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ABSTRACT: Enzymes provide the critical means by which to catalyze almost all biological reactions in a controlled manner. Methods to harness and exploit their properties are of strong current interest to the growing field of biotechnology. In contrast to depending upon recombinant genetic approaches, a growing body of evidence suggests that apparent enzymatic activity can be enhanced when located at a nanoparticle interface. We use semiconductor quantum dots (QDs) as a well-defined and easily bioconjugated nanoparticle along with *Escherichia coli*-derived alkaline phosphatase (AP) as a prototypical enzyme to seek evidence for this process in a de novo model system. We began by first assessing whether the relatively large dimeric AP protein (∼100 kDa) can be assembled onto two differentially sized green and red CdSe/ZnS core/shell QDs in a ratiometric and structurally controlled manner; such assembly is necessary to minimize heterogeneity within the bioconjugate and provide intimate control over the experimental format. For this, analysis is undertaken using both structural modeling and physicochemical characterization techniques including dynamic light scattering and agarose gel electrophoresis; these all provide strong supporting evidence for controlled AP attachment to the QDs. The enzymatic activity of AP-QD bioconjugates assembled on the different QDs and displaying variable AP:QD ratios was then assayed against equivalent amounts of freely diffusing enzyme controls in both conventional excess substrate formats and a varying enzyme–fixed substrate format that is more amenable in general to concentration-limited nanoparticle conjugates. The resulting experimental data were then analyzed in the context of the Michaelis–Menten model and compared. The results show a general equivalency between the two assay formats while also providing evidence for an increase in apparent AP activity of ca. 25% when attached to the QDs. Some discussion is provided on the underlying mechanisms that may contribute to the enhanced activity along with the implications of this work toward future research.

INTRODUCTION

Enzymes, nature’s catalysts, are highly efficient at accelerating biochemical processes with precise specificity.1,2 This has made enzymes of considerable interest for utilization in a myriad of biotechnological applications, including as part of sensors; remediation materials; bioreactors; nanomedicine; theranostics; artificial biosynthesis; food, biofuel, and industrial product preparation processes; and molecular biology reagents, to name but a few.3–5 To adapt enzymes for these purposes, researchers have employed recombinant DNA engineering techniques to enhance their stability and activity for use across this wide range of reaction space.3–6 6 However, protein engineering techniques, including the widely utilized error-prone polymerase chain reaction and in vitro recombination techniques, are oftentimes tedious, time-consuming, and expensive along with providing limited improvement in results because of the need to conserve critical protein structures for specific “lock and key” fitting and function within the catalytic site.9 To utilize enzymes for specific applications, they are very often immobilized onto surfaces and, more recently, nanoparticles.4,5

Research into immobilizing enzymes onto support structures has been underway for more than half a century. The enzyme support structure or carrier is typically an insoluble material that is designed to capture the enzyme at an interface and
facilitate later separation if needed along with controlling placement for subsequent use.\textsuperscript{10–13} Covalent and adsorption-based immobilization techniques have also been used in part to improve the operational efficiency of enzymes in environments that possess pH, temperature, and substrate concentration ranges beyond those typically experienced in native enzymatic habitats.\textsuperscript{14,15} However, in a significant number of examples, immobilization reduces enzyme activity in many of these environments, as the native configuration of the protein is perturbed.\textsuperscript{16,17} In an effort to improve the activity of immobilized enzymes, recent work has focused on utilizing nanoparticles as carriers for enzymes. Nanoparticles have been shown to promote both the retention of the native nanoparticles as carriers for enzymes. Nanoparticles have been immobilized enzymes, recent work has focused on utilizing nanoparticles suspended in solution) are suggested to increase the apparent activity of immobilized versus free enzyme as they experience diffusion limitations that are inherently less than that of immobile solid or porous enzyme carriers and are capable of being displayed in a higher localized density. Various nanoparticulate materials including those composed of gold,\textsuperscript{19–21} luminescent semiconductor nanocrystals (i.e., quantum dots),\textsuperscript{22–24} polystyrene,\textsuperscript{25,26} iron oxide,\textsuperscript{27} and calcium phosphate\textsuperscript{28} have all been used as enzyme carriers.

The use of quantum dots (QDs) as mobile enzyme carriers offers several key advantages over use of conventional nanoparticles for such studies, including defined sizes with low polydispersity and excellent photophysical properties; wide utility in Förster resonance energy transfer (FRET)-based nanosensors; retention of biological activity; and well-established reproducible and homogeneous surface chemistry modifications that promote QD conjugate stability while allowing control over the attached biomolecule’s orientation, position, and valence (i.e., number of biomolecules attached per QD) in many cases.\textsuperscript{29–33} There are a handful of recent reports regarding the immobilization of enzymes onto QDs including the use of organophosphorus hydrolase,\textsuperscript{34} glucose oxidase,\textsuperscript{35–38} and luciferase.\textsuperscript{39} These studies demonstrated promising results regarding peptide and nerve agent detection, diabetes management, in vivo animal imaging, and sensing of enzymatic inhibitors. There are also examples in which QD materials have been bioconjugated with endoglucanase and exoglucanase for cellulose digestion and ferredoxin:NADP+ oxidoreductase for understanding component elements of photosynthesis.\textsuperscript{23,24} However, a fundamental analysis regarding the combined effects of enzyme-to-QD ratio and QD size on immobilized enzyme behavior still needs to be more fully explored.

Our prior work in this context analyzed enzymatic activity around QD–peptide substrate bioconjugates by monitoring changes in FRET resulting from the proteolytic activity of the enzyme trypsin.\textsuperscript{40} This was accomplished by conjugating a Cy3 acceptor dye to the QD donor through a peptidyl linkage that contains a single arginine residue as a cleavage site, which when subsequently cut by trypsin, disassociates the Cy3 from the QD and disengages FRET. This work was extended to also monitor trypsin activity around the QD in a time-gated fashion along with monitoring the simultaneous activity of two proteases in which one activated the second from proenzyme to active species in both a direct and time-gated manner.\textsuperscript{41,42} In particular, apparent trypsin activity on the QD–peptide substrates appeared to be significantly enhanced, manifesting an enzymatic efficiency that was ∼5× over interactions with freely diffusing substrate alone.\textsuperscript{40} In this work, we investigate the converse configuration in a de novo system by immobilizing the enzyme alkaline phosphatase (AP) directly to the QD nanocrystal and subsequently monitoring the behavior of immobilized enzyme versus that of freely diffusing enzyme controls. The ratio and spatial arrangement of immobilized enzyme on the QDs is first carefully controlled by using polyhistidine (His)\textsubscript{n}-sequences appended to the AP termini which coordinate to the QD surface. Two distinctly sized QDs are used to probe the potential effects that surface area/curvature has on enzyme activity while the valence of AP to QD is discretely varied. Full enzyme progress curves for varying AP-QD valences are acquired in both conventional excess substrate formats along with a varying enzyme–fixed substrate format. Results from this hybrid inorganic–protein bioconjugate system again provide evidence for apparently enhanced enzymatic activity at a nanoparticle interface.

