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Avidin: A Natural Bridge for Quantum Dot-Antibody Conjugates

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Abstract: We describe the preparation and characterization of bioinorganic conjugates in which luminescent semiconductor CdSe−ZnS core−shell nanocrystal quantum dots (QDs) were coupled to antibodies through the use of an avidin bridge adsorbed to the nanocrystal surface via electrostatic self-assembly. Avidin, a highly positively charged protein, was found to adsorb tightly to QDs modified with dihydrolipoic acid, which gives their surface a homogeneous negative charge. QD conjugation to biotinylated antibodies subsequently is readily achieved. Fluoroimmunoassays utilizing these antibody conjugated QDs were successful in the detection of protein toxins (staphylococcal enterotoxin B, cholera toxin). QD-antibody conjugates formed in such a facile manner permit their use as a common immuno reagent, and in the development of multianalyte detection.

Introduction

Colloidal semiconductor CdSe−ZnS core−shell nanocrystal quantum dots (QDs) are luminescent inorganic fluorophores that have the potential to overcome some of the functional limitations encountered by organic dyes in fluorescence labeling applications. Small organic dyes commonly used for diagnostics applications and in biological imaging[1] have characteristics that limit their effectiveness in these applications. Problems with organic fluorophores include narrow excitation bands and broad emission spectra, which can make detection of multiple light emitting probes difficult due to spectral overlap, and low resistance to chemical degradation and photodegradation.[2] Fluorescent QDs have size-dependent tunable photoluminescence (PL) with broad excitation spectra and narrow emission bandwidths (full width at half maximum of ∼30−45 nm) that span the visible spectrum,[3−7] which allows for simultaneous excitation of several particle sizes at a single wavelength. In addition QDs have high photochemical stability, excellent resistance to chemical degradation and photodegradation, and a good fluorescence quantum yield. Luminescence emission from QDs is detected at concentrations comparable to organic dyes by using conventional fluorescence methods, and individual QDs and QD-bioconjugates are easily observable by confocal microscopy.[8,9]

In a departure from covalent chemistry techniques,[10−14] we have recently developed a novel conjugation strategy based on electrostatic interactions between negatively charged dihydrolipoic acid (DHLA)-capped CdSe−ZnS core−shell QDs and a positively charged leucine zipper[15] interaction domain appended onto the C-terminus of engineered recombinant proteins.[8,16−18] QD-antibody conjugates, prepared using an adaptor protein that

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bridges the inorganic fluorophores and antibodies, were employed in fluoroimmunoassays.[17,18] In the present work we show the general utility of that approach by the use of avidin (a highly positively charged tetramer) as a bridge between QDs and biotinylated antibodies to form QD-antibody conjugates.

Avidin, a glycoprotein found in avian egg white, is a homotetramer with a molecular mass of 68 000 daltons.[19,20] Avidin interacts stoichiometrically with biotin, binding one biotin per subunit. Due to the specific and high affinity interaction (Kd = 10^{-15} M; ref 20) between avidin and biotin, the avidin−biotin system is a well-known and powerful tool for research and analysis. The interaction between biotin and biotin binding proteins (such as avidin) has been widely exploited in various applications including immunoassays, selections, and purification schemes. Biotin-modified antibodies, proteins, and DNAs are commercially available or can be easily prepared. Conjugating QDs with avidin potentially permits the attachment of these fluorescent inorganic particles to any biotinylated protein or DNA.

Here we describe the preparation of bioconjugates of CdSe−ZnS QDs with avidin that can then be used to form QD-antibody conjugates by reaction with biotinylated antibodies. To facilitate purification of the conjugated QDs, a mixed surface conjugation strategy was employed. In this method both avidin and maltose binding protein genetically fused with a positively charged leucine zipper interaction domain (MBP-zb; refs 8 and 16) were adsorbed to the QD surface (Figure 1A). While avidin tightly binds the biotinylated antibody, the MBP-zb permits removal of unbound antibody through affinity chromatography. Thus, QD-antibody conjugates can be easily formed and purified for use in fluorescence-based assays. We show both direct and capture fluoroimmunoassays employing QD-antibody conjugates formed through an avidin bridge. We also show preliminary results utilizing these antibody conjugated QDs in simultaneous analysis of two protein toxins as a prelude to future multiplex applications.

