

## Research Article

# Fluorescence Resonance Energy Transfer in Quantum Dot-Protein Kinase Assemblies

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In search of viable strategies to identify selective inhibitors of protein kinases, we have designed a binding assay to probe the interactions of human phosphoinositide-dependent protein kinase-1 (PDK1) with potential ligands. Our protocol is based on fluorescence resonance energy transfer (FRET) between semiconductor quantum dots (QDs) and organic dyes. Specifically, we have expressed and purified the catalytic kinase domain of PDK1 with an N-terminal histidine tag [His<sub>6</sub>-PDK1( $\Delta$ PH)]. We have conjugated this construct to CdSe-ZnS core-shell QDs coated with dihydrolipoic acid (DHLA) and tested the response of the resulting assembly to a molecular dyad incorporating an ATP ligand and a BODIPY chromophore. The supramolecular association of the BODIPY-ATP dyad with the His<sub>6</sub>-PDK1( $\Delta$ PH)-QD assembly encourages the transfer of energy from the QDs to the BODIPY dyes upon excitation. The addition of ATP results in the displacement of BODIPY-ATP from the binding domain of the His<sub>6</sub>-PDK1( $\Delta$ PH) conjugated to the nanoparticles. The competitive binding, however, does not prevent the energy transfer process. A control experiment with QDs, lacking the His<sub>6</sub>-PDK1( $\Delta$ PH), indicates that the BODIPY-ATP dyad adsorbs nonspecifically on the surface of the nanoparticles, promoting the transfer of energy from the CdSe core to the adsorbed BODIPY dyes. Thus, the implementation of FRET-based assays to probe the binding domain of PDK1 with luminescent QDs requires the identification of energy acceptors unable to interact nonspecifically with the surface of the nanoparticles.

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## 1. INTRODUCTION

The outstanding photophysical properties of semiconductor quantum dots (QDs) have encouraged the development of binding assays, based on fluorescence resonance energy transfer (FRET), for the detection of a diversity of biorelevant analytes [1–5]. Some of these sensing protocols are aimed at the investigation of protein-ligand interactions relying on FRET in QD-protein-dye assemblies. For example, the maltose binding protein (MBP) was expressed with a C-terminal oligohistidine segment to promote its adsorption on the surface of CdSe-ZnS core-shell QDs coated with dihydrolipoic acid (DHLA) [6, 7]. By conjugating the dark quencher QSY9 to  $\beta$ -cyclodextrin, it was shown that titration of the solution containing QD-bound MBP with  $\beta$ -cyclodextrin-QSY9 effectively quenched the nanoparticle luminescence. Such a system was further demonstrated to function as a prototype nanosensor. In fact, the QD luminescence could be re-

stored on subsequent titration with maltose, which competitively displaced  $\beta$ -cyclodextrin-QSY9 from the sugar binding pocket. In principle, a similar strategy could be invoked for the detection of compounds that competitively displace an enzyme-bound dye-labeled substrate for use in high-throughput drug screening assays.

Discovering potent and selective inhibitors of individual members of the protein kinase superfamily has proved to be a long and arduous task. Whereas a large number of high affinity inhibitors have been identified, the majority of such compounds exhibit broad specificity since they typically bind in the ATP-binding cleft shared among all kinase enzymes [8–10]. The matter is further complicated by the fact that the protein kinase superfamily is the largest enzyme family with an estimated 518 members in the human genome [11]. The broad specificity kinase inhibitors are commonly identified in high-throughput enzymatic screening assays, whereby kinase activity is monitored by phosphorylation-induced

fluorescence changes in model peptide substrates. Such enzymatic assays demand active forms of the kinase, which require one or more critical residues to be phosphorylated. Inspection of X-ray structures available for the catalytic domains of different kinases in their phosphorylated and active forms shows highly homologous structures, especially in the ATP-binding cleft [12]. However, known structures of catalytic kinase domains in their unphosphorylated and inactive forms reveal greater divergence of conformational space, which may better accommodate selective binding of compounds that stabilize inactive conformations [12]. Thus, we sought to determine whether competitive displacement of dye-labeled ATP from protein kinase-QD assemblies could be used as a sensitive assay for the detection of compounds that bind either active or inactive forms of kinases.