\section{EXPERIMENTAL METHODS}

\textbf{Quantum Dots.} CdSe/ZnS (core/shell) QDs with emission centered at ca. 525 and 625 nm were synthesized and solubilized with dihydrolipoic acid-based zwitterionic compact ligand (DHLA-CL4) as described in detail elsewhere.\textsuperscript{43} The zwitterionic portion of DHLA-CL4 consists of a single tertiary amine (pK\textsubscript{a} 9.06) which terminates in two alkyl carboxyl groups (pK\textsubscript{a} values of 3.29 and 3.93). QDs functionalized with this ligand demonstrate biocompatibility, high quantum yields, and long-term stability across a broad range of pH (5–13) while still retaining the ability to be functionalized with proteins and peptides through either covalent attachment or metal-affinity coordination.\textsuperscript{43}

\textbf{Transmission Electron Microscopy.} Structural characterization of as-prepared QDs was carried out using a JEOL 2200-FX analytical high-resolution transmission electron microscope (TEM) with a 200 kV accelerating voltage. Samples for TEM were prepared by spreading a drop (5–10 μL) of the filtered QD dispersion (filtered using 0.25 μm Millipore syringe filters) onto ultrathin carbon/holey support grid (Ted Pella, Inc.) and letting it dry. The concentration of QDs in the deionized water used was typically ∼1 μM for the 625 QDs and ∼5 μM for the 525 QDs. Individual particle sizes were measured using a Gatan Digital Micrograph (Pleasanton, CA); average sizes along with standard deviations were extracted from analysis of at least 50–100 nanoparticles.

\textbf{Alkaline Phosphatase Preparation.} The gene encoding the \textit{Escherichia coli} AP was amplified from the pECAN45 expression vector detailed in Swain et al. using Taq DNA polymerase (New England Biolabs) and primers designed to incorporate BamHI and XhoI restriction sites at the 5′ and 3′ ends, respectively.\textsuperscript{44} PCR product was purified using the Qiagen PCR Purification kit (QIAGEN). Amplified AP gene and PET22 vector were digested with BamHI and XhoI (New England Biolabs, NEB) for 4 h. The vector and the 1383 basepair PCR product were then purified from an agarose gel following electrophoresis using a QIAGEN Gel Extraction Kit (QIAGEN). The DNA fragments were ligated using T4 DNA Ligase (NEB) at a 1:3 PET22 vector:insert ratio overnight at 16 °C. Ligation products were transformed into TOP10 cells (Invitrogen) and plated on selection media. Individual clones were sequenced by Eurofins MWG Operon. Correct clones were transformed into \textit{E. coli} strain BL21 (DE3) cells (Invitrogen) for protein expression.
AP expression was induced with isopropyl β-D-1-thiogalactopyranoside (IPTG), and protein was purified from the periplasm of expression cultures using an osmotic shock protocol and immobilized metal affinity chromatography as previously described. Briefly, 500 mL volumes of Terrific Broth containing ampicillin were inoculated with 1:100 volume of an overnight culture. The culture was grown at 37 °C for 3 h until the optical density at 600 nm was between 0.8–1.0. The culture was then induced with 1 mM IPTG and transferred to 30 °C for 3 h. The cells were then pelleted via centrifugation and resuspended in cold osmotic shock buffer (0.1 M Tris–HCl pH 7.3, 0.75 M sucrose) containing 0.1 mg/mL hen egg lysozyme (Sigma). The cell suspension was placed on ice, and 1 mM EDTA was added dropwise to twice the volume. Following a 15 min incubation, MgCl2 was added to a final concentration of 10 mM. Bacterial spheroplasts were pelleted, and the soluble material was transferred to a 50 mL Falcon tube. A 500 μL bed volume of Ni-NTA Sepharose resin (GE Healthcare) was added to the solution, and the solution was incubated at 4 °C on a Dynal Rotisserie for a minimum of 3 h. Batch washes of the resin were performed with 60 bed volume equivalents of wash buffer (20 mM phosphate buffer pH 7.3, 0.4 M NaCl, 25 mM imidazole) and low speed centrifugation a total of three times. AP was eluted from the Ni-NTA column with wash buffer containing 500 mM imidazole. The 1 mL of eluate was further purified on a Superdex G75 10/300 GL column (GE Healthcare) using a BioRad BioLogic DuoFlow system and 1X phosphate buffered saline (137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, pH 7.4, PBS) as the running buffer. Purified AP was quantitated using a Nanodrop 1000 spectrophotometer and frozen in 20% glycerol for long-term storage at −80 °C.

Protein expression typically yielded 15–25 μg/L of purified protein.

**Assembly of AP-QD Bioconjugates.** A 2 nM purified AP enzyme solution was created with AP buffer (100 mM NaCl, 100 mM Tris-Cl, 50 mM MgCl2, 1% Tween 20 in H2O, pH 9.5) within a 1.5 mL Eppendorf tube. The enzyme solution was vortexed for 10 s at 500 rpm and microcentrifuged for 5 s to ensure solution homogeneity. Molar equivalents of QDs were added to the enzyme/buffer solution to create enzyme-to-QD ratios of 0:1, 2:1, 12:1, and 24:1. Enzyme-only control solution had no QDs added.

**Agarose Gel Electrophoresis.** AP was allowed to self-assemble to 525 nm QDs at increasing stoichiometric ratios (0–8 AP per QD). Each reaction contained 5 picomoles of QD; the assemblies were incubated at room temperature for 30 min to facilitate assembly, and the total volume for each assembly was 20 μL. Assemblies were run on a precast 1.5% agarose low electroendosmosis (EEO) gel in 1X Tris–Borate–EDTA buffer, pH 8.3. The gel was visualized with a BioRad Gel Image Analyzer to monitor QD migration.

