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Fluoroimmunoassays Using Antibody-Conjugated Quantum Dots

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Summary

Luminescent colloidal semiconductor nanocrystals (quantum dots) are robust inorganic fluorophores that have the potential to circumvent some of the functional limitations encountered by organic dyes in sensing and biotechnological applications. Quantum dots exhibit size-dependent tunable, narrow fluorescence emission spectra that span the visible spectrum and have broad absorption spectra. This allows simultaneous excitation of several particle sizes at a single wavelength with emission at multiple wavelengths. Quantum dots also provide a high-resistance threshold to chemical degradation and photodegradation. We have developed a conjugation strategy for the attachment of antibodies to quantum dots based on electrostatic interactions between negatively charged dihydrolipoic acid (DHLA)-capped CdSe-ZnS core-shell quantum dots and positively charged proteins (natural or engineered) that serve to bridge the quantum dot and antibody. This chapter details the materials and methods for synthesis of the DHLA-capped CdSe-ZnS core-shell quantum dots, the construction and preparation of recombinant proteins, the conjugation of antibodies to quantum dots, and the use of antibody-coated quantum dots in a fluoroimmunoassay.

Key Words

Quantum dots; fluoroimmunoassay; nanocrystals; dihydrolipoic acid; leucine zipper.

1. Introduction

Luminescent colloidal semiconductor nanocrystals (quantum dots) provide an alternative to conventional organic fluorophores for use in a variety of biotechnological applications. The CdSe-ZnS core-shell quantum dots used in our studies exhibit size-dependent tunable photoluminescence with narrow emission bandwidths (full width at half maximum of 25–45 nm) that span the visible spectrum along with broad absorption spectra, which allow simultaneous excitation of several particle sizes at a single wavelength ***(1–5)***. In addition,

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quantum dots have high photochemical stability, and a good fluorescence quantum yield. Photoluminescence from these quantum dots can be detected at concentrations comparable to standard fluorescent organic dyes using conventional fluorescence methods ***(6)***.

We have developed protocols for the conjugation of quantum dots to antibodies for use in fluoroimmunoassays for the detection of proteins or small molecules. Our conjugation strategy is based on electrostatic self-assembly between negatively charged dihydrolipoic acid (DHLA)-capped CdSe-ZnS core-shell quantum dots and positively charged proteins (natural or engineered) that serve to bridge the quantum dot and antibody ***(7,8)***. To facilitate easy separation of the desired quantum dot–antibody product from unlabeled antibody, we employ a mixed surface strategy in which both an antibody-bridging protein and a purification tool protein are immobilized on each quantum dot. This electrostatic noncovalent self-assembly approach to conjugate luminescent quantum dots with proteins extends and complements existing quantum dot-labeling methods ***(9,10)***. Conjugate preparation is simple, highly reproducible, and easily achieved.

We engineered proteins to interact with DHLA-capped quantum dots by appending a positively charged leucine zipper ***(11)*** interaction domain onto the C-terminus of recombinant proteins. Antibodies were conjugated to quantum dots either through the use of an engineered bridging protein consisting of the immunoglobulin G (IgG)-binding β2 domain of streptococcal protein G modified by genetic fusion with the positively charged leucine zipper interaction domain (PG-zb), or through the use of the positively charged protein avidin. A genetically engineered maltose-binding protein appended with the charged leucine zipper (MBP-zb) was used as a purification tool in conjunction with both types of bridging proteins. By using affinity chromatography, excess unconjugated antibody can be separated from the complete quantum dot immunoreagent. **Figure 1** shows schematic representations of the mixed-surface quantum dots with antibodies coupled using the engineered PG-zb or avidin as a bridge. Protocols for conjugation of quantum dots to antibodies using this scheme, as well as the use of antibody-conjugated quantum dots in fluoroimmunoassays for the detection protein targets, are described in the following sections.

2. Materials

2.1. Synthesis of Quantum Dots

1. Selenium (99.99%).
2. Dimethyl cadmium ($CdMe_2$).
3. Trioctylphosphine (TOP) (90–95%).
4. Trioctylphosphine oxide (TOPO).
5. Inert gas (nitrogen or argon).

