

Luminescent Quantum Dot-Adaptor Protein-Antibody Conjugates for Use in Fluoroimmunoassays

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A method for the preparation and characterization of bioinorganic conjugates made with highly luminescent semiconductor CdSe–ZnS core-shell quantum dots (QDs) and antibodies for use in fluoroimmunoassays is presented. The conjugation strategy employs two routes: 1. Use of an engineered molecular adaptor protein, attached to the QDs via electrostatic/hydrophobic self-assembly, to link the inorganic fluorophore with antibodies, and 2. use of avidin, also electrostatically self-assembled onto the nanocrystal surface, which allows QD conjugation to biotinylated antibodies via avidin–biotin binding scheme. With this approach, the average number of antibodies conjugated to a single QD can be varied. In addition, we have developed a simple purification strategy based on mixed composition conjugates of the molecular adaptor and a second “inert” two-domain fusion protein that allows the use of affinity chromatography. QD/adaptor-antibody conjugates were successfully employed in fluoroimmunoassays for the detection of small molecule analytes, 2,4,6-trinitrobenzene (TNB) and hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX). We also demonstrate the use of QD/avidin-antibody conjugates for fluoroimmunoassays using a model protein system.

Introduction Colloidal semiconductor nanocrystals (quantum dots, QDs) are spherical particles in a size regime dominated by strong quantum confinement of the charge carriers. This confinement lifts the degeneracy of the carrier states within the conduction and valence bands, and increases the effective band gap energy significantly with decreasing particle size, resulting in size dependence of several properties, such as absorption and photoluminescence (PL) spectra [1–3]. Colloidal QDs with surfaces ligated with a variety of functional capping groups can be dispersed in organic solvents, and water-compatible QDs have been prepared either directly by using aqueous-compatible surface caps, or indirectly by embedding the particles in micron-size beads and functionalizing the surface of the beads with ligands to make them water compatible [4–8]. Luminescent colloidal QDs have the potential to circumvent some of the functional limitations encountered by organic dyes in biotechnological applications. Organic fluorophores have characteristics that limit their effectiveness for certain applications. These limitations include narrow excitation bands and broad emission bands with red spectral tails, and many organic dyes exhibit low resistance to photodegradation [9]. In contrast, CdSe–ZnS core-shell QDs exhibit narrow PL with full width at half-maximum (FWHM) of ~30–45 nm that spans the visible spectrum, and they have broad absorp-

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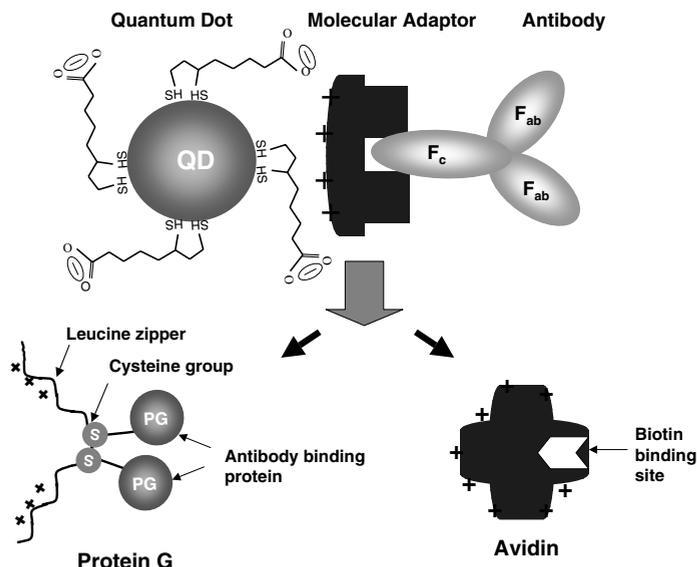


Fig. 1. Upper part: schematic representation of a QD/adaptor-antibody conjugate. Bottom left part: the S-S linked PG-zb homodimer acts as a molecular adaptor to connect the DHLA-capped QD to the Fc region of an antibody. Bottom right part: schematics of an avidin protein. The highly specific binding between avidin and biotin allows conjugation of QD/avidin to biotinylated IgGs

tion spectra that allow excitation of several color QDs with a single wavelength [1, 6, 8]. These nanoparticles also have a high PL quantum yield and high resistance to photodegradation.

