Capping of CdSe–ZnS quantum dots with DHLA and subsequent conjugation with proteins

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Published online 28 September; corrected online 30 November 2006 (details online); doi:10.1038/nprot.2006.184

We provide a detailed protocol for designing water-soluble CdSe–ZnS quantum dots (QDs) based on cap exchange of the native hydrophobic shell with dihydrolipoic acid (DHLA) ligands, and the preparation of functional QD bioconjugates for use in immunoassays. Our conjugation strategy is based on non-covalent self-assembly between DHLA-capped QDs and protein appended with either an electrostatic attachment domain (namely, the basic leucine zipper) or a polyhistidine tag. These bioconjugates combine the properties of the QD and attached biomolecule to create structures with desirable luminescent and biologically specific properties. This method also allows the preparation of mixed surface conjugates, which results in the conjugates gaining multiple biological activities. Conjugation of DHLA-capped QDs to maltose binding protein (MBP), the immunoglobulin-G-binding β2 domain of streptococcal protein G (PG) and avidin will be described. MBP and PG were modified by genetic fusion with either a charged leucine zipper or a polyhistidine interaction domain.

INTRODUCTION

Luminescent quantum dots (QDs) are versatile inorganic probes that have unique spectroscopic properties, including narrow and size-tunable photoemission profiles coupled with broad absorption spectra. Their broad absorption spectra allow the flexibility to efficiently excite QD samples at wavelengths that are far removed from their emission spectra, which translates into large experimental Stokes shifts. This is particularly beneficial for the implementation of multiplexed assays in which multiple distinct QD samples can be simultaneously excited with a single line that is far from their emission spectra, which simplifies the experimental conditions and signal deconvolution. Considerably larger than molecular dyes, the nanometer size QDs have much larger absorption cross-sections than commonly used organic dyes, and offer accessible surface area for the subsequent attachment of molecules[1–3].

Recently, there has been a growing emphasis on assembling customized composite metal-organic nanostructures that merge disparate yet desirable properties into a stable hybrid nanoparticle. In general, colloidal QDs are not intrinsically compatible with biological environments due to the presence of hydrophobic capping groups on their surfaces. However, this limitation can be overcome by using a variety of post-synthesis strategies that include encapsulating the native hydrophilic QDs with a layer of amphiphilic molecules that interdigitate with the existing hydrophobic groups, or completely replacing the native surface ligands with hydrophilic moieties (referred to as a 'cap exchange')[2–7]. Once stabilized in an aqueous medium, QDs can be further processed to attach biomolecules to their surfaces.

In this protocol, we describe several practical approaches that we have developed for constructing QD–protein bioconjugates based on non-covalent self-assembly and their subsequent use to design and implement immunoassays specific for the detection of targeted molecules[8,9]. Other conjugation approaches using carbodiimide-based covalent reactions or attachment of biotinylated proteins to streptavidin-coated QDs have been reported (see summary in **Table 1**). Each of these approaches has inherent advantages and disadvantages, which will guide the choice of conjugation method to use, depending on the specific application and desired properties of the nanoparticle conjugate. The present conjugation method has the advantage that it does not rely on a particular linking chemistry and the constraints that this may bring. For example, the use of the conventional covalent cross-linking approach—based on 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) condensation applied to QDs capped with COOH-terminated ligands (including dihydrolipoic acid (DHLA))—results in aggregate build-up, which can be attributed to a reduction in QD water