**Dynamic Light Scattering and ζ-Potential.** Dynamic light scattering (DLS) measurements were carried out using a CGS-3 goniometer system equipped with a HeNe laser illumination at 633 nm and a single-photon counting avalanche photodiode for signal detection (ALV, Langen, Germany). The autocorrelation function was performed by an ALV-5000/EPP photon correlator (ALV, Langen, Germany) and analyzed using Dispersion Technology Software (DTS, Malvern Instruments Ltd, Worcestershire, UK). QDs (625 nm; 20–50 nM in 0.1X PBS buffer, 525 nm; 50–100 nM in 0.1X PBS buffer, pH 7.4) and enzyme (1.5–3.0 μM) were prefiltered through 0.25 μm syringe filters prior to DLS measurements to remove dust or impurities in the sample. To measure the effect of enzyme conjugation on the hydrodynamic size of NPs, we used the prefiltered QD solution mixed with the desired amount of enzyme (4–30 times that of QD concentration) with and without substrates (∼10 μM). Sample temperature was maintained at 20 °C. For each sample, the autocorrelation function was the average of three runs of 10 s each and then repeated at different scattering angles (within 80° and 110°). CONTIN analysis was then used to extract number versus hydrodynamic size profiles for the dispersions studied. For zeta-potential (ζ-potential) measurement, laser doppler velocimetry (LDV) measurements were performed using a ZetaSizer NanoSeries equipped with a HeNe laser source (λ = 633 nm) (Malvern Instruments Ltd, Worcestershire, UK) and an avalanche photodiode for detection, controlled with DTS software. Micromolar concentration solutions of QDs or QD–bioconjugates were loaded into disposable cells, and data were collected at 25 °C. Three runs of the measurements were performed for each sample to achieve the ζ-potential. All samples were prepared in 0.1 × PBS buffer pH 7.4 with similar concentration as DLS measurement.

**AP-QD Enzymatic Assays.** A Tecan Infinite M1000 dual monochromator multifunction plate reader equipped with a xenon flash lamp (Tecan, Research Triangle Park, NC) was used to monitor AP-QD bioconjugate activity. Spectral acquisition was carried out at 400 Hz flash frequency using 386 nm excitation and 448 nm emission. The temperature of the plate during measurements was held constant at 25 °C to ensure enzyme activity was not affected by temperature fluctuations. A shaking sequence (20 s every 5 min) was preprogrammed into the plate reader to ensure proper substrate–enzyme mixing. AP-QD bioconjugates were pipetted into a Corning flat-bottom black polystyrene 96 well non-binding plate, and 4-methylumbelliferyl phosphate (MUP, Sigma-Aldrich) substrate was added to each solution to a total volume of 100 μL immediately before spectral acquisition. For the excess substrate progress curves, enzyme concentration was fixed at 62.5 pM while equivalent molar ratios of QDs were added to the solution to create enzyme-to-QD ratios of 0:1, 2:1, 12:1, and 24:1. In each experiment, run simultaneously on the same well plate, the substrate concentration was varied among 1, 2.5, 5, 10, 25, 50, 100, 250, 500, and 1000 μM for each enzyme-to-QD ratio. For the varying enzyme–fixed substrate progress curves, the enzyme concentration was varied among 1, 2.0, 3.9, 7.8, 15.6, 31.3, 62.5, 125, 250, 500, and 1000 μM while equivalent molar ratios of QDs were added to the solution to create enzyme-to-QD ratios of 0:1, 2:1, 12:1, and 24:1. In each of these experiments, run simultaneously on the same well plate within the Tecan, all enzyme concentrations were tested for each enzyme-to-QD ratio with a fixed substrate concentration of 10 μM. Each experiment was performed in at least triplicate. For converting photoluminescence (PL) intensity into units of activity, predigested MUP was diluted to provide the appropriate calibration curve.

**AP-QD Spatial Model.** All models were constructed using coordinates available in the protein data bank (PDB) for AP (entry 1AJA). Models for the proteins as they might assemble on a QD were constructed using Chimera 1.7. To estimate the maximum loading of the protein onto a QD, the relationship between the QD and the protein must be considered. The protein is first docked to the QD surface and oriented using the (His)6 tail, which establishes an approximate orientation of the protein relative to the QD
surface. When the protein is viewed from above, the dimensions can be measured and the area of an ellipse that approximates this area calculated. A side view of two or more proteins on the QD surface can then be used to determine where the proteins are going to come into contact with each other during assembly. Depending on protein shape and orientation, this could be at or above the QD surface. In the case of AP, this was 43 Å above the QD surface because of the protein’s wedge shape. These distances plus the radius of the QD are used to calculate a surface area of a sphere representing the potential cross-sectional contacts between protein molecules at the point above the QD surface (Table 1). The protein’s asymmetrical shape gives rise to the values used here. Because the cross section of the protein at this point has been determined, the ratio of areas can be used to estimate the maximum loading of protein; most simplistically, fitting spheres representing the AP around a larger sphere representing the QD. For AP, a molecule with high asymmetry, this yields estimates of 13 and 25 molecules for the 525 (diameter, 4.2 nm) and 625 (9.2 nm) QDs, respectively (Table 1).

### Table 1. QD Surface Area and Predicted Enzyme Loadings Obtained from the Spatial Models

<table>
<thead>
<tr>
<th>QD diameter (nm)</th>
<th>QD enz. offset surface area (nm²)</th>
<th>QD enz. offset surface area range (nm²)</th>
<th>Enzyme load (average)</th>
<th>Enzyme loading range</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.2 ± 0.5</td>
<td>523</td>
<td>483–564</td>
<td>13</td>
<td>12–14</td>
</tr>
<tr>
<td>9.2 ± 0.8</td>
<td>995</td>
<td>887–1087</td>
<td>25</td>
<td>22–27</td>
</tr>
</tbody>
</table>