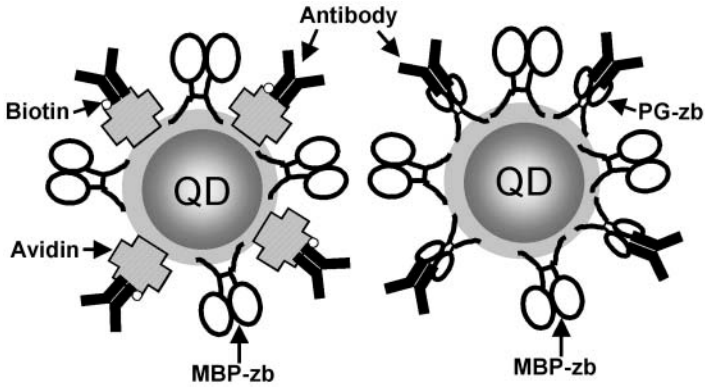


Fig. 1. Schematic of a mixed-surface quantum dot–antibody conjugate in which avidin bridges CdSe-ZnS core-shell nanocrystal quantum dot (capped with a negatively charged DHLA surface) and biotinylated antibody (**Left**). Schematic of a mixed-surface composition quantum dot–antibody conjugate in which the PG-zb (IgG-binding β2 domain of streptococcal protein G modified by genetic fusion with a dimer-forming positively charged tail) acts as a molecular adaptor to connect DHLA-capped CdSe-ZnS core-shell with Fc region of the IgG. **(Right)** In both quantum dot constructs, the MBP-zb (maltose-binding protein appended with the dimer-forming positively charged tail) serves as a purification tool for separating quantum dot–IgG conjugate away from excess IgG through affinity chromatography using crosslinked amylose resin. The exact numbers of avidin, PG-zb, and MBP-zb per quantum dot are not known; the image is not drawn to scale.

6. Glove box.
7. Schlenk line.
8. Solvents (hexane, toluene, butanol, ethanol, methanol, dimethylformamide [DMF]).
9. Diethylzinc ($ZnEt_2$).
10. Hexamethyldisilathiane (TMS_2S).
11. Tthioctic acid.
12. Potassium-*tert*-butoxide (KTB).
13. Ultrafree centrifugal filtration device, molecular weight cutoff of 50,000 (Millipore, Bedford, MA).
14. DHLA. This is prepared from distilled thioctic acid by borohydride reduction ***(12)***.

2.2. Construction of DNA Vector and Expression of Protein

1. pMal-c2 plasmid (New England Biolabs, Beverly, MA).
2. Cloning enzymes (polymerases and endonucleases).
3. QIAquick gel extraction kit (Qiagen, Valencia, CA)
4. pBad/HisB protein expression kit (Invitrogen, Carlsbad, CA).
5. *Escherichia coli* TOP 10 (Invitrogen).

6. Luria Broth Base (LB, Invitrogen).
7. Ampicillin.
8. Isopropyl β-D-thiogalactoside (IPTG).
9. L-(+)Arabinose (Sigma, St. Louis, MO).

2.3. Purification of Protein

1. Buffer A: 100 m*M* NaH_2PO_4, 10 m*M* Tris, 6 *M* guanidine HCl; adjust pH to 8.0 using NaOH.
2. NiNTA resin (Qiagen).
3. Oak Ridge polypropylene centrifuge tubes (50 mL).
4. Buffer B: 100 m*M* NaH_2PO_4, 10 m*M* Tris, 8 *M* urea; adjust pH to 8.0 with NaOH *immediately prior to use*.
5. Buffer C: 100 m*M* NaH_2PO_4, 10 m*M* Tris, 8 *M* urea; adjust pH to 6.3 with NaOH *immediately prior to use*.
6. Phosphate-buffered saline (PBS): 200 m*M* NaCl, 2.7 m*M* KCl, 8.2 m*M* Na_2HPO_4, 4.2 m*M* NaH_2PO_4, 1.15 m*M* K_2HPO_4, pH 7.4.
7. Buffer D: 50 m*M* NaH_2PO_4, 300 m*M* NaCl, 250 m*M* imidazole; adjust pH to 6.5 with HCl.
8. Dialysis tubing (12- to 14-kDa cutoff).
9. Centriprep and/or Centricon (Millipore).
10. Syringe filter (0.22 μ) compatible with protein samples.

2.4. Immunoassays

1. Borate buffer: 10 m*M* sodium borate, pH 9.0.
2. Amylose affinity resin (New England Biolabs).
3. Maltose (Sigma).
4. Small columns (such as Bio-Spin columns or Micro-Bio-Spin columns; Bio-Rad, Hercules, CA).
5. PBS (*see* **Subheading 2.3., item 6**).
6. 96-Well white microtiter plates (FluoroNunc™ Plates MaxiSorp™ surface, Nalge Nunc, Rochester, NY).
7. Fluorescence microtiter plate reader.
8. Appropriate antibodies and antigens.

3. Methods

3.1. Synthesis of Quantum Dots

3.1.1. CdSe Core

1. Prepare a 1 *M* stock solution of TOP:selenide (TOP:Se) by dissolving 7.9 g of Se (99.99%) into 100 mL of TOP (90–95%) (*see* **Note 1**).
2. Add 170–250 μL of $CdMe_2$ and 3.5–4 mL of 1 *M* TOP:Se to about 15 mL of TOP.
3. Mix under inert atmosphere in a glove box.
4. Load into a syringe equipped with a large-gage needle for injection. Store in the glove box until **step 9**.