The human phosphoinositide-dependent protein kinase-1 (PDK1) is particularly well suited to initiate development of QD-mediated competitive binding assays. PDK1 is a member of the AGC subfamily of serine-threonine protein kinases and is comprised of an N-terminal catalytic kinase domain and a C-terminal pleckstrin homology (PH) domain [13]. Baculovirus-mediated expression in Sf9 insect cells and affinity purification of the recombinant catalytic domain construct of PDK1 (residues 51–359) with an N-terminal His<sub>6</sub> tag is well established, and this construct [His<sub>6</sub>-PDK1( $\Delta$ PH)] exhibits high solution stability [14–16]. His<sub>6</sub>-PDK1( $\Delta$ PH) catalyzes autophosphorylation of Ser-241 located in its activation or T-loop, which is the only post-translational modification required for its kinase function [14–16]. Most interestingly, Ser-241 monophosphorylated His<sub>6</sub>-PDK1( $\Delta$ PH) remains relatively inactive, and it is ultimately activated by allosteric interaction with a phosphorylated hydrophobic motif (HM) at the C-terminus of its protein substrate [14–16]. The phosphorylated HM has been termed the “PDK1 interacting fragment” or PIF, and it binds to the PIF pocket on the N-lobe of the PDK1 kinase domain [17–20]. Kinetic studies indicate that His<sub>6</sub>-PDK1( $\Delta$ PH) approximates a Rapid Equilibrium Random Bi Bi System so that it readily forms a high-affinity complex with ATP in the absence of protein substrate [15], as exemplified by the X-ray structure reported for the binary enzyme-nucleotide complex [14]. In this article, we report the ability of His<sub>6</sub>-PDK1( $\Delta$ PH) to adsorb on the surface of CdSe-ZnS core-shell QDs coated with DHLA and the interactions of the resulting assemblies with a compound incorporating an ATP ligand and a BODIPY chromophore within its molecular skeleton.

## 2. MATERIALS AND METHODS

CdSe-ZnS core-shell QDs were prepared following literature procedures [21, 22] and then coated with DHLA according to an established protocol [23]. His<sub>6</sub>-PDK1( $\Delta$ PH) was expressed using the Bac-to-Bac Baculovirus Expression System (Invitrogen, Inc., Carlsbad, Calif, USA) and purified as reported earlier [24]. BODIPY TR adenosine 5'-triphosphate (BODIPY-ATP) was purchased from commercial sources (Invitrogen, Inc.). Visible absorption spectra were recorded with a Varian Cary 100 Bio spectrometer using quartz cells with a path length of 0.5 cm. Emission spectra were recorded

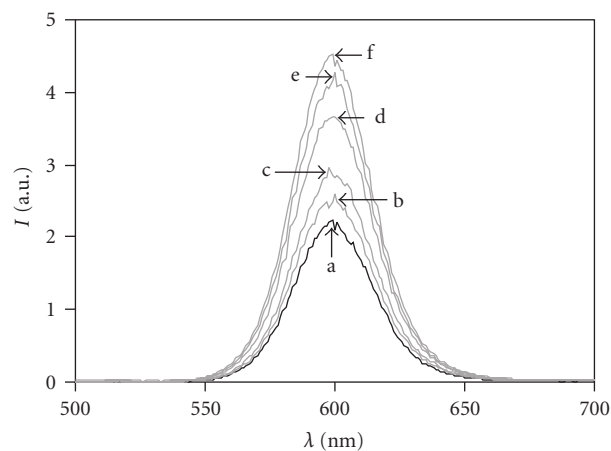


FIGURE 1: Emission spectra of CdSe-ZnS core-shell QDs ( $0.1 \mu\text{M}$  in borate buffer,  $\text{pH} = 7.4$ ,  $T = 20^\circ\text{C}$ ,  $\lambda_{\text{EX}} = 442 \text{ nm}$ ) coated with DHLA in the absence (a) and presence of  $0.5$  (b),  $1.0$  (c),  $2.0$  (d),  $5.0$  (e), and  $10.0$  (f)  $\mu\text{M}$  of His<sub>6</sub>-PDK1( $\Delta$ PH).

with a Varian Cary Eclipse spectrometer in aerated solutions and are uncorrected with the exception of those shown in Figure 8.