**Data Analysis.** Data analysis utilized the generalized Michaelis–Menten (MM) model

$$E + S \xrightarrow{k_1} ES \xrightarrow{k_{-1}} E + P$$

where enzyme (E) and substrate (S) interact with an association rate ($k_1$) and disassociation rate ($k_{-1}$) to form enzyme substrate (ES) complex. The intermediary ES is converted into product (P) at the rate $k_{\text{cat}}$ also known as the enzyme turnover number. The Briggs–Haldane condition or steady-state approximation for the MM formalism is one in which $[S]_0 \gg [K_m]$ and $d[ES]/dt = 0$ (i.e., the rate of ES formation equals the rate of ES breakdown), so that $-d[S]/dt \approx d[P]/dt$ is valid. Thus, by assuming homogeneous conditions, the kinetics can be reduced to

$$\frac{d[P]}{dt} \equiv \frac{d[S]}{dt} = \frac{V[S]}{K_m + [S]}$$

such that $V \equiv k_{\text{cat}}[E]_0$ is the maximum possible reaction rate, referred to as $V_{\text{max}}$ here, $[E]_0$ the starting enzyme concentration, and $K_m \equiv (k_{-1} + k_{\text{cat}})/k_1$ is the Michaelis–Menten constant. $k_{\text{cat}}$ can then be easily computed as

$$k_{\text{cat}} = \frac{V_{\text{max}}}{[E]_0}$$

If total enzyme concentration in each solution is held constant, $k_{\text{cat}}$ is then directly proportional to $V_{\text{max}}$. Some other more common assumptions about the MM model which help simplify the subsequent analysis include that the enzyme is...
stable over the experimental time, that the reaction is nonreversible, and that P does not inhibit the enzymatic activity. For the “excess substrate” assay format, results were obtained by performing nonlinear regression analysis of the initial portion of the progress curves (i.e., initial rate curve). For the “varying enzyme–fixed substrate” assays, data was processed using an integrated version of the MM formula as described in detail in the Supporting Information. It is worth noting that although we use the standard MM descriptors to characterize our AP-QD bioconjugate activity, these values should be considered as apparent values (i.e., $K_{\text{Mapp}}$) because it is not clear that the assumption of the MM model holds true for nanoparticle–enzyme bioconjugates.\textsuperscript{5,40,50}

## RESULTS

**Alkaline Phosphatase, Quantum Dot Assembly, and Enzymatic Assay.** Alkaline phosphatase (AP, EC 3.1.3.1) is a dimeric enzyme that is produced commercially and used to remove phosphate groups from DNA and other biological substrates such as peptides as part of preparation protocols in molecular biology. It is also used for signal transduction by hydrolyzing fluorescent substrates as part of assays. Mechanistically, AP accomplishes dephosphorylation via a proposed three-step catalysis that involves key metal ions associated with the catalytic site.\textsuperscript{51} AP was selected for this study in part because it is easily expressed recombinantly in bacterial expression systems (MW $\sim$100 kDa, formed from two $\sim$49 kDa dimers) without requiring post-translational modification and can be stored for long periods of time in glycerol and buffer at $-20$ °C without compromising activity. Equally important, it was readily available in our lab.

For AP bioconjugation to the QDs, we again rely on (His)$_n$-driven metal affinity coordination.\textsuperscript{52–56} It has been repeatedly demonstrated that the imidazole side-chains on (His)$_n$ sequences can spontaneously coordinate to the surface of QDs functionalized with a variety of different ligands and coatings.\textsuperscript{52–56} This high-affinity ($K_d \sim 10^{-9}$ M) interaction allows for ratiometric control over the number of (His)$_n$- appended moieties assembled per QD simply through the molar amounts mixed together along with orientational control within the conjugate assembly without the need for further purification in many cases.\textsuperscript{33,57–60} Such orientational control is exceedingly important in these studies as it minimizes conjugate structural heterogeneity which could impair activity while complicating analysis. In addition, the discrete ratiometric control of the assemblies would allow us to assay a range of AP-QD valences which can potentially provide insight into underlying kinetic activity. Gratuitously, AP was already premodified with a C-terminal (His)$_6$ which was originally inserted into its sequence for purification over Ni-chelate media.

The two CdSe/ZnS QDs utilized here include 525 and 625 nm emitting dispersions. The 525 nm green-emitting QDs have a diameter of $4.2 \pm 0.5$ nm, whereas the 625 nm red-emitting QDs have a diameter of $9.2 \pm 0.8$ nm (representative TEMs in Figure 2); this corresponds to surface areas of $\sim 55.4$ and 265.8 nm$^2$ along with estimated curvatures (K, where $K = 1$/radius R) of $\sim 0.47$ and 0.22 nm, respectively. Although the QDs are faceted, they still display fairly spherical shapes overall. To render them colloidally stable and biocompatible for enzymatic assays, the QDs are surface functionalized with DHLA-CL4 ligands (Figure 1c).\textsuperscript{61} These compact-zwitterionic ligands provide for long-term stability across a wide pH and ionic range, and these properties have already been utilized in several different studies.\textsuperscript{61,62} To assay AP activity, we utilize a commercially available 4-methylumbelliferyl phosphate substrate (see Figure 1 for structure).\textsuperscript{63} Enzymatic removal of the phosphate converts substrate to a fluorescent methylumbelliferyl (MU) product with an emission at $\sim 448$ nm which is not masked by PL from the QD although both will be excited at the 386 nm wavelength used (the QD more so in fact). Given this, along with the long

![Image](https://example.com/image.png)
intrinsic QD lifetimes, resonance energy transfer from the MU to the QDs is not an issue here. AP-QD bioconjugate enzymatic activity was monitored using two slightly different assay formats. The first is the commonly utilized “excess substrate” format in which a fixed concentration of AP-QD bioconjugate is assayed against increasing concentrations of MUP such that $[S]_0 \gg K_M$ while still meeting the Briggs–Haldane steady-state assumptions.\(^5\),\(^40\),\(^49\) The second assay format used is that of “varying enzyme—fixed substrate” in which varying concentrations of AP-QD bioconjugates are assayed against a fixed concentration of MUP. In previous work evaluating enzyme activity on peptide substrates attached to QD surfaces,\(^40\) it was observed that, for assaying a protease having a micromolar $K_M$ value, true excess substrate conditions could not be met for that format because of QD—peptide bioconjugate material limitations, i.e., high micromolar to low millimolar concentrations of QD bioconjugate are not physically attainable. This led to the use of a varying enzyme—fixed substrate format which depends upon integrating the assay data and a more elaborate analysis. It is important to point out that this format was actually used by Michaelis and Menten in their seminal work.\(^64\) The current work affords us a unique opportunity to perform a side-by-side comparison of both assay formats and to evaluate their validity in the context of monitoring enzymatic activity at a nanoparticle interface. Data from assays in which we monitored enzymatic activity for various enzyme/substrate concentrations in either format over an extended period are referred to as progress curves here.