We have recently developed a novel conjugation strategy, based on electrostatic interactions between negatively charged dihydrolipoic acid (DHLA)-capped CdSe–ZnS QDs and a positively charged leucine zipper interaction domain appended onto the C-terminus of engineered recombinant proteins [6, 7]. We have also extended that approach to using avidin (a positively charged tetramer) to form QD/avidin conjugates [10].

In this paper, we describe the formation of QD/antibody conjugates using either a molecular adaptor protein that employs the immunoglobulin G (IgG)-binding B2 domain of streptococcal protein G (PG) appended with a leucine zipper interaction domain, or using avidin to form QD/antibody conjugates with biotinylated IgGs via avidin–biotin binding (Fig. 1). We describe the use of antibody-conjugated QDs in plate-based immunoassays for the detection of ng quantities of the TNT-surrogate, TNB-fluorescein, and the explosive hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) in aqueous samples. We also demonstrate direct detection of mouse IgG employing QD/avidin bioconjugates formed using biotinylated Rabbit-anti-mouse IgG.

Materials Preparation and Experimental Protocols

Nanocrystal preparation Highly luminescent CdSe–ZnS quantum dots with quantum yields of ~30%–50% were prepared via the pyrolysis of organometallic reagents in a coordinating solvent made of a mixture of trioctyl phosphine and trioctyl phosphine oxide (TOP/TOPO) [1, 11]. Water-soluble CdSe–ZnS nanocrystals were prepared using a stepwise procedure [6]. After synthesis of the TOP/TOPO-capped CdSe core, a rela-

tively thick ZnS-overcoating of 5–7 monolayers was grown on the particle surface [11]. The native capping groups were then exchanged with dihydrolipoic acid groups by suspending ~150 mg of size-selected nanocrystals (2–3 times) in ~500 μ l dihydrolipoic acid and equilibrating at ~70 °C for 1–2 h. The mixture was then diluted in 1–2 ml of dimethylformamide (DMF), and deprotonation of the terminal-COOH groups was carried out using potassium-tert-butoxide (K-t-butoxide) [6]. The resulting precipitate, consisting of the nanoparticles together with released TOP/TOPO, was sedimented by centrifugation, and then redispersed in water and further purified using a membrane filtration device to remove excess solubilized TOP/TOPO, salts, and DMF. The final clean and stable aqueous QD dispersions showed the emission characteristics of the initial nanocrystals and a PL yield of 10%–20%. Four colors of nanocrystals were used in various aspects of the present work: $\lambda_{\text{max}} = 520$ nm (core radius of 14 Å), $\lambda_{\text{max}} = 555$ nm (core radius of 17.5 Å), $\lambda_{\text{max}} = 570$ nm (core radius of 19 Å) and $\lambda_{\text{max}} = 590$ nm (core radius of 21.5 Å).

Molecular adaptor proteins The gene encoding of the two-domain protein G-basic leucine zipper (PG-zb) fusion protein was constructed using standard gene assembly and cloning techniques as previously described [12]. PG-zb protein consists of the B2 IgG-binding domain of streptococcal protein G (PG) linked with a C-terminal anchor domain containing the poly-Asn linker from the pMal plasmid series (New England Biolabs), a dimer-promoting cysteine, the basic leucine zipper, and a hexahistidine tag. The purified PG-zb protein was about 80% S–S linked dimer and 20% monomer. Hen egg white avidin was purchased from Pierce and used without further purification. Avidin was first mixed with the maltose binding protein appended with the basic leucine zipper tail (MBP-zb) in borate buffer at pH 9 to create a mixed solution at the desired protein ratio, and the mixture was added to solution of QDs resulting in the formation of QD/avidin conjugates at a ratio: QD:avidin:MBP-zb of 1:2:6.

Formation and purification of QD/antibody conjugates QD/antibody conjugates were formed using mixed-surface precursors in order to allow facile purification of the final products from excess antibody. In all cases, DHLA-capped quantum dots were first incubated with various concentrations of the chosen molecular adaptor (PG-zb or avidin) together with comparable amounts of a derivative of E. coli Maltose Binding Protein (MBP-zb) [6]. By occupying some of the surface sites of each final QD-conjugate, the MBP-zb allowed conjugate purification using immobilized cross-linked amylose affinity medium (New England Biolabs). All QD/protein conjugation work was carried out in 10 mM sodium borate pH 9 (borate buffer). In practice, after formation of QD/adaptor protein precursors, they were allowed to react with an excess of the desired IgG for approximately 1 h at room temperature. For conjugates using PG-zb adaptors, reaction mixtures containing mixed surface QD-conjugates were applied onto 0.5 ml amylose columns, unbound IgG was removed by washing with ca. 2 ml HEPES buffer (10 mM HEPES, 50 mM NaCl, 1 mM EDTA, pH 8), and the QD/antibody conjugates were then eluted with the same buffer containing 10 mM maltose. For conjugates using avidin adaptors, excess biotinylated antibody was allowed to react for ca. 1 h with QD/avidin-MBP-zb precursors previously immobilized on an amylose column before washing with 2 ml PBS buffer and eluting with 10 mM maltose in PBS. Under the conditions used for the present experiments, an average of three antibodies were conjugated with each nanocrystal in affinity-purified preparations [12].