5. Load 20–30 g of TOPO (90%) into a 100-mL three-neck flask.
6. Use a Schlenk line to heat TOPO to 150–180°C for 2 h under vacuum while stirring. This dries and degases the TOPO.
7. Backfill with inert gas (typically nitrogen or argon).
8. Raise the temperature to 300–350°C in preparation for precursor injection.
9. Remove the flask from the heating source. Retrieve the syringe from the inert chamber (glove box) and quickly inject the syringe content into the 100-mL flask.
10. Keep the temperature below 200°C for a few minutes (to avoid growth) and take an absorption spectrum. The spectrum should show resolved features with the peak of the first transition (band edge absorption) usually located approx 490 nm.
11. Raise the temperature to 280–300°C. These higher temperatures allow growth and annealing of the quantum dots.
12. During growth, periodically remove samples and take their ultraviolet (UV)/visible absorption spectra. Monitor the position of the first absorption peak and its relative width; this is usually indicative of a sample's size distribution. If spectra indicate that growth has stopped, raise the temperature by several degrees (if desired).
13. Once the location of the first absorption peak reaches a wavelength indicative of a desired size, drop the temperature to below 100°C to arrest crystal growth.
14. Store the growth solution in a mixture of butanol and hexane (or toluene).

3.1.2. Purification

To isolate quantum dots with TOP/TOPO-capping ligands and to obtain a sample with a more narrow size distribution, CdSe quantum dots are often purified using size-selective precipitation, which makes use of preferential Van der Waals interactions ***(1)***.

1. Retrieve a fraction of the growth solution (usually containing a mixture of quantum dots, TOP, TOPO, butanol, and hexane [or toluene]).
2. Slowly add a "bad" solvent for the TOP/TOPO-capped nanocrystals, such as methanol or ethanol.
3. Precipitate the mixture.
4. Redisperse the precipitate in hexane or toluene.
5. Precipitate again using methanol or ethanol.

These steps should provide solutions of quantum dots with very low concentrations of free TOP/TOPO ligands. Repeating this operation without inducing macroscopic precipitations can substantially reduce the overall size distribution of the quantum dots; however, it reduces product yield ***(1)***.

3.1.3. ZnS Overcoating

In the mid-1990s, a few reports ***(4,5)*** showed that overcoating CdSe quantum dots with ZnS improved quantum yields to values of 30–50%. This is owing to the fact that passivating the quantum dots with an additional layer made of a

wider band-gap semiconductor provides a better passivation of surface states and results in a dramatic enhancement of the fluorescence quantum yield.

The procedure for overcoating colloidal CdSe quantum dots with a thin layer of ZnS can be carried out as follows: A dilute solution of quantum dots (containing Cd concentrations of approx 0.5 mmol or smaller) is dispersed in a TOPO-coordinating solvent. The temperature of the solution is raised to about 150°C but kept lower than 200°C to prevent further growth of the quantum dots. A dilute solution of Zn (or Cd) and S precursors is then slowly introduced into the hot stirring quantum dot solution. A typical ZnS overcoating includes the following steps:

1. Mount a round-bottomed flask (100 mL or larger) along with a separate addition funnel.
2. Load 20–30 g of TOPO into the round-bottomed flask and let it dry and degas (as described in **Subheading 3.1.1., step 6**) for 2 to 3 h under vacuum.
3. Add purified CdSe quantum dot solution (dispersed in hexane or toluene) at 70–80°C to a final Cd concentration of 0.5 mmol or smaller.
4. Evaporate the solvent under vacuum.
5. Increase the temperature of the quantum dot/TOPO solution to between 140 and 180°C, depending on the initial core radius (lower temperature for smaller core size).
6. In parallel, add equimolar amounts of $ZnEt_2$ and TMS_2S precursors that correspond to the desired overcoating layer for the appropriate CdSe nanocrystal radius to a vial containing 4 to 5 mL of TOP. Use an inert atmosphere (e.g., a glove box) to carry out this operation, because precursors are volatile and hazardous.
7. Load the Zn and S precursor solution from **step 6** into a syringe (in the glove box).
8. Retrieve the syringe containing the solution from the inert chamber and transfer the content to the addition funnel.
9. Slowly add through the addition funnel the Zn/S precursor solution to the quantum dot/TOPO solution at a rate of about 0.5 mL/min (about 1 drop every 3–5 s).
10. Once the addition is complete, lower the solution temperature to 80°C, and leave the mixture undisturbed for several hours.
11. Add a small amount of solvent (e.g., butanol and hexane), and precipitate the ZnS-overcoated quantum dots with methanol to recover the quantum dot product.

3.1.4. DHLA Cap and Water Solubilization

Water-soluble CdSe-ZnS nanoparticles, compatible with aqueous conjugation conditions, can be prepared using a stepwise procedure. A relatively thick ZnS overcoating of five to seven monolayers should be used to prepare the water-compatible quantum dots.

1. Purify TOP/TOPO-capped CdSe-ZnS core-shell quantum dots by two to three rounds of size-selection precipitation (*see* **Subheading 3.1.2.**).