**Alkaline Phosphatase—Quantum Dot Bioconjugate Characterization.** Prior to confirming and characterizing AP assembly onto the QDs, we began by constructing a spatial model of how many enzymes could fit on both distinctly sized QDs. This helps provide upper boundaries for bioconjugate assembly ratios while also visualizing the conjugate structure as well; the latter is something that has helped repeatedly with both design of QD bioconjugate-based assays and with interpreting results.\(^50\),\(^65\) The modeling process uses an approach in which the three-dimensional protein structure is obtained from crystallographic coordinates, and then an estimate of the sphere the protein occupies is made and fitted to the different sized spheres representing the QD sizes (taken from TEM imaging) in silico (see Experimental Methods for a full description). The loading of protein spheres on the QD surfaces allow for an estimate of enzyme loading ratios. The resultant structural models presented in Figure 2 show that an average maximum of 13 (enzyme loading range, 12–14) and 25 (enzyme loading range, 22–27) AP enzymes can fit on the 525 and 625 nm QDs, respectively, under ideal conditions (see also Table 1). Furthermore, this structural modeling is important to understanding AP orientation on the QD following assembly. The AP-(His)$_6$ motif is attached at the C-termini of each of the monomers that make up the full-functional AP dimer, which places the catalytic site on the other side of the protein. Thus, when proteins assemble to the QD surfaces by (His)$_6$-driven metal affinity coordination, the resultant enzyme orientation is one in which the catalytic sites face outward from the bioconjugate structure. Such an outward facing enzyme orientation is conducive to enzyme—substrate binding events as the enzymatic binding pocket is not blocked by the QD itself or by neighboring immobilized enzymes to incident substrate.

Agarose gel electrophoresis was then used to confirm AP assembly to the QDs; see Figure 3 for representative results with the 525 nm QDs. The indicated ratios of AP per QD, ranging from 0.25:1 to 8:1, were mixed with the QDs in PBS buffer and allowed to assemble followed by separation in a 1× TBE gel, as described in the Experimental Methods. At defined time points, the agarose gel was visualized to monitor the effects of increasing AP-QD assembly on bioconjugate migration. Clearly, as more AP moieties are assembled to the QD surface, the resulting composite structure becomes larger, thereby impeding its migration through the agarose gel when an electric field is applied. Distinct bands are seen in the gel for AP-QD ratios of 0.25:1 to 3:1 starting after 10 min but are most noticeable after 15 min. As expected, assembly follows a Poisson distribution process in which the majority of the QDs will have the number of indicated enzymes attached only at higher concentrations, i.e., $\geq 4:1$ ratio.\(^33\),\(^66\) At lower ratios, however, a statistically significant number of the QDs will assemble with distinct enzyme subpopulations that are slightly below or above the target AP-QD ratio. Indeed, this process is epitomized by the “1:1” ratio in which three distinct populations of roughly equal intensity can be seen corresponding to the prediction of assemblies showing ratios of 0, 1, and 2 AP per QD. Similar results were obtained with AP assembly to the 625 nm QDs (data not shown). Because we would use samples in which the bioconjugate concentration would be quite low, we also utilized FRET between a dye-labeled AP and the QDs to confirm that the conjugate would remain assembled and intact even when diluted below 1 pM concentration, see Supporting Information. The high-avidity and long-term stability of the AP-QD conjugates arises primarily from the presence of two-distinct (His)$_6$ sequences as presented and displayed on each intact dimeric protein. It should also be noted that we cannot ascertain if both motifs are fully bound to the QD surface. Analogous high-affinity binding has been noted before for the QD assembly of protein constructs displaying multimeric, bundled, or dendritic (His)$_6$ motifs.\(^33\),\(^52\),\(^53\),\(^55\)

Lastly, we used dynamic light scattering (DLS) and ζ-potential analysis to provide further insight into assembly of the AP-QD bioconjugates (see Experimental Methods for details).

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**Figure 3.** Agarose gel electrophoretic separation of QD-AP conjugates. The indicated increasing stoichiometric ratios of AP were allowed to self-assemble to 525 nm QDs coated with DHLA-CL4 (0—8 AP per QD) as indicated at the top of the figure for each well. The resulting assemblies were separated on a 1.5% weight/volume agarose gel in 1× Tris–borate–EDTA buffer. Lanes 1 and 10 contain only QD with no AP, serving as controls for reference of QD migration. Arrows on the bottom left indicate the migration position of QDs assembled with 0, 1, and 2 AP per QD starting from the bottom.
Table 2. Hydrodynamic Diameters and ζ-Potential of Select QD-AP Conjugates

<table>
<thead>
<tr>
<th>sample</th>
<th>hydrodynamic diameter, $H_D$ (nm)</th>
<th>ζ-potential (mV)</th>
<th>surface charge</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>no substrate 10 μM substrate</td>
<td>no substrate 50 μM substrate</td>
<td>charge density, $\sigma$ (C/m²)</td>
</tr>
<tr>
<td>525 QD only</td>
<td>8.5 ± 0.4</td>
<td>−32.4 ± 1.4</td>
<td>−1.6 × 10⁻²</td>
</tr>
<tr>
<td>AP:525 QD (4:1)</td>
<td>11.2 ± 1.8</td>
<td>−27.5 ± 1.0</td>
<td>−1.3 × 10⁻²</td>
</tr>
<tr>
<td>AP:525 QD (10:1)</td>
<td>15.1 ± 0.6</td>
<td>−18.1 ± 1.0</td>
<td>−8.6 × 10⁻³</td>
</tr>
<tr>
<td>AP:525 QD (30:1)b</td>
<td>15.6 ± 0.3</td>
<td>−15.8 ± 0.7</td>
<td>−7.5 × 10⁻³</td>
</tr>
<tr>
<td>625 QD only</td>
<td>11.2 ± 0.3</td>
<td>−28.6 ± 1.1</td>
<td>−1.2 × 10⁻²</td>
</tr>
<tr>
<td>AP:625 QD (4:1)</td>
<td>14.3 ± 0.6</td>
<td>−18.7 ± 6.1</td>
<td>−7.7 × 10⁻³</td>
</tr>
<tr>
<td>AP:625 QD (10:1)</td>
<td>16.0 ± 0.4</td>
<td>−15.8 ± 3.2</td>
<td>−6.4 × 10⁻³</td>
</tr>
<tr>
<td>AP:625 QD (30:1)</td>
<td>16.9 ± 0.4</td>
<td>−13.6 ± 4.8</td>
<td>−5.5 × 10⁻³</td>
</tr>
<tr>
<td>enzyme-only (30)</td>
<td>7.6 ± 0.3</td>
<td>8.6 ± 0.4</td>
<td>−</td>
</tr>
</tbody>
</table>

The standard deviation was calculated after repeated measurements (n = 3−5). $H_D$ was obtained from dynamic light scattering (DLS) experiments, and ζ-potential was obtained from laser doppler velocimetry (LDV) measurements as described. All experiments were performed in 0.1 × PBS (pH 7.4).

b This assembly ratio corresponds to excess unconjugated enzyme present; therefore, values derived are from only the conjugate. QD surface charge density was estimated from the measured ζ-potential with no substrate present. Number of negative charges per QD was calculated from the surface charge density by using electron charge and surface area of QD-CL4 (see Supporting Information).