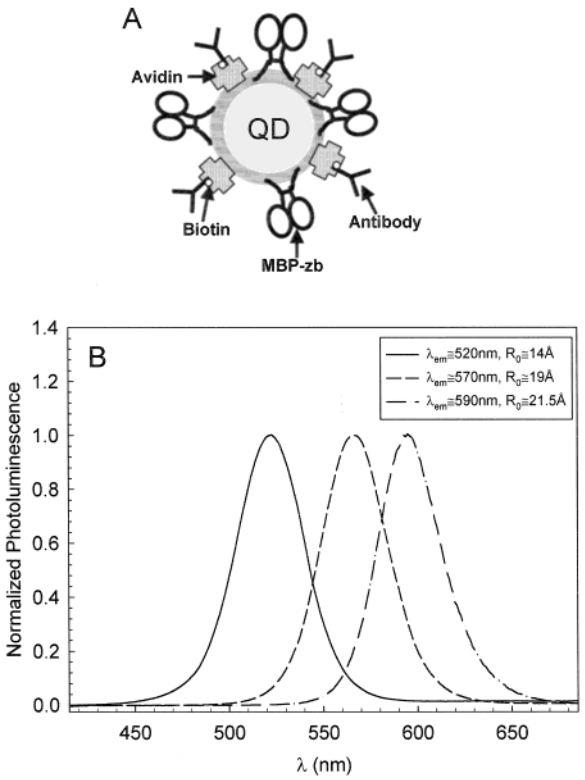

Figure 1. (A) Cartoon of a mixed surface QD-antibody conjugate in which avidin bridges the nanocrystal and biotinylated antibody. The MBP-zb is used for purification of the complex. Exact numbers of avidin and MBP-zb per QD are not known; not drawn to scale. (B) Photoluminescence spectra of three sizes of CdSe−ZnS quantum dots (radius, R_0, of 14, 19, and 21.5 Å emitting at 520, 570, and 590 nm, respectively) used in this study.

Experimental Procedure

Preparation of QD Immuno Conjugates for Bioassays. CdSe−ZnS quantum dots capped with dihydrolipoic acid (DHLA) were prepared and characterized as previously described.[8,16] QDs with emission maximum of 520, 570, and 590 nm were used in these experiments (Figure 1B).

A mixed surface composition strategy was employed to enable rapid and easy removal of excess unconjugated antibody after coupling to QDs. Mixed surface QDs were prepared by incubating DHLA-capped QDs with avidin (Pierce) and a recombinant fusion protein consisting of maltose binding protein appended with a positive leucine zipper interaction domain (MBP-zb; refs 8 and 16). Avidin (216 pmol) and MBP-zb (432 pmol) were mixed in 200 μL of 10 mM sodium tetraborate solution, pH 9 (Borate buffer). QDs (108 pmol) were added to the protein solution, and the mix was incubated for 15 min at room temperature. An additional 216 pmol of MBP-zb was added to the QD protein solution, followed by a second 15 min incubation to saturate the QDs with protein. This procedure yielded self-assembled mixed surface QDs, free of obvious aggregates.

Conjugation to biotinylated antibodies and purification of QD-antibody products was achieved by using mixed surface QDs immobilized on an amylose resin (New England Biolabs) column. Approximately 100 pmol of intermediates prepared as above were loaded on a 0.5 mL column of cross-linked amylose resin, and the resin was washed with 1 mL of PBS (120 mM NaCl, 2.7 mM KCl, 10 mM phosphate buffer; pH 7.4). Biotinylated antibody (20 μg) was flowed into the column, and the flow stopped for a 1 h incubation period. This procedure allowed saturation of avidin binding sites with antibody while preventing free diffusion of nascent conjugates thereby avoiding the formation of cross-linked immunoprecipitates. Excess antibody was then washed through the column with 2 mL of PBS. QD-antibody conjugates were released upon addition of 10 mM maltose in PBS. The same procedure was used to test strepavidin and NeutrAvidin's (Pierce) ability to couple QDs to biotinylated antibodies. Biotinylated Immunoglobulin G (IgG) antibodies used in these conjugates were prepared as described previously;[22] IgG preparations were incubated for 30 min with a long-chain derivative of biotin *N*-hydroxysuccinimidyl ester (EZ-Link NHS-LC-biotin; Pierce) at a 5:1 (biotin:IgG) molar ratio in 50 mM borate, pH 9, 40 mM NaCl. Labeled protein was separated from unincorporated biotin on a sizing column (Bio-Gel P10; Bio-Rad).