2. Suspend 100–500 mg of purified TOP/TOPO-capped quantum dots in 300–1000 µL of freshly prepared DHLA. Heat the mixture to 60–80°C for a few hours, while stirring.
3. Dilute the quantum dot solution in 3–5 mL of DMF or methanol.
4. Deprotonate the terminal lipoic acid-COOH groups by slowly adding excess KTB. A precipitate is formed, consisting of the nanoparticles and released TOP/TOPO reagents.
5. Sediment the precipitate by centrifugation and discard the supernatant solvent.
6. Disperse the precipitate in water. The quantum dots with the new DHLA caps should disperse well in the water.
7. *Optional:* Conduct centrifugation or filtration of the dispersion (using a 0.5-µm disposable filter) to permit removal of the TOP/TOPO and provide a clear dispersion of the alkyl-COOH-capped nanocrystals.
8. Use an ultrafree centrifugal filtration device (M_W cutoff of approx 50,000) to separate the DHLA-capped quantum dots from excess hydrolyzed KTB and residual DMF. This will also remove the TOP/TOPO if **step 7** is skipped.
9. Repeat the centrifugation cycle using the centrifugal filtration device four times, taking up the quantum dot solution in water using a concentration/dilution of 10:1.
10. Disperse the final material in deionized water or buffer at basic pH.

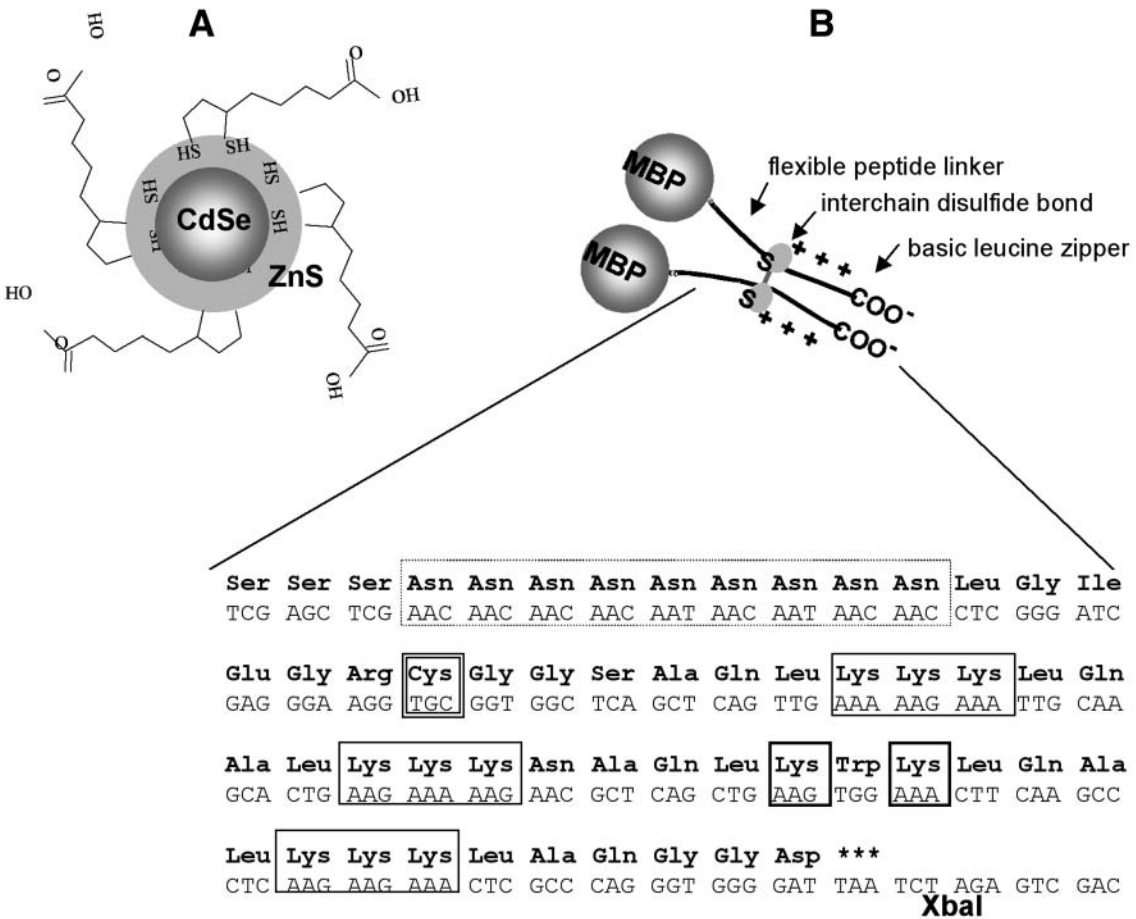
Dispersions of quantum dots in aqueous suspension with concentrations of 5–30 µ*M* are prepared using this approach. The aqueous quantum dot suspensions are stable for months if stored at 4°C.

3.2. Construction of DNA Vector and Expression of Protein

3.2.1. Construction of MBP-zb DNA Vector and Expression of Protein

The coding DNA sequence for the two-domain maltose-binding protein–basic zipper fusion protein (MBP-zb) was constructed using standard gene assembly and cloning techniques. **Figure 2** illustrates the idealized MBP-zb dimer and the detailed nucleotide coding and primary amino acid sequences of the version of MBP-zb lacking a HIS tail.