## 3. RESULTS AND DISCUSSION

The emission spectrum (a in Figure 1) of CdSe-ZnS core-shell QDs coated with DHLA in borate buffer ( $\text{pH} = 7.4$ ) shows an intense band centered at  $600 \text{ nm}$  with a quantum yield of  $0.2$ . The addition of His<sub>6</sub>-PDK1( $\Delta$ PH) to this dispersion affects the emissive behavior of the nanoparticles. Specifically, the luminescence increases with the concentration of His<sub>6</sub>-PDK1( $\Delta$ PH) (a–f in Figure 1) in agreement with the adsorption of the protein on the surface of the QDs. Indeed, literature reports [6] demonstrate that the coating of CdSe-ZnS core-shell QDs with histidine-tagged proteins leads to a luminescence enhancement, as a result of the significant change in the local environment around the emissive inorganic particles. In particular, the plot (see Figure 2) of the emission intensity of our QDs at  $600 \text{ nm}$  against the relative concentration of His<sub>6</sub>-PDK1( $\Delta$ PH) shows that saturation is reached at a protein/QD ratio of ca.  $30$ . Under these conditions, the luminescence quantum yield of the nanoparticles is  $0.4$ .

The absorption spectrum (a in Figure 3) of BODIPY-ATP shows an intense band for the fluorescent component of this dyad in the visible region. This absorption is positioned in the same range of wavelengths where our QDs emit (b in Figure 3) with an overlap integral of  $5.3 \cdot 10^{-13} \text{ M}^{-1} \text{ cm}^3$ , suggesting that the BODIPY dye can accept the excitation energy of these nanoparticles. The overlap integral ( $J$ ) was calculated from the emission intensity ( $I$ ) of the quantum dots, the molar extinction coefficient ( $\epsilon$ ) of the BODIPY dye and the wavelength ( $\lambda$ ) using

$$J = \frac{\int_0^\infty I \epsilon \lambda^4 d\lambda}{\int_0^\infty I d\lambda}. \quad (1)$$

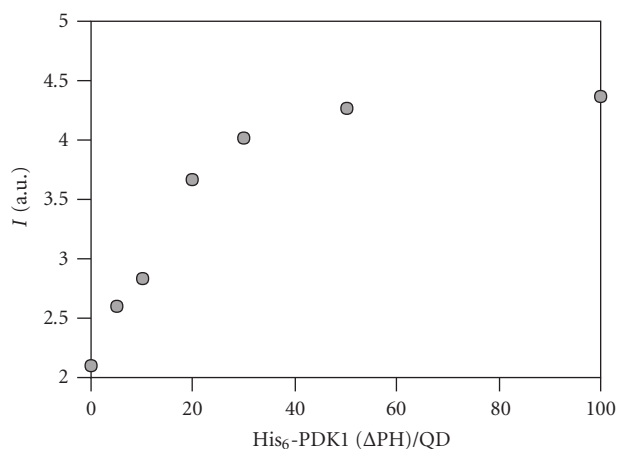


FIGURE 2: Emission intensity at 600 nm of CdSe-ZnS core-shell QDs ( $0.1 \mu\text{M}$  in borate buffer,  $\text{pH} = 7.4$ ,  $T = 20^\circ\text{C}$ ,  $\lambda_{\text{EX}} = 442 \text{ nm}$ ) coated with DHLA against the ratio between the concentration of His<sub>6</sub>-PDK1(ΔPH) and that of the QDs.

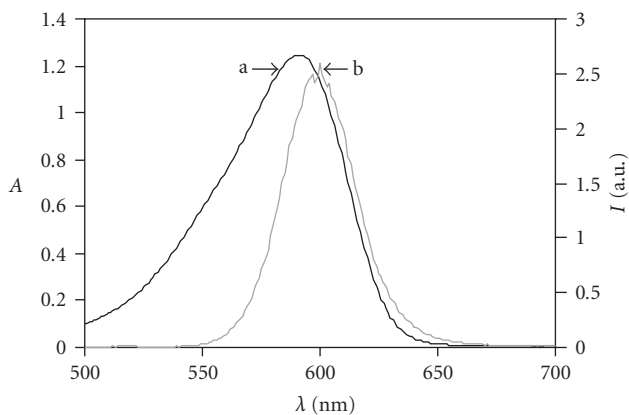


FIGURE 3: Absorption spectrum (a) of BODIPY-ATP ( $30 \mu\text{M}$ ) and emission spectrum (b) of CdSe-ZnS core-shell QDs ( $0.1 \mu\text{M}$ ) and His<sub>6</sub>-PDK1(ΔPH) in borate buffer ( $\text{pH} = 7.4$ ,  $T = 20^\circ\text{C}$ ,  $\lambda_{\text{EX}} = 442 \text{ nm}$ ).