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Determination of Antibody/QD Ratios. To quantitate the number of IgG molecules bound per QD, Cy5-labeled biotinylated IgG was used as a tracer in the coupling procedure. Biotinylated IgG was labeled with 2.8 molecules of Cy5 per IgG according to the manufacturer's (Pharmacia-Amersham) instructions. QD-IgG was then prepared as described above by using dye labeled antibody. The number of IgG per QD-IgG was calculated by using the Cy5 absorbance at 650 nm (ϵ = 250 000 M^{-1} cm^{-1}) of the purified product assuming no loss of QDs during the column purification. The nanocrystal absorption at 650 nm was negligible.

Direct Fluorimetric Assays. Wells of opaque white microtiter plates (Maxisorb, Nunc) were coated overnight (4 °C) with 50 μL aliquots of serial dilutions of goat IgG (Rockland), staphylococcal enterotoxin B (SEB, Toxin Technologies Inc.), or cholera toxin (Calbiochem) dissolved in 0.1 M $NaHCO_3$ (pH 8.6). Each experiment included a nonspecific binding control in which only 50 μL of buffer was added to wells. After removing excess antigen or blank solutions from wells, plates were blocked at room temperature for 2−3 h with PBS containing 4% (w/v) powdered nonfat milk. Plates were then washed 2 times with TBST (50 mM Tris-HCl, 105 mM NaCl, pH 7.5 with 0.1% Tween 20) and 50 μL of avidin-bridged QDs conjugated to the appropriate antibody [Rabbit anti-goat IgG (Rockland), Sheep anti-SEB IgG (Toxin Technologies Inc.), Rabbit anti-cholera toxin IgG (Biogenesis)] was added to test and control wells which were incubated for 1−2 h with gentle shaking at room temperature. Unbound QD-antibody was discarded and wells were washed 2−3 times with PBS buffer, followed by automated fluorescence measurement with a SpectraFluor Plus microtiter plate reader (Tecan). A 25 nm band-pass filter was used for excitation at 310 nm and a 530 nm long pass was used to measure photoluminescence.

Toxin Sandwich Immunoassay. Wells of microtiter plates were coated overnight with 100 μL of polyclonal goat anti-cholera toxin IgG (Biogenesis) or mouse monoclonal anti-SEB antibody (Mab 2b, Igen) 5 or 10 μg/mL respectively in 0.1 M $NaHCO_3$ (pH 8.6), then blocked and washed as described above. Serial dilutions of toxin (cholera toxin or SEB) in PBS were added to the wells (50 μL/well) and incubated for 1−1.5 h. After each well was washed twice with TBST, QDs conjugated with polyclonal rabbit anti-cholera toxin or polyclonal sheep anti-SEB (50μl/well) were added to anti-cholera toxin or anti-SEB wells respectively, and the plates were incubated for another 1−2 h. Wells were then washed and photoluminescence measured as above.

Dual Analyte Detection. Wells of microtiter plates were coated overnight with a saturating amount of toxin (5 μg/mL of cholera toxin or 10 μg/mL of SEB) and blocked and washed as described above. QD conjugates with anti-cholera toxin and anti-SEB antibodies were prepared as described above with use of 520 and 590 nm emitting QDs, respectively. Both QD-anti-cholera toxin and QD-anti-SEB conjugates were added separately and together to wells coated with either cholera toxin, SEB, or buffer controls. Plates were read as described above by using 25 nm band-pass filters centered at 535 and 595 nm to collect the emission from each population of QD-conjugate.

Results and Discussion

Coupling of Antibodies and QDs with Biotin Binding Proteins. Mixtures of avidin and MBP-zb proteins added in 2-fold and 6-fold molar excess respectively to DHLA-capped CdSe−ZnS QDs in solution yielded mixed surface QDs with no macroscopic aggregates or precipitated material. These avidin and MBP-zb coated QDs retained both the spectroscopic properties of the nanocrystals (absorption and photoluminescence) and the binding abilities of both avidin and MBP. It was critical to immobilize the mixed surface QD intermediate on a column before adding biotinylated antibody for the formation of the QD-antibody product. If antibody is added to the intermediate in solution a precipitate forms due to the biotinylated antibodies cross-linking QDs.