1. Amplify DNA coding for the basic zipper from the plasmid pCRIIBasic (kindly supplied by H. C. Chang of Harvard University; ***[13]***) using polymerase chain reaction (PCR) with the following conditions: 25 cycles (30 s at 94°C, 90 s at 60°C, and 90 s at 72°C) using primers 1 and 2 (primer 1: 5′-TGCGGTGGCT CACTCAGTTG-3′; primer 2: 5′-GCTCTAGATTAATCCCCACCTGGGCGAG TTTC-3′) and pfu DNA polymerase (Stratagene).
2. Digest the amplified DNA with *Xba*I endonuclease.
3. Ligate into the *Xmn*I/*Xba*I sites within the polylinker downsteam of the *mal E* gene in the commercially available pMal-c2 vector to produce the plasmid pMBP-zb.


A
B
CdSe
ZnS
MBP
MBP
flexible peptide linker
interchain disulfide bond
basic leucine zipper
COO-
COO-
Ser Ser Ser Asn Asn Asn Asn Asn Asn Asn Asn Asn Leu Gly Ile
TCG AGC TCG AAC AAC AAC AAC AAT AAC AAT AAC AAC CTC GGG ATC
Glu Gly Arg Cys Gly Gly Ser Ala Gln Leu Lys Lys Lys Leu Gln
GAG GGA AGG TGC GGT GGC TCA GCT CAG TTG AAA AAG AAA TTG CAA
Ala Leu Lys Lys Lys Asn Ala Gln Leu Lys Trp Lys Leu Gln Ala
GCA CTG AAG AAA AAG AAC GCT CAG CTG AAG TGG AAA CTT CAA GCC
Leu Lys Lys Lys Leu Ala Gln Gly Gly Asp ***
CTC AAG AAG AAA CTC GCC CAG GGT GGG GAT TAA TCT AGA GTC GAC
XbaI

The coding sequence for the C-terminus of MBP-zb (**Fig. 2**) was remodeled using standard DNA manipulation and cloning techniques to include a short spacer element linked to a hexahistidine affinity tag. The finally obtained C-terminus in pMBP-zb-his was identical to the C-terminal sequence of PG-zb shown in **Fig. 3**.

The following protocol for protein expression can be used with either the pMBP-zb or pMBP-zb-his vector construct. We performed the majority of our work using the pMBP-zb-his vector. The protein purification protocol detailed in **Subheading 3.3.** is for the his-tag-containing protein.

1. Inoculate 10 mL of LB medium (100 µg/mL of ampicillin) with a single colony of *E. coli* (strain TOP 10; Invitrogen) freshly transformed with the MBP-zb-his vector.
2. Grow with shaking at 37°C overnight (about 15 h).
3. Inoculate 5 mL of the overnight culture into 0.5 L of LB (100 µg/mL of ampicillin).
4. Continue to grow at 37°C until an OD_{600} of about 0.5 is reached. Induce protein production by adding IPTG (from a 1 *M* sterile stock) to a final concentration of 1 m*M*.
5. Grow an additional 2 h at 37°C with shaking.
6. Pellet the cells by centrifugating 4,000 rpm at 4°C, and store the resulting cell pellet frozen at –80°C.

3.2.2. Construction of PG-zb DNA Vector and Protein Expression

The two-domain protein G-basic leucine zipper (PG-zb) fusion protein was constructed using standard gene assembly and cloning techniques. **Figure 3** shows a schematic representation and the coding sequence of the PG-zb construct.

1. Use PCR to amplify the β2 IgG-binding domain of streptococcal protein G (PG; ***[14]***) and to introduce sites for cloning with the following conditions: 25 cycles (45 s at 94°C, 45 s at 55°C, and 45 s at 72°C) using primers GNCO199 (CAACGCTAAAATCGCCATGGCTTACAAACTTGTTATTAAT) and GSAC199 (GGTACCAGATCACGAGCTCTCAGTTACCGTAAAGGTCTT); *Nco*I, *Sac*I, and *Kpn*I sites are underlined.

Fig. 2. *(previous page)* **(A)** Schematic of CdSe-ZnS core-shell nanoparticle with DHLA surface capping groups; **(B)** schematic of S-S-linked MBP-zb homodimer and detail showing nucleotide and primary amino acid sequence of C-terminal basic leucine zipper interaction domain. Poly-Asn flexible linker is boxed with dashed lines, unique engineered cysteine is double boxed, and lysine residues contributing to net positive charge of leucine zipper are single boxed. (Reprinted from **ref. *6***. Copyright [2000] American Chemical Society.)