Thus, the supramolecular association of the ATP fragment of the dyad with the His<sub>6</sub>-PDK1(ΔPH) coating of the QDs can be exploited to bring the BODIPY fluorophore in close proximity to the nanoparticles and encourage the transfer of energy from the emissive CdSe core to the organic dye (a in Figure 4). Indeed, the addition of increasing amounts of BODIPY-ATP to the His<sub>6</sub>-PDK1(ΔPH)-QD conjugate alters significantly the emission spectrum (a–g in Figure 5). In particular, the emission band of the QDs at 600 nm fades with an increase in the concentration of BODIPY-ATP. Concomitantly, a second band for the BODIPY fluorophore grows at 624 nm in agreement with the expected transfer of energy from the nanoparticles to the organic dye upon excitation.

In principle, the addition of a ligand able to displace BODIPY-ATP from the complementary recognition site of the His<sub>6</sub>-PDK1(ΔPH)-QD conjugate should result in the physical separation of the nanoparticle donor from the BOD-

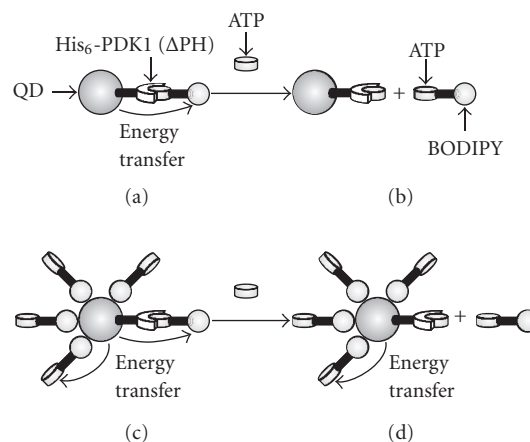


FIGURE 4: The supramolecular association of BODIPY-ATP with His<sub>6</sub>-PDK1(ΔPH)-QD encourages the transfer of energy from QD to BODIPY upon excitation (a). ATP displaces BODIPY-ATP from the recognition site of His<sub>6</sub>-PDK1(ΔPH)-QD and is expected to prevent energy transfer (b). The nonspecific adsorption of BODIPY-ATP on the surface of QD, however, can also encourage energy transfer (c). Under these conditions, the displacement of BODIPY-ATP by ATP is not sufficient to prevent energy transfer.

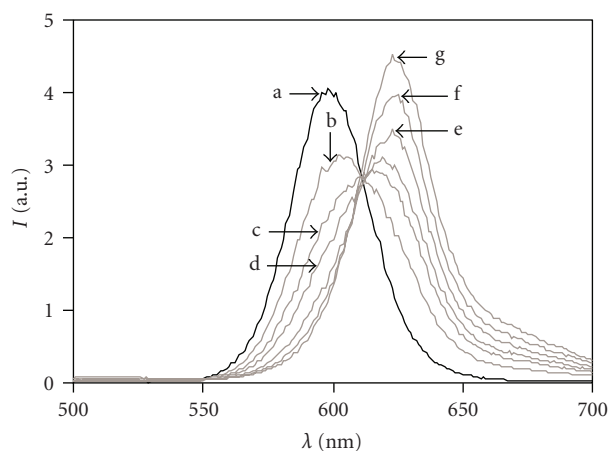


FIGURE 5: Emission spectra of a dispersion of CdSe-ZnS core-shell QDs ( $0.1 \mu\text{M}$ ) and His<sub>6</sub>-PDK1(ΔPH) ( $10 \mu\text{M}$ ) in borate buffer ( $\text{pH} = 7.4$ ,  $T = 20^\circ\text{C}$ ,  $\lambda_{\text{EX}} = 442 \text{ nm}$ ) in the absence (a) and presence of 1.0 (b), 3.0 (c), 5.0 (d), 10.0 (e), 20.0 (f), and 30.0 (g)  $\mu\text{M}$  of BODIPY-ATP.