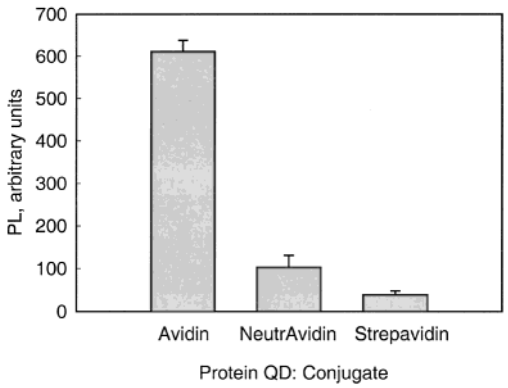

Figure 2. Comparison of the ability of three biotin binding proteins to cross-link QDs and a biotinylated anti-goat antibody. Proteins were tested for their ability to form conjugates capable of binding to wells with saturating adsorbed goat IgG. Measurements were done in triplicate and error bars represent standard deviations. QDs emitting at 570 nm were used.

To determine the average number of IgG present per avidin coated QD we examined QD-antibody conjugates formed with IgG dual labeled with Cy5 and biotin. On average there were 0.5 antibodies per QD using the conjugation conditions and antibody concentration described in the materials section. These results indicate that each antibody labeled QD has only one IgG, suggesting the interactions of these QD-antibody conjugates are dominated by antibody affinity, rather than overall conjugate avidity. Ideally the conjugation conditions could be optimized by changing the amounts of avidin and antibody to produce a reagent with one antibody per QD. Factors that may affect QD-antibody stoichiometry, including the mode of interaction of avidin and the leucine zipper interaction domain with the charged nanoparticle surfaces, are not well characterized.

Two additional biotin-binding proteins, streptavidin and NeutrAvidin, were also examined for their ability to cross-link QDs to biotinylated antibodies. The effective bridging activity of each protein was determined by measuring the ability of QDs conjugated with biotinylated anti-goat antibody (as described in the Experimental Procedure) to bind to goat IgG adsorbed to wells of microtiter plates (Figure 2). In identical direct binding assays, avidin-coated QDs produced signals at least 6 times larger than NeutrAvidin and about 15 times larger than streptavidin. This strongly suggests positively charged hen egg avidin (pI 10.0) adsorbs to the negatively charged DHLA capped QDs much more effectively than NeutrAvidin or strepavidin, which have pIs of 6.3 and 5.0, respectively. This is consistent with our previous findings[8] that the driving force for protein adsorption onto QDs is charge−charge interactions. Increasing ionic strength of the binding mixture, however, did not seem to affect the interaction between avidin and the QDs as there was no significant change in photoluminescence upon the addition of up to 1.0 M NaCl (data not shown). Apparently the multiple salt bridges formed between the avidin and QDs are resistant to ionic strength effects.

Direct Binding Fluoroimmunoassays. Direct binding experiments showed that QD-antibody conjugate constructed through bridging avidin was able to bind to goat IgG immobilized in microtiter plate wells (Figure 3). Binding was assessed by measuring the photoluminescent signal from directly

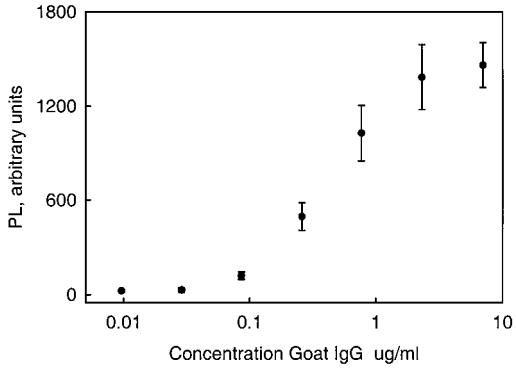

Figure 3. Direct binding of QD-anti-goat antibody conjugates to adsorbed goat IgG. Wells coated with varying concentrations of goat IgG (9.6 ng/mL to 7 μg/mL) were probed with biotinylated anti-goat antibody conjugated to avidin coated QDs. Measurements were done in triplicate and error bars represent standard deviations. QDs emitting at 570 nm were used.