Figure 4. AP-QD excess substrate assays. Representative progress curves collected using an excess substrate format for a fixed concentration of AP enzyme (62.5 nM) immobilized on (a) 525 QDs with an enzyme-to-QD ratio of 2, (b) 625 QDs with an enzyme-to-QD ratio of 2, and (c) free control in solution without QDs. (Top row, a−c) PL emission corresponding to reactions containing substrate concentrations: 1 (brown), 2.5 (red), 5 (orange), 10 (green), 25 (light green), 50 (light blue), 100 (blue), 250 (dark blue), 500 (purple), and 1000 μM (pink). (Bottom row, d−f) Corresponding nonlinear regression analysis of the initial rate ($V_o$) derived from the progress curves fitted to the Michaelis−Menten equation for the same assays.

Table 2 shows representative results from this analysis in which both DLS and ζ-potential corroborate ratiometric AP attachment to the QD surface. Indeed, QD hydrodynamic diameter ($H_D$) increases approximately 3−7 nm with AP conjugation, which is consistent with the enzyme’s size or $H_D$. $H_D$ also increases proportionally for both sized QDs as enzyme-to-QD ratio increases. Interestingly, inclusion of 10 μM MUP substrate increases the overall $H_D$ by 10−16%, suggesting some potential enzyme conformational changes in the presence of substrate; such conformational changes during catalysis have been noted previously.67 Alternatively, these changes along with the decreases in ζ-potential also noted, may represent bioconjugate capture of substrate or product by its surrounding hydration layer (vide infra). Adding the same amount of MUP to QDs alone did not alter the $H_D$ confirming the absence of nonspecific interactions (data not shown). The surface ζ-potential, a key indicator of colloidal stability in solution, shows relatively high values (>25) for both sized unassembled QDs.
Potentials start at higher negative values that become more positive as the enzyme-to-QD ratio increases and as substrate is added to the solution. This shift to a more positive ζ-potential is supported by the estimated isoelectric point (pI) of 6.2 from the AP sequence (http://protcalc.sourceforge.net/). Table 2 also includes the extrapolated surface charge density per unit area for the QDs and conjugates along with the number of negative charges per QD, highlighting the shift to a more positive overall charge. We also noted that the QD PL profile did not change with AP bioconjugation nor during subsequent assays (data not shown).

Excess Substrate Assays. Full enzyme progress curves were first acquired in the “excess substrate” format for enzyme-to-QD ratios of 2:1, 12:1, and 24:1 for both the 525 and 625 nm QDs, see Figure 4 for representative data for the 2:1 ratio. Although the 24 AP-QD ratio for the 525 QDs exceeds the estimated AP assembly ratio, this was still included to look for potential synergistic effects between AP that is assembled to QDs and those freely diffusing in the sample. In these assays, the enzyme concentration was fixed at 62.5 μM, while the QD component was adjusted to give the indicated ratios, and the assay substrate concentration was increased in 10 distinct increments ranging between 1 and 1000 μM. This range prevents substrate concentration from being an experimental rate-limiting factor in that it is present in considerable excess across the assay. This experimental approach is also inherently beneficial in that it allows the enzyme concentration to remain fixed while the AP display ratio or valency on the QD is the only variable to be altered. Figure 4a–c shows representatives changes in product PL intensity monitored at 448 nm as the 2:1 AP-QD bioconjugates are assayed against the range of substrate concentration versus equivalent amounts of free enzyme control. The assay time frame is sufficient for almost all the substrate concentrations to be converted to product, as evidenced by their asymptotic flattening off to a consistent intensity at later time points.

The resulting PL from the assays was converted into product concentration by comparison to a calibration curve of preformed product and fitting to a quadratic conversion equation (see Supporting Information). This allowed for subsequent conversion into units of activity as shown in the representative plots of Figure 4d–f. Nonlinear regression analysis of the initial portion of the progress curves (i.e., initial rate curve) allowed for estimation of the $V_{\text{max}}$, $k_{\text{cat}}$, and the Michaelis constant ($K_M$) values. The plots in Figure 5 present a comparison between each of the AP-QD ratios tested and free enzyme controls for each of these three kinetic descriptors. For all the reported parameters, enzyme immobilized on the smaller 525 nm QDs induced a larger apparent increase in both $V_{\text{max}}$ and $k_{\text{cat}}$ as these are somewhat reflective of the same data. In comparison to freely diffusing enzyme controls in solution, these are both enhanced by approximately 23% for the 525 nm enzyme-to-QD ratio of 2:1, 15% for the ratio of 12:1, and then this remains approximately constant at ~14% for the ratio of 24:1. In contrast, these values decrease to approximately 3%, 4%, and 7% for each of these assembly ratios on the larger 625 nm QDs, respectively. More interesting is the comparison among the derived $K_M$ values. Here, the increases versus the free enzyme-only control are of a much smaller magnitude. These approximately constant $K_M$ values demonstrate that any potential leaching or interaction of QD cadmium and zinc ions does not occur or does not inhibit substrate–enzyme binding under these conditions. Furthermore, this pattern of $V_{\text{max}}/k_{\text{cat}}$ values for enzyme “on” and “off” the QD corroborates that the enzyme–QD binding configuration occurs as predicted to the protein backbone, which positions the enzyme binding pocket and active site away from the QD interface, minimizing nearest neighbor interactions; i.e., enzyme immobilized on the QD is not hindered and is fully active.

Varying Enzyme–Fixed Substrate Assays. Full progress curves were also acquired using the varying enzyme–fixed substrate format, see Figure 6. Here, substrate concentration was fixed at 10 μM while the enzyme concentration was incremented across a range spanning 1–1000 μM. The enzyme component was simultaneously adjusted to maintain conjugates displaying ratios of 2:1, 12:1, and 24:1 AP as the enzyme concentration changed. During initial assays, a noticeable downward drift was observed in the product signal at AP-QD concentrations which were higher than that used above (see Figure 6a for representative examples). This was also present in the enzyme-only control samples, revealing that it did not originate from QD presence. As a result of this, reanalysis of the
excess substrate data above showed that it also occurred here but at such a slight rate that it could be essentially ignored without altering the results to any extent. We speculate that this drift represents some type of nonspecific interaction of the AP with the fluorescent product which degraded or altered the latter to a secondary product. Indeed, the substrate concentrations in these assays was toward the lower end of that used above, suggesting that it may also occur after most of the primary substrate was consumed. A modified version of the data processing and MM model was developed that incorporated and accounted for the nonspecific product drift and degradation at higher enzyme concentrations (see Supporting Information). Resultant PL from these enzymatic assays was converted into the corresponding product (MU) and substrate (MUP) concentrations and fitted according to the above modified MM model (Figure 6a–c).