Fluoroimmunoassays using QD/antibody conjugates All assays were conducted in a 96-well microtiter plate format using opaque white plates (Maxisorb, Nunc). QD/IgG bioconjugates prepared using the PG-zb adaptor were used in model competition assays to analyze for TNB-fluorescein and RDX. Microtiter plate wells were coated overnight (4 °C) with a saturating concentration of trinitrobenzenesulfonate-modified ovalbumin (TNB-ovalbumin) in 0.1 M sodium carbonate pH 8.5. Buffer with no TNB-ovalbumin was used to assess non-specific binding in control wells. After removing excess TNB-ovalbumin or buffer blank solutions, wells were blocked at 4 °C for 2 h with PBS containing 4% (w/v) powdered non-fat milk, then washed two times with PBST (PBS with 0.1% Tween 20). The derivatized ovalbumin served as a surface-adsorbed capture agent for QD/anti-TNB conjugates in competition with dissolved TNB-fluorescein (the fluorescein moiety is not utilized in the present assays). QDs conjugated with either of two available anti-TNB antibodies, namely 11B3 [13] or A1.1.1 (Strategic Biosolutions), were added to test and control wells and incubated for 1–2 h. A 40 µM stock solution of TNB-fluorescein (synthesis will be published elsewhere) was used to make up a dilution series of 4 µM to 4 nM of the TNT surrogate in HEPES buffer. 50 µl of each TNB-fluorescein dilution was added to appropriate test and control wells followed immediately by 50 µl of QD/11B3 (or QD/A1.1.1), then incubated at room temperature for 1–2 h. Wells were then washed and the photoluminescence measured using a SpectraFluor Plus microtiter plate reader (Tecan). A 25 nm band pass filter was used for excitation at 310 nm, and either a long pass filter with lower cut-off at 530 nm or a 25 nm band pass filter centered at 535 nm were used for luminescence data collection. All samples (plus negative controls) were prepared in triplicate and average values at each concentration were calculated.

The plate-based assay using QD-conjugates to detect RDX [hexahydro-1,3,5-trinitro-1,3,5-triazine] was essentially carried out following the procedure described above, after BSA-RDX conjugates were immobilized on wells of microtiter plates. QDs were conjugated with the monoclonal anti-RDX antibody W2.1 (Strategic Biosolutions), again using PG-zb as adaptor. RDX stock solution (2 µg/ml) was prepared by transferring 1 µl of a 1 mg/ml RDX solution (Radian international) in acetonitrile to a glass tube containing 0.5 ml HEPES buffer, appropriate dilutions were made, and samples used immediately for competition assays. 100 µl samples of QD/W2.1 conjugates and RDX dilutions (50 µl QD-conjugates and 50 µl of RDX solution) were added to test and control wells, and incubated at room temperature for 1–2 h. Wells were then washed and the PL intensity was measured using the above reader.

For direct binding assays using QD-conjugates made using avidin as adaptor, 50 µl aliquots of mouse IgG at several concentrations (1 µg/ml–16.5 ng/ml) or the same volume of buffer with no IgG were adsorbed onto plate wells overnight at 4 °C. After blocking the wells, 50 µl aliquots of QD/Rabbit-anti-mouse IgG conjugates were added to the wells and let incubate for 1–2 h, followed by removal of excess QD/antibody conjugates and washing (three times). Residual luminescence was then measured as described above.