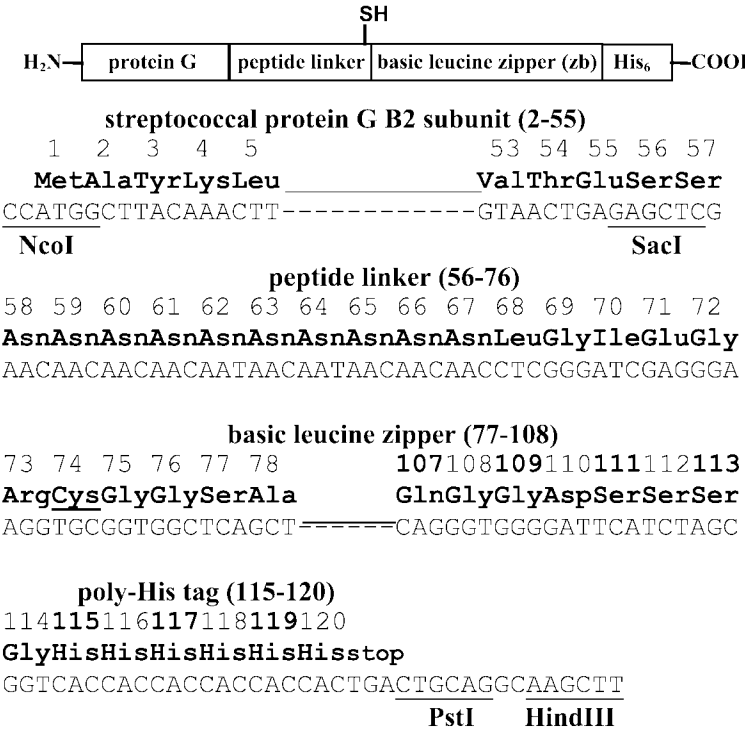


Fig. 3. Schematic of PG-zb protein with DNA and translated protein sequences of relevant region of pBadG-zb. (Reprinted in part from **ref. *7***. Copyright [2002] American Chemical Society.)

2. Extract the *Nco*I-*Kpn*I fragment containing the PG coding sequence from a 2% agarose gel (QIAquick gel extraction kit; Qiagen) and ligate into the *Nco*I/*Kpn*I sites of expression vector pBad/HisB (Invitrogen) to produce the plasmid pBadG.
3. Ligate the purified *Sac*I-*Hin*dIII DNA fragment (from pMBP-zb-his) containing the coding sequences for the poly-Asn linker (from the pMal plasmid series; New England Biolabs), a dimer-promoting cysteine, the basic leucine zipper, and the C-terminal hexahistidine tag into the *Sac*I/*Hin*dIII sites of pBadG to produce pBadG-zb.
4. Select or screen for the appropriately cloned insert.

The following protocol details protein expression for the PG-zb protein.

1. Inoculate 10 mL of LB medium (50 µg/mL of ampicillin) with a single colony of *E. coli* (strain TOP 10; Invitrogen) freshly transformed with pBadG-zb.
2. Grow with shaking at 37°C overnight (about 15 h).

3. Dilute the overnight 1/100 into LB medium (50 μg/mL of ampicillin).
4. Grow with shaking at 37°C until an OD_{600} of approx 0.5 is reached. Induce protein production with the addition of L-(+)arabinose to a final concentration of 0.002% (w/v).
5. Grow an additional 2 h at 37°C with shaking.
6. Pellet the cells by centrifugating at 4,000 rpm, and store the resulting cell pellet frozen at –80°C.

3.3. Purification of Protein

The following protocol can be used for protein purification of both the MBP-zb and PG-zb proteins containing the his tail. This is a denaturing protein preparation and serves to eliminate the copurification of nucleic acids and significant amounts of very active protease(s) that occur under nondenaturing conditions using the cytoplasmic protein fraction from cell lysis and a metal affinity column chromatography.

1. Resuspend the frozen cell pellet from the 500-mL *E. coli* culture (approx 2.5 g of wet cells) in 12.5 mL of denaturing buffer A. Stir the resuspended cells vigorously for 1 h at room temperature using a magnetic stirrer (avoid excess foaming).
2. Place the suspension in a polypropylene Oak Ridge tube (50 mL), and centrifuge at 15,000 rpm for 30 min at room temperature (4°C is also fine).
3. Transfer the supernatant to a clean tube. Add 6 mL of NiNTA resin (about 50% slurry) previously equilibrated two times with 15 mL of buffer A. Tumble the suspension for 45 min at room temperature.
4. Load a 1- to 1.5-cm-diameter column with the protein-charged resin and allow the fluid to flow through. Collect and save the flow-through for possible later analysis.
5. Wash the settled resin in the column two times with 15 mL of buffer A.
6. Wash the resin two times with 15 mL of buffer B.
7. Wash the resin four times with buffer C.
8. Wash the resin four times with 15 mL of PBS buffer (pH 7.4).
9. Elute the protein at about 1 mL/min with 26 mL of buffer D. Collect 1- to 2-mL fractions. Store the fractions at 4°C until dialysis.
10. Analyze the fractions by absorption A_{280} and A_{260} and/or sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).
11. Dialyze the pooled purified fractions against 4 L of PBS at 4°C for 2 to 3 h, change the buffer, and dialyze against 4 L of fresh PBS overnight.
12. Centrifugally concentrate the dialyzed protein solution to about 2 to 3 mL using Centriprep and/or Centricon devices at 4°C.
13. Pass the concentrated material through a buffer-washed 0.22-μ*m* syringe filter into a sterile plastic tube for storage. Aliquots can be stored short term at 4°C or quick frozen in a dry ice–ethanol bath for storage at –80°C.
14. Read the final A_{280} on the properly diluted sample. The molar extinction coefficients for the MBP-zb and PG-zb dimers are 166,200 and 35,400 $M^{-1}cm^{-1}$,

respectively. Run a denaturing SDS-PAGE gel ± reducing agent on the final concentrated product. Estimate and record the approximate covalent monomer/dimer ratio based on the gel results.