There are several caveats to the use of excess substrate formats for monitoring enzymatic activity; one of which is that $[S]_0 > 3 K_M$ should be met. If this is not satisfied, the resulting activity curves will only reflect the ratio $V_{max}/K_M \propto k_{cat}/K_M$. The $k_{cat}/K_M$ ratio is generally referred to as the specificity constant and is used as a measure of enzymatic efficiency as it is an effective second-order rate constant and overall quite useful descriptor.\textsuperscript{5,40} Table 3 presents a comparison of the $k_{cat}/K_M$ values derived from both assay formats. Gratifyingly, the values for the enzyme-only experiments are essentially the same at 5.6 ± 0.4 and 6.0 ± 0.3 for excess substrate and enzyme, respectively. Following on this, the values for the 525 QDs at both 2:1 and 12:1 and the 625 QDs at 24:1 AP display ratio are
Table 3. Comparison of the $k_{\text{cat}} K_m^{-1}$ Second-Order Rate Constants

<table>
<thead>
<tr>
<th>sample</th>
<th>$k_{\text{cat}} K_m^{-1}$ ($\mu$M$^{-1}$ s$^{-1}$)</th>
<th>varying [S] fixed [E] excess</th>
<th>varying [E] fixed [S]</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP:525 QB (2:1)</td>
<td>6.5 ± 0.2</td>
<td>7.3 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>AP:525 QB (12:1)</td>
<td>6.2 ± 0.4</td>
<td>6.0 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>AP:625 QB (2:1)</td>
<td>5.8 ± 0.4</td>
<td>7.8 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>AP:625 QB (12:1)</td>
<td>5.8 ± 0.3</td>
<td>8.4 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>AP:625 QDs (24:1)</td>
<td>5.8 ± 0.3</td>
<td>6.8 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>enzyme-only</td>
<td>5.6 ± 0.4</td>
<td>6.0 ± 0.3</td>
<td></td>
</tr>
</tbody>
</table>

also essentially the same between formats within experimental error. The 625 QDs at 2:1 and 12:1 ratio samples show slight differences, with the values derived for the varying enzyme–fixed substrate format being on average ~40% larger. It is possible that some of these differences may originate from the presence of the secondary reaction which gave rise to the drift described above. In comparison to the activity of enzyme-only experiments, values for the enzyme on QD assays are on average ~17% larger (range of ~16–40%).

CONCLUSIONS

Similar to that demonstrated for enzymes, there are many potential applications for nanoparticle–enzyme constructs, including probes for monitoring cellular activity; signal transduction in assays; bioremediation materials; biocatalysis and biosynthesis; and utility as an intimate part of theranostic nanomaterials. The phenomenon of enhanced enzymatic activity at the nanoparticle interface is something that has been repeatedly observed with many different combinations of both enzymes and nanoparticle materials and thus provides these materials with even more potential. For example, Konwarh and co-workers immobilized keratinase onto PEG-coated superparamagnetic nanoparticles and observed a 4-fold increase in enzyme activity compared to free enzyme in solution. Wang and co-workers created a CaHPO$_4$-$\alpha$-amylase nanoparticle conjugate and also observed an increase in catalytic activity compared to that of free enzyme. A variety of enzymes have also been attached to gold nanoparticles for various applications and have manifested enhanced kinetic activity. Bioconjugating similar CdSe/ZnS QD materials with endoglucanase, exoglucanase, or ferredoxin:NADP$^+$ oxidoreductase enzymes also yielded increases in relevant kinetic parameters compared to that of free enzyme in solution. There have also been initial attempts to model enhanced activity. Building from a system consisting of $\alpha$-chymotrypsin covalently attached to polystyrene nanoparticles ranging in size from 110 to 1000 nm, Jia and co-workers exploited collision theory to explain the increase in $k_{\text{cat}}$ of enzyme activity attached to the nanoparticle versus that free in solution. They observed that changes in particle size and viscosity of reaction media affect the activity of the particle-attached enzyme. Our efforts here are more modest and represent an initial foray into attaching enzymes to QDs for the purposes of observing and confirming enhanced activity. Specifically, we focused on four basic questions: (1) Could the average number of enzymes attached to the QD (i.e., ratio) be controlled? (2) Could the enzymes be controllably attached to QDs in a homogeneous manner? (3) Would these enzymes manifest enhanced activity when attached to the QDs as compared to the equivalent amount free in solution? (4) Could we use this system to provide a side-by-side comparison of conventional excess substrate assay formats versus that of using varying enzyme–fixed substrate?

For these purposes, we utilize the QDs as a model nanoparticle with defined size and low polydispersity and specifically exploit the ability to bioconjugate them in a controlled manner. In terms of controlled and homogeneous AP assembly to QDs, all the characterization and assay data we collected in conjunction with structural modeling suggest that this is indeed so. Examination of the AP sequence and crystal structure reveals that the (His)$_6$ sequences are distal or opposite of the binding site. This constrains the enzyme to assemble to the QDs with the catalytic site facing away from the conjugate and clearly available, which is later confirmed in the activity assays. As a dimer, AP is a relatively large protein molecule (~100 kDa); however, its "wedge"-like shape allows for a relatively high density assembly on the QD. Indeed, this assembly density matches that seen for more globular proteins with less than half this mass. The presence of two (His)$_6$ motifs insures that the QD assembly is high affinity and stable even at dilute concentrations. Analysis using agarose gel electrophoresis and DLS confirms the control over assembly valence. Overall, these results again confirm that judicious placement of a (His)$_6$ sequence onto a protein terminus along with subsequent use for QD assembly can provide for ratiometric and orientational control within the conjugate.

Second, the assays reveal enhancement of activity when AP is attached to the QDs in comparison to freely diffusing enzyme control samples. Although enhancement is present in all cases, the magnitude of this enhancement is relatively modest with a maximum of only ~25% increase reached (note, this maximum increases to ~40% if one considers the results from the excess substrate format to be the more accurate results). Lastly, comparison of the conventional excess substrate assay format to that of varying enzyme–fixed substrate reveals a general equivalency among the two. This is an important finding in that we anticipate the latter format to be utilized for many nanoparticle–substrate and nanoparticle–enzyme conjugate systems in which materials are limiting, and this helps confirm that data obtained in either format is generally equivalent and can be compared.