Results and Discussion Figure 2 shows that soluble TNB-fluorescein competed effectively with adsorbed TNB-ovalbumin for binding with QD/11B3 and QD/A1.1.1 conjugates. A systematic decrease of PL intensity with increasing TNB-fluorescein concentration was measured with conjugates formed using either antibody. The signal reached

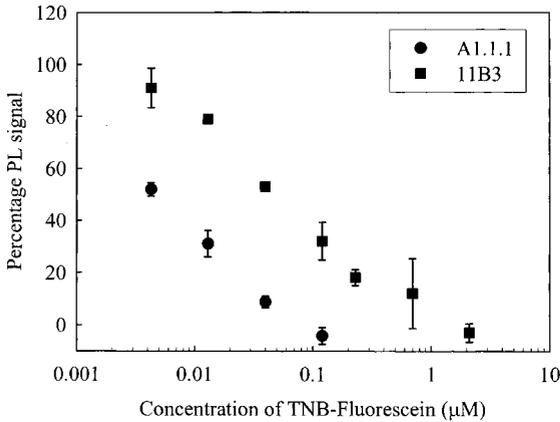


Fig. 2. Competitive assay for the detection of TNB-fluorescein employing both QD/PG-zb-11B3 and QD/PG-zb-A1.1.1 conjugates, where inhibition of QD/antibody conjugate binding to TNB-ovalbumin by free TNB-fluorescein was investigated. PL intensity was measured (in percentage values) for varying concentrations of QD/PG-zb-anti-TNB-binding to wells coated with a saturating amount of TNB-ovalbumin (10 µg/ml). 100% indicates signal in the absence of TNB-fluorescein

background values at the higher soluble analyte concentrations, i.e., ~0.1 µM and 5 µM for QD/A1.1.1 and QD/11B3 conjugates, respectively. TNB-fluorescein at concentrations as low as 1 nM can be detected in the competitive assay using QD/anti-TNB conjugates, with a slightly greater sensitivity seen for QD/A1.1.1 conjugates.

Soluble RDX similarly competes with adsorbed RDX-BSA for binding to QD/W2.1 conjugates (Fig. 3). Measurable decay in the PL intensity was observed at ~5 nM of RDX, with signal reaching background values at ~5 µM.

Figure 4 shows the results of a direct binding assay employing QD/avidin as the precursor conjugate; the measured PL intensity is plotted as a function of mouse IgG concentration initially added to wells for adsorption. The dynamic range of this assay spans two orders of magnitude. Similar behavior was observed previously in experiments where QD/PG-zb-IgG conjugates were used in similar direct and sandwich-format assays for analysis of staphylococcal exenterotoxin B (SEB) [12].

In a previous study [12], we examined the ability of QD/11B3 conjugates to bind to surface adsorbed TNB-ovalbumin at saturating concentrations, and found that the measured PL increased linearly with increasing QD/11B3 concentration. Our present assays

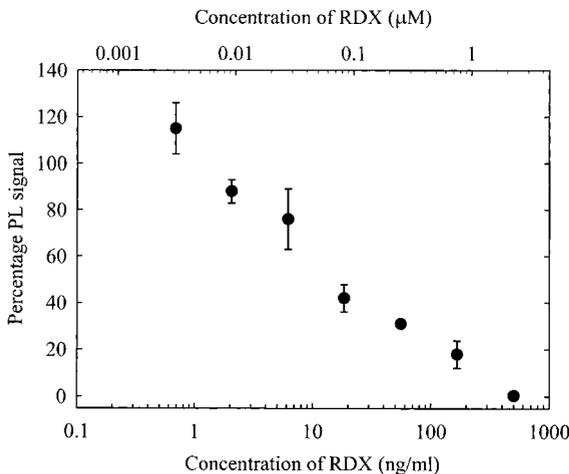


Fig. 3. Competitive assay for the detection of RDX employing QD/PG-zb-anti-RDX conjugates, where inhibition of QD-conjugates binding to BSA-RDX by free RDX was investigated. The PL intensity was measured for varying concentrations of QD/anti-RDX binding to wells coated with a saturating amount of BSA-RDX. 100% indicates signal in the absence of RDX

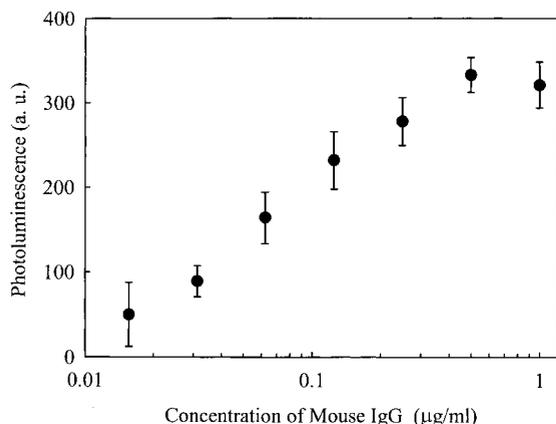


Fig. 4. Direct fluoroimmunoassay, employing QD/avidin-Rabbit-anti mouse conjugates. Wells were coated with various concentrations of mouse antibody. The PL intensity was measured as a function of increasing concentration of mouse IgG. Data are shown for QDs emitting at 570 nm. Similar data were collected for QDs emitting at other wavelengths

for the detection of TNB-fluorescein and RDX (and TNT in Ref. [12]) employing QD/antibody conjugates were built on those results. In the present microtiter plate-based competition assay, we used soluble TNB-fluorescein and showed that free TNB-fluorescein competed effectively with adsorbed TNB-ovalbumin for binding with QD/11B3 and QD/A1.1.1 conjugates, as indicated by the systematic decrease in fluorescence emission observed in the presence of increasing concentrations of TNB-fluorescein (Fig. 2). Competitive displacement of QD/antibody conjugates was complete at relatively smaller concentration of TNB-fluorescein when using QD/A1.1.1, which indicates that A1.1.1 antibody has a higher affinity for this analog than does the 11B3 antibody.

When forming the QD/antibody conjugates used in the present study we took two seemingly independent approaches. In one approach the antibodies were attached to the QD label via the asymmetric PG-zb adaptor protein, which tightly self-assemble onto the nanocrystal surface, while offering its functional N-terminal domain for interaction with antibodies. Furthermore, the readily formed S-S linked homodimer strengthens the PG-IgG interactions, due to orientation of the N-terminal domains with respect to the QD surface (see Fig. 1) [6]. In the other approach, avidin permits one to use known approaches to conjugate the nanocrystals to antibodies via the conventional high affinity avidin-biotin binding scheme [9].

Although QD/antibody conjugates were prepared using two different molecular adaptors to link nanocrystals to the immunoglobulins, we believe that the basic assembly mechanisms for conjugates are identical. Both approaches rely on charge-charge interactions between the protein adaptor and the QD surface. The PG-zb adaptor utilizes a highly charged, carefully engineered C-terminal domain for attachment to the modified nanocrystal surface, while its functional domain remains available for interaction with antibodies. As we predicted, the net positive charge that resides on the avidin tetramer allows an analogous electrostatic interaction with QD surfaces, while permitting antibody conjugation via high affinity avidin-biotin binding [9]. It should be emphasized that the procedure we have developed for reaction of QD/avidin precursor with biotinylated IgG requires formation of the conjugates under immobilized conditions and with a saturating concentration of antibodies, since under soluble conditions essentially quantitative precipitates are formed due to polyvalent interactions possible in a freely diffusing system.

Using avidin as the bridging protein takes advantage of the highly specific and very stable avidin–biotin binding phenomenon that is virtually ubiquitous in biotechnological applications. This strategy might be extended to conjugate QDs with any biotinylated species (protein, polymer, or small molecule analog). The present results demonstrate that under the proper conditions naturally occurring and inexpensive avidin can be employed using the electrostatic self-assembly conjugation scheme we developed using engineered recombinant proteins and DHLA-capped semiconductor nanocrystals.

Finally, preparation of conjugates with mixed surface composition, by reaction with various ratios of either MBP-zb:PG-zb or MBP-zb:avidin, should allow control of the average number of antibodies in each QD-bioconjugate, while filling the remaining binding sites with an “inert” coating. Ultimately, this approach may permit titration of the number of antibodies conjugated to a QD down to the limit of one IgG per nanoparticle, a valuable feature in applications where antibody affinity rather than avidity is desirable.

Conclusions In the present study we showed that QD/IgG conjugates prepared using a molecular adaptor protein, either protein G with a basic leucine zipper attachment or avidin, can be used in fluoro-immunoassays. QD/IgG complexes with emission wavelengths varying from the green to orange depending on the QD size were employed interchangeably in the present assays, and sensitivity of detection comparable to organic fluorophores was obtained. Based on some of the other unique properties of these nanoparticles, such as high resistance to photobleaching and their ability to be excited over essentially a continuum of wavelengths, QD-antibody conjugates promise to be particularly useful in the development of fluoroimmunoassays and in immunosensor devices.

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