoc solid-phase peptide synthesis from a Rink-amide solid support on an aapptec ACT Apex 396 Peptide Synthesizer. The peptides were cleaved and deprotected with 95:2.5:2.5 trifluoroacetic acid (TFA)/H_2O/triisopropylsilane for 3 h and precipitated and washed with cold diethyl ether. The crude peptides were purified to >98%, as determined by LC−MS, on a semipreparative C_{18} HPLC column using a water/acetonitrile mobile phase containing 0.1% (v/v) TFA.

An aliquot of 3 μL of a 100 μM peptide solution in 10 mM sodium borate (pH 9.6) was added to 100 μL of 100 nM aqueous QDs (prepared as in ref 15). The mixture was vortexed briefly and allowed to stand at room temperature for 1 h, after which 10 μL of a 1% (w/v) solution of bovine serum albumin (≥99%, Sigma) in PBS was added. The mixture was then diluted in 25 mM HEPES (pH 7.5), 10 mM $MgCl_2$, and subjected to 3× 10-fold concentration/dilution cycles by centrifugal dialysis. The inclusion of BSA to a final concentration of 0.1% (w/v) provided enhanced colloidal stability and was necessary to avoid nonspecific binding.

Gel Electrophoresis. MPA-capped QDs (20 μL aliquots, 100 nM) in 10 mM sodium borate (pH 9.6) were incubated for 1 h with different volumes of peptide solution (1 μM) to achieve peptide/QD ratios ranging from 0−50:1. Glycerol (100%) was then added to the solutions to reach a final concentration of 5% (v/v) prior to gel electrophoresis. The peptide conjugates were run on 1% (w/v) agarose gels in 1× TAE buffer for 30 min at 10 V/cm and imaged under 365 nm illumination. The QDs exhibited retarded electrophoretic mobility in the presence of peptide, although the mobility did not change at ratios ≥30:1, suggesting saturation of free binding sites on the QD surface and providing a rough approximation of the final stoichiometry of the conjugate (Supporting Information, Figure S1)

Antibody Labeling. Monoclonal antiphosphotyrosine (Sigma, clone PT-66) was buffer exchanged into azide-free PBS by spin dialysis and incubated with a 10-fold molar excess of Alexa Fluor 647 succinimidyl ester (Invitrogen) in dimethylformamide (final DMF concentration <1%) for 1 h. The antibody was purified from excess-dye by spin dialysis until the retentate exhibited no further dye-specific absorption. The ratio of dye/protein was calculated according to the manufacturer's instructions and the conjugate was stored protected from light at 4 °C until use.

Enzyme Reactions and Photoluminescence. Purified recombinant Src and Abl were obtained from SignalChem and CalBiochem, respectively. Enzyme reactions were carried out in polypropylene microtubes. The reaction mixtures consisted of 10 μL enzyme, 20 μL peptide−QD (30 nM final concentration), and 10 μL ATP (100 μM final concentration) in 25 mM HEPES (pH 7.5), 10 mM $MgCl_2$, and 0.1% (w/v) BSA. The reactions were carried out for 1 h at 30 °C and quenched with 10 μL of EDTA (100 mM). A 2 μL portion of the antibody-dye conjugate was then added to reach a final QD/antibody ratio of 4:1. The mixture was incubated for an additional 30 min at room temperature prior to recording photoluminescence spectra. Steady-state spectra were recorded on a Jobin Yvon FluoroMax-4 Flourimeter. The spectra were corrected for variations in lamp and detector intensity with files from Jobin Yvon. Lifetimes were measured as described.[24]

Inhibitor Titration. Stock solutions of staurosporine (Calbiochem) were prepared in anhydrous DMSO and stored at −20 °C prior to use. Reactions were carried out in triplicate in 384-well black clear bottom plates (Nunc) with 2-fold dilutions of staurosporine in assay buffer. Reactions contained 5 μL of Abl (5 U), 4 μL of peptide−QD conjugate (30 nM), 1 μL ATP, and 10 μL of staurosporine stock. As staurosporine is an ATP-competitive inhibitor, the concentration of ATP was held at the apparent K_m of 12.5 μM. Quenching and antibody detection was carried out as described, and the photoluminescence intensity at 605 and 670 nm was measured on a Spectra Max Gemini XS fluorescence microplate reader.

Acknowledgment. Work at the Molecular Foundry was supported by the Office of Science, Office of Basic Energy Sciences, of the U.S. Department of Energy under Contract No. DE-AC02-05CH11231. M.M.S. thanks the EPSRC and ERC Grant "Naturale" for financial support.

Supporting Information Available: Details of QD surface modification and supporting results. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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