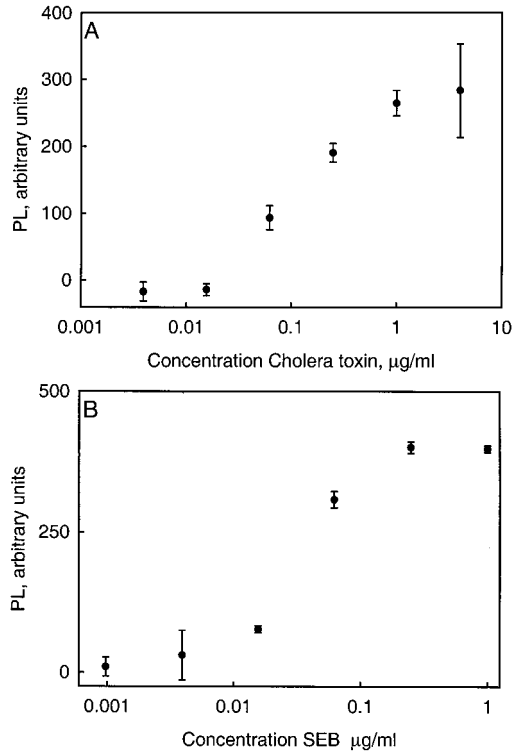

Figure 4. Sandwich fluoroimmunoassay for detection of cholera toxin and SEB. QDs emitting at 570 nm were used. (A) Results of a sandwich assay where varying concentrations of cholera toxin (4 ng/mL to 4 μg/mL) were captured by anti-cholera toxin antibody immobilized on wells of a plate. Signal generation was accomplished by using QD-anti-cholera toxin conjugate. (B) Results of a sandwich assay where varying concentrations of SEB (0.98 ng/mL to 1 μg/mL) were captured by anti-SEB antibody immobilized on wells of a plate. Signal generation was accomplished by using QD-anti SEB conjugate.

captured QD-anti-goat antibody. In these experiments, the fluorescent signal for bound conjugate was measured over a concentration range of goat IgG from 9.6 ng/mL to 7 μg/mL. The lowest concentration of goat IgG that gave meaningful signal over background was approximately 250 ng/mL. This experiment suggested that these QD-antibody conjugates could be used in direct detection fluoroimmunoassays. Analogous direct binding assays also showed that adsorbed protein toxins (cholera toxin and SEB) were able to be detected with use of QD-antibody conjugates (data not shown).

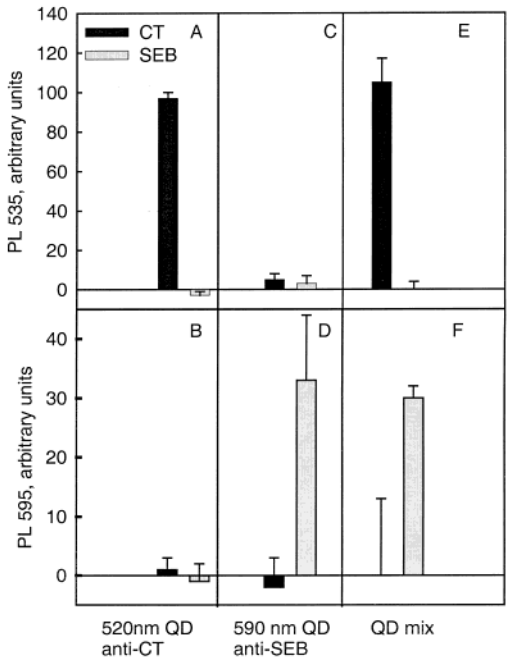

Figure 5. Dual analyte detection using anti-cholera toxin and anti-SEB coated QDs. QD-conjugated antibodies were examined alone and in combination for their ability to bind to wells coated with saturating amounts of cholera toxin (5 μg/mL) or SEB (10 μg/mL). Black bars represent the signal from cholera toxin coated wells, gray bars from SEB coated wells. Each single component reagent was prepared to have approximately the same concentration of that color QDs as was in the mixed sample. All measurements were done in triplicate and error bars represent standard deviations. (A) Anti-cholera toxin-QD conjugate incubated with cholera toxin and SEB coated wells. Signal read using the 535 nm filter. (B) Same wells as in Panel A. Signal read using the 595 nm filter. (C) Anti-SEB-QD conjugate incubated with cholera toxin and SEB coated wells. Signal read using the 535 nm filter. (D) Same wells as Panel C. Signal read using the 595 nm filter. (E) Mix of anti-cholera toxin-QD and anti-SEB-QD conjugates incubated with cholera toxin and SEB coated wells. Signal read using the 535 nm filter. (F) Same wells as Panel E. Signal read using the 595 filter.