It is probably fair to assert that the mechanism(s) by which AP enzymatic activity is enhanced at the QD interface is complex. That the $K_M$ values are the least perturbed of all the kinetic descriptors suggests that, at least in this case, it is not an enhancement related to increased binding site affinity with the substrate. Rather it is more likely to be a complex interplay of kinetic interactions that affect the association and disassociation rates ($k_1$ and $k_{-1}$) which in turn act to increase the overall catalytic rate. This complexity may be further exacerbated by additional factors such as nanoparticle size, diffusion constraints, curvature, and surface ligand character. Typically, enzymes immobilized on larger macroparticles or surfaces have shown reduced enzyme catalytic rates and efficiencies due to changes in tertiary structure, steric hindrance, and diffusion limitations. The unique physicochemical attributes of nanoparticles including relatively small size with high curvature in combination with enzymatic attachment by its terminus seem to preclude issues associated with loss of activity due to changes in tertiary structure or wholesale denaturation. At least in this case. Similar reasoning should hold true for the exceedingly rare possibility of structure being responsible for the increases in activity. It is also important to
consider that the current AP-QD conjugates do not represent a system of freely diffusing enzyme and substrate nor is it a bulk interface; rather, it represents a novel intermediary between the two but which is not properly described by either.\textsuperscript{5,40} Indeed, the complexity of this system is epitomized by the S25 QD 2:1 ratio conjugates which display the highest activity but at the lowest ratio of active enzyme on the QDs; the next highest values obtained were for the S25 enzyme-to-QD ratios of 12 and 24, respectively. The 625 QD conjugates display far lower increases in comparison, although here an increasing trend that correlates with ratio is seen, similar to what has been reported.\textsuperscript{54} This suggests the possibility of a co-operative effect between the enzymes on the surface of QDs with the higher density and lower enzyme–enzyme distance, yielding the higher catalytic efficiency. However, confounding this conjecture, the trend does not hold true for the S25 QDs and here it is indeed opposite. Nanoparticle size thus appears to be an important determinant, at least in this system, for enhanced activity. We suspect that the relatively large size of the AP itself in combination with that of the larger 625 QD may have accessed an upper boundary for enhanced activity, future studies are planned to investigate this with other enzymes and nanoparticles. Critically, AP does not appear to lose any function or activity when attached to the QDs in all the examples tested; this is something that we partially attribute to our controlled and homogeneous assembly. This may not have held true for heterogeneous assembly using carbodiimide chemistry, for example.\textsuperscript{5,79,80} Moreover, previous AP-QD bioconjugates assembled using biotin–avidin chemistry showed marked losses in activity compared to that of free enzyme.\textsuperscript{81} We also see no evidence of either cooperative or detrimental effects from having both the enzyme conjugates and free enzyme present in the same sample (e.g., S25 QD 24:1 ratio).

Some insight into the mechanisms which may potentially contribute to the current observations may be drawn from the work of Yan and co-workers with enzymes tethered to DNA origami tiles.\textsuperscript{82} Here, discrete glucose oxidase (GOx)/horseradish peroxidase (HRP) enzyme pairs, which function in coupled reactions, were organized on the DNA at different distances. Enhanced activity was observed at close enzyme spacing (<20 nm) which rapidly dropped off substantially with larger separations putatively due to Brownian diffusion. However, inserting a noncatalytic protein between the GOx and HRP, even at larger separation distances, resulted in significantly enhanced activity. The intermediary protein was believed to act as a bridge and connect the separated hydration shells of the enzymes into a functional continuum that facilitated substrate transfer. It is well-known that nanoparticles have surrounding hydration layers with complex properties such as pH and ion gradient that are still not fully understood.\textsuperscript{79,83,84} Although still a hypothesis, one possibility is that the AP-QD bioconjugate could present a hydration layer that functionally acts to capture substrate, and indeed the DLS and z-potential measurements do show some corollorating increases in size and changes in charge when substrate is added to the assembly. Alternatively, the localized capture of substrate to the conjugate could be based on electrostatic or hydrophobic interactions with portions of the ligand, the enzyme, or both, in addition to other complex interactions.\textsuperscript{55,85} The enhanced $V_{\text{max}}$ and $k_{\text{cat}}$ could then be attributed to a high local concentration of substrate around the enzyme–QD bioconjugate. This translates to the available amount of localized substrate for the enzyme immobilized on the QDs being higher than that seen by free enzyme in solution, and this serves to enhance the overall turnover number and maximal velocity.

The current results suggest that an enzyme (selected literally at random) can demonstrate some enhanced activity when placed at a nanoparticle interface. The results also suggest that there are many closely related factors to explore in order to understand this process. Thus, a system in which only one variable can be altered within a series of experiments would prove ideal for study as it may help limit the complexity of the system. Attaching enzymes to QDs and other nanoparticles via a (His)$_n$ linker can potentially help make this type of system a reality as it can provide control over enzyme display orientation and ratio while other variables such as nanoparticle size or curvature are changed. It will also be interesting to evaluate a system in which QD attachment results in decreased performance to ascertain if the QD–conjugate might maintain much higher activity at conditions not favorable to the free enzyme. In contrast to the large size of the AP, future studies will focus on evaluating far smaller enzymes conjugated to QDs as this should provide for an increase in the number of catalytic sites displayed around the conjugate and perhaps increase the magnitude of enhanced activity. Such a model system will potentially allow for expansion to a wide range of nanoparticle sizes and shapes along with being more tractable to in-depth kinetic and molecular modeling. Knowledge derived from such systems will help in the understanding of localized enzymatic activity and contribute toward the goal of ex vivo artificial biosynthesis.\textsuperscript{54}

\section*{ASSOCIATED CONTENT}

\subsection*{Supporting Information}

AP monomer sequence in pET22 with (His)$_6$ added and pelB removed; plot of ratio of sensitized AlexaFluor 647 emission to 625 nm QD donor PL from AP-QD bioconjugates that underwent serial dilution (Figure S1); and additional description of (i) quadratic conversion equation, (ii) modified MM model for AP fixed substrate–excess enzyme dilution curves, and (iii) surface charge density and the number of negative charges per QD-CL4. This material is available free of charge via the Internet at http://pubs.acs.org.

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\subsection*{Notes}

The authors declare no competing financial interest.

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