3.4. Immunoassays

3.4.1. Preparation of MBP-zb/Avidin/IgG-Coated Quantum Dots

This section describes the preparation of antibody-conjugated quantum dots using avidin to bridge quantum dots and biotinylated antibody. Quantum dots are mixed with avidin and a purification tool protein (MBP-zb). The protein-coated quantum dots are loaded onto an amylose column and biotinylated antibody is bound to the avidin quantum dots on the column. The MBP-zb facilitates purification of the final quantum dot/antibody reagent from excess free antibody. The left panel of **Fig. 1** shows a representation of the MBP-zb/avidin/IgG-coated quantum dot product produced through this protocol.

1. To 200 µL of borate buffer add 0.43 nmol of MBP-zb, 15 µg of avidin (about 0.22 nmol), and 0.1 nmol of quantum dots. Mix gently and incubate at room temperature for about 15 min (*see* **Notes 2** and **3**).
2. Add a second aliquot of 0.22 nmol of MBP-zb to the dot-protein mix. Mix gently and incubate for another 15 min at room temperature. At this point, the quantum dots can be stored overnight in a refrigerator.
3. Prepare an amylose column by mixing the resin and transferring 0.5 mL/column.
4. Wash the column with 1 mL of buffer (PBS).
5. Add the MBP-zb/avidin-coated quantum dots to the top of the column.
6. Wash the column with 1 mL of PBS.
7. Add biotinylated antibody (20 µg) to the top of the column. Let it just run into the resin and cap the column to stop the flow (*see* **Note 4**).
8. Add 50 µL of PBS to the top of the resin so that it does not dry. Allow the biotinylated antibody to interact with the quantum dot–conjugate on the column for about 1 h.
9. Remove the cap, let the PBS on top run into the column, and then wash the resin with 1 mL of PBS.
10. Elute the MBP-zb/avidin/IgG-coated quantum dots with 1 mL of 10 m*M* maltose in PBS. The elution can be monitored with a hand-held UV light (365 nm). These MBP-zb/avidin/IgG-coated quantum dots can be stored in a refrigerator for at least a few days before use.

3.4.2. Preparation of PG-zb/MBP-zb/IgG-Coated Quantum Dots

This section describes the preparation of antibody-conjugated quantum dots prepared using the engineered protein PG-zb to bridge quantum dots and IgG antibody. Quantum dots are mixed with PG-zb, the purification tool protein (MBP-zb), and IgG. The protein-coated quantum dots are loaded onto an

amylose column used to purifiy the final quantum dot/antibody reagent from free antibody. The right panel of **Fig. 1** shows a representation of the PG-zb/MBP-zb/IgG-coated quantum dot product produced via this protocol.

1. To 200 µL of borate buffer add 0.25 nmol of MBP-zb, 0.22 nmol of PG-zb, and 0.1 nmol of quantum dots. Mix gently and incubate at room temperature for about 15 min (*see* **Note 5**).
2. Add a second aliquot of 0.33 nmol of MBP-zb to the dot-protein mix. Mix gently and incubate for another 5 min at room temperature.
3. Add about 35 µg of IgG to the quantum dot-protein mix. Incubate at 4°C for 1 h (*see* **Note 6**).
4. Prepare an amylose column by mixing the resin and transferring 0.5 mL/column.
5. Wash the column with 1 mL of buffer (PBS).
6. Add the MBP-zb/PG-zb/IgG quantum dots to the top of the column.
7. Wash the column with 2 mL of PBS.
8. Elute the MBP-zb/PG-zb/IgG-coated quantum dots with 1 mL of 10 m*M* maltose in PBS. The elution can be monitored with a hand-held UV light.

3.4.3. Protocol for Sandwich Immunoassays

Figure 4 shows data from a sandwich immunoassay for cholera toxin. Goat anti-cholera toxin (Biogenesis, Kingston, NH) was used as the capture antibody adsorbed onto the wells of plates. Rabbit anti-cholera toxin antibody (Biogenesis) was coupled to 550-nm emitting quantum dots using the PG-zb conjugation strategy detailed in **Subheading 3.4.2.** Concentrations of cholera toxin down to 3 ng/mL were visualized in this assay. Control wells in which no cholera toxin was added to capture antibody-coated wells were also examined.