Sandwich Assay Format for Toxin Detection. A sandwich assay format was also explored as this is the method of choice for the analysis of unknown samples. QDs conjugated to antitoxin antibodies via avidin bridges were used in this format for detection of cholera toxin and SEB. Anti-cholera toxin capture antibody was adsorbed to wells of a microtiter plate. A series of dilutions of cholera toxin were applied to wells containing adsorbed capture antibody, followed by QD-polyclonal rabbit anti-cholera toxin conjugate as the signal-producing reagent. Fluorescent signal from bound rabbit-anti-cholera toxin conjugate was measured over a range of concentrations of toxin from 4 ng/mL to 4 μg/mL (Figure 4A). The lowest concentration of cholera toxin that gave positive signal over background was approximately 60 ng/mL; the signal increased until saturation was reached at about 1 μg/mL.

QD- sheep-anti-SEB antibody conjugates were similarly used in a sandwich assay (Figure 4B). The lowest concentration of SEB that gave useful signal over background was approximately 15 ng/mL, and the signal increased until saturation was reached at about 250 ng/mL.

This is the first demonstration of the use of avidin-conjugated DHLA-QDs in sandwich fluoroimmunoassays, and one of only a few of the uses for QDs in sandwich fluoroimmunoassays.[12,17] While the limits of detection achieved in these experiments are

not superior to conventional immunoassays, these experiments show that QDs can be used as a fluorescent tag for this application.

Dual Detection. A major advantage of QDs over conventional fluorophore labels is different color nanocrystals can be excited at a single wavelength. We examined the anti-SEB and anti-cholera toxin QD-antibody conjugates prepared via an avidin linker in two analyte tests aiming at eventual multianalyte detection.

Anti-cholera toxin antibody was conjugated to 520 nm emitting QDs, and anti-SEB antibody was conjugated to 590 nm emitting QDs. These conjugates are spectrally separable by using the Tecan plate reader. Figure 5A shows QD-anti-cholera toxin conjugates added to wells coated with saturating amounts of SEB or cholera toxin and examined with a 535 nm filter. Signal was observed only in wells coated with cholera toxin, indicating that there is no cross reactivity between the anti-cholera toxin antibody and SEB. When observed with a 595 nm filter these same wells show no signal (Figure 5B). Similarly, no signal was observed when QD-anti-SEB conjugate was added to wells coated with saturating amounts of SEB or cholera toxin and examined with use of a 535 nm filter (Figure 5C). This demonstrates that there was minimal cross talk between the 520 and 590 nm emitting QD conjugates. When the wells containing QD-anti-SEB conjugate were examined with use of a 595 nm filter (Figure 5D), the signal was observed for only the SEB coated wells, showing that the anti-SEB antibody has no cross reactivity toward cholera toxin. A mix of anti-cholera toxin conjugated QDs and anti-SEB conjugated QDs was incubated on wells coated with cholera toxin or SEB and imaged by using the 535 or 595 filters. When assayed with the 535 nm filter, only signal from the cholera toxin coated wells was observed (Figure 5E). Using the 595 filter generates signal from only the SEB coated wells (Figure 5F).

We were limited to simultaneously screening for only two toxins using two-color QDs because the filters available for our plate reader are incapable of finer color resolution. Our results suggest that improved instrumentation will allow QD-antibody conjugates to be used in multianalyte assays in both direct and sandwich formats with at least 3−5 different toxins and associated colored QD conjugates. The advantages of combining multiple assays in a single microtiter well include savings in reagents and consumables, decreased sampling errors, and higher throughput compared with single-assay systems.

Conclusions

We have prepared QD-avidin conjugates that effectively and tightly bind biotinylated antibodies and tested them in direct, capture, and dual component fluoroimmunoassays. Preparing QDs with associated avidin allows conjugation of QDs with any biotin containing antibody, protein, DNA, or synthetic polymer. QD resistance to photobleaching, their ability to be excited at many wavelengths, and their narrow emission wavelengths combined with the ready availability of a wide range of biotinylated chemicals, biomolecules, and antibodies make avidin conjugated QDs useful for additional bioanalytical applications.

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