IPY acceptor and, therefore, suppress the energy transfer processes (b in Figure 4). Consistently, the addition of increasing amounts of ATP to a solution of the complex formed between BODIPY-ATP and His<sub>6</sub>-PDK1(ΔPH)-QD causes a decrease in emission intensity with a concomitant hypsochromic shift (a–f in Figure 6). Instead, the titration of a dispersion of the QDs ( $0.1 \mu\text{M}$ ) with ATP (0–30  $\mu\text{M}$ ) has no influence on their emission spectrum. The deconvolution of the final spectrum (a in Figure 7) shows the observed emission to be the sum of two distinct bands (b and c in Figure 7). One of them (b in Figure 7) is centered at 600 nm, corresponds to the emission

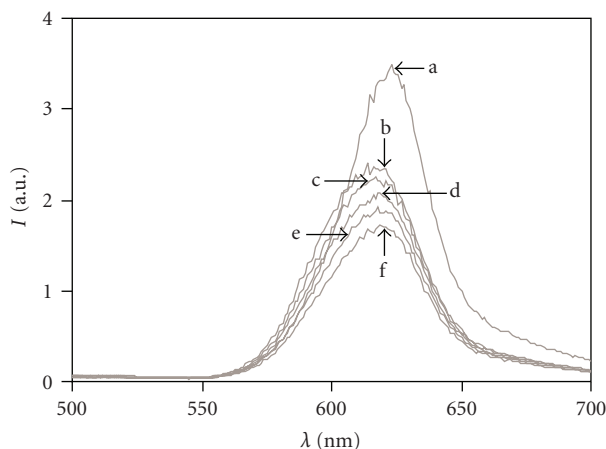


FIGURE 6: Emission spectra of a mixture of CdSe-ZnS core-shell QDs ( $0.1 \mu\text{M}$ ), His<sub>6</sub>-PDK1( $\Delta\text{PH}$ ) ( $10 \mu\text{M}$ ) and BODIPY-ATP ( $10 \mu\text{M}$ ) in borate buffer ( $\text{pH} = 7.4$ ,  $T = 20^\circ\text{C}$ ,  $\lambda_{\text{EX}} = 442 \text{ nm}$ ) in the absence (a) and presence of 1 (b), 3 (c), 11 (d), 31 (e), and 301 (f)  $\mu\text{M}$  of ATP.

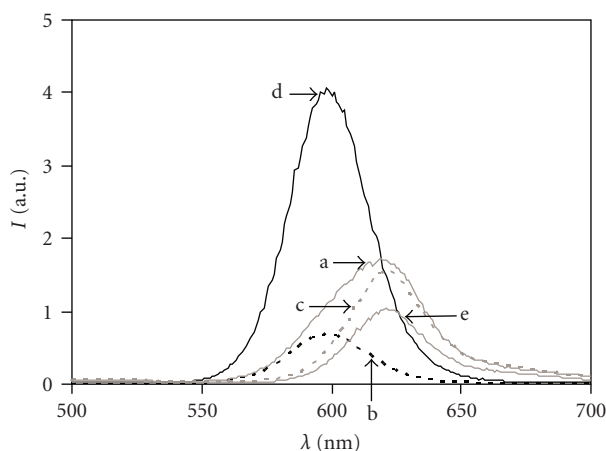


FIGURE 7: Emission spectra of a mixture of CdSe-ZnS core-shell QDs ( $0.1 \mu\text{M}$ ), His<sub>6</sub>-PDK1( $\Delta\text{PH}$ ) ( $10 \mu\text{M}$ ), BODIPY-ATP ( $10 \mu\text{M}$ ) and ATP ( $301 \mu\text{M}$ ) (a) CdSe-ZnS core-shell QDs ( $0.1 \mu\text{M}$ ) (d) and BODIPY-ATP ( $10 \mu\text{M}$ ) (e) in borate buffer ( $\text{pH} = 7.4$ ,  $T = 20^\circ\text{C}$ ,  $\lambda_{\text{EX}} = 442 \text{ nm}$ ). Deconvolution (b and c) of trace a.

of the QDs, and its intensity is significantly smaller than that recorded for a dispersion of the QDs alone (d in Figure 7) under otherwise identical conditions. The other band (c in Figure 7) is centered at 624 nm, corresponds to the emission of the BODIPY dyes, and its intensity is greater than that recorded for BODIPY-ATP alone (e in Figure 7) under otherwise identical conditions. Thus, the QDs recover their luminescence only in part, even after the addition of a relatively large amount of ATP, and still sensitize the emission of the BODIPY dyes under these conditions. These observations suggest that a fraction of the BODIPY-ATP conjugates remains associated with the QDs even in the presence of an excess of ATP (c and d in Figure 4). In agreement with this interpretation, the addition of increasing amounts of

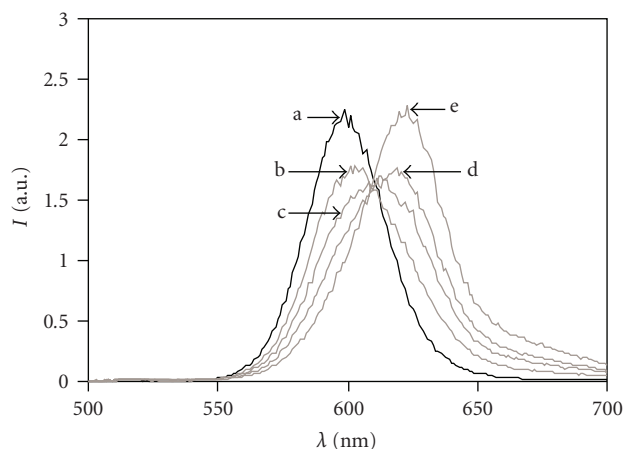


FIGURE 8: Emission spectra of CdSe-ZnS core-shell QDs ( $0.1 \mu\text{M}$  in borate buffer,  $\text{pH} = 7.4$ ,  $T = 20^\circ\text{C}$ ,  $\lambda_{\text{EX}} = 442 \text{ nm}$ ) in the absence (a) and presence of 1.0 (b), 3.0 (c), 5.0 (d), and 10.0 (e)  $\mu\text{M}$  of BODIPY-ATP.

BODIPY-ATP to a dispersion of QDs leads to the disappearance of the nanoparticle emission at 600 nm with the concomitant appearance of the BODIPY emission at 624 nm (a-e in Figure 8). Hence, the BODIPY-ATP can accept the excitation energy of the QDs despite the absence of the His<sub>6</sub>-PDK1( $\Delta\text{PH}$ ) coating around the nanoparticles. These results suggest that the BODIPY-ATP conjugate can adsorb non-specifically on the QDs, presumably, as a result of interactions between the chromophoric component and the surface of the nanoparticles.

#### 4. CONCLUSIONS

The incubation of CdSe-ZnS core-shell QDs, coated with DHLA, and His<sub>6</sub>-PDK1( $\Delta\text{PH}$ ) in borate buffer ( $\text{pH} = 7.4$ ) leads to the adsorption of proteins on the surface of the nanoparticles with a concomitant luminescence enhancement. The dependence of the emission intensity on the protein concentration suggests that an average of ca. 30 proteins adsorb on each QD. The exposure of the His<sub>6</sub>-PDK1( $\Delta\text{PH}$ )-QD conjugate to a BODIPY-ATP dyad brings the inorganic nanoparticle in close proximity to the BODIPY chromophore, encouraging the transfer of energy from the former to the latter. Consistently, the emission band of the QDs fades, as the concentration of BODIPY-ATP increases, with the concomitant growth of an emission band for organic dye. The competitive binding of ATP, however, restores the nanoparticle luminescence only in part. Indeed, control experiments show the occurrence of energy transfer between QDs lacking the His<sub>6</sub>-PDK1( $\Delta\text{PH}$ ) shell and BODIPY-ATP. These observations suggest that the organic chromophore adsorbs nonspecifically on the surface of the nanoparticles. Thus, the development of binding assays for PDK1 based on QDs and FRET demands the identification of strategies to prevent the direct adsorption of the energy acceptor on the surface of the nanoparticle donor.

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