1. Coat plates overnight at 4°C with 10 µg/mL of appropriate capture antibody diluted into 0.1 *M* sodium bicarbonate, pH 8.6, using 100 µL/well.
2. The next day remove the capture antibody and block the plates with 4% (w/v) powdered nonfat milk in PBS. Blocking can be done for 1 h at room temperature or for several hours at 4°C.
3. After blocking, wash the plates twice with PBS plus 0.1% Tween-20 (PBST).
4. Add 100 µL of antigen solution (diluted in PBS) to the wells of the plates, usually a dilution series with a highest value of 1–10 µg/mL of antigen. Remember to include control wells with no antigen. Test and control wells should be plated in at least triplicate.
5. Rock the plates gently at room temperature for 1 h.
6. Wash the plates twice with PBST.
7. Add 50–100 µL of antibody-conjugated quantum dot reagent (in PBS). Usually the quantum dots eluted in **Subheadings 3.4.1.** or **3.4.2.** are diluted to three to four times the elution volume.
8. Rock the plates gently at room temperature for 1 h.

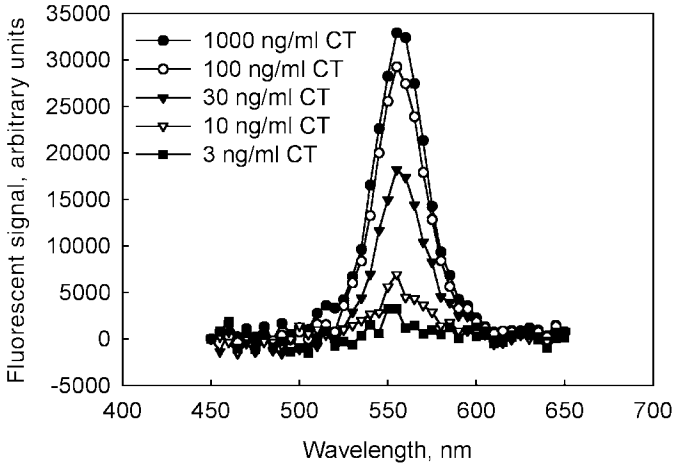


Fig. 4. Sandwich assay for detection of cholera toxin (CT). Goat anti-cholera toxin (Biogenesis) was adsorbed onto wells of the 96-well plate as a capture antibody. Concentrations of cholera toxin ranging from 3 to 1000 ng/mL were incubated with the capture antibody. Rabbit anti-cholera toxin antibody (Biogenesis) coupled to 550-nm emitting quantum dots using the PG-zb adaptor (detailed in **Subheading 3.4.2.**) was used as the signal-producing reagent. Each concentration was measured in triplicate, and the average signal is shown.

9. Wash the plates twice with borate buffer (pH 9.0), and add a final 50–100 µL of borate buffer to the plates (*see* **Notes 7** and **8**).
10. Read the fluorescence in a fluorescent microplate reader. We use an excitation of 330 nm; however, the broad excitation spectra of the quantum dots allows a wide choice of excitation. Choice of emission setting/filter depends on the emission spectra of the quantum dots used in the assay. If the control wells have a high nonspecific signal from the quantum dots, wells may be washed several more times with the borate buffer to reduce further nonspecific signal.

4. Notes

1. Peng and colleagues ***(15,16)*** have developed a modified organometallic synthesis that is less dependent on the purity of the TOPO and avoids the use of pyrophoric $CdMe_2$ precursor. High-purity TOPO and controlled amounts of cadmium-coordinating ligands such as hexylphosphonic acid or tetradecylphosphonic acid, are combined in the preparation flask. Cadmium compounds (e.g., cadmium oxide or cadmium acetate) are added at a relatively low temperature (140°C). The mixture is heated to generate Cd^{2+} ions before the addition of TOP:Se results in nanocrystal nucleation and growth.
2. Mercaptoundecanoic acid- and mercaptoacetic acid-capped CdSe-ZnS quantum dots have also worked with this conjugation method based on electrostatic

self-assembly. Any cap that leads to a negatively charged surface on the quantum dot should also work.

3. Recently, it has been observed that the MBP appended with a 5-HIS tail can be conjugated to CdSe-ZnS core-shell DHLA-capped quantum dots and used as a purification tool protein.
4. Biotinylated antibody is bound to avidin-coated quantum dots on the column to prevent the formation of crosslinked aggregates.
5. By varying the amount of PG-zb per quantum dot, the number of antibodies per quantum dot can be tuned. Problems with the amylose purification however, can arise if there are too few of the purification proteins (MBP-zb) per quantum dot.
6. If less antibody is added than there are available PG-zbs for binding antibody, generic IgG (goat IgG) can be added to the reagent before use to prevent free PG-zbs on the quantum dot surface from binding to capture antibody in a sandwich assay.
7. These quantum dots contain cadmium and selenium in an inorganic crystalline form. Dispose of quantum dot waste in compliance with applicable local, state, and federal regulations for disposal of this kind of material.
8. The quantum dots may be stabilized by washing with borate buffer containing 1% bovine serum albumin and reading the plates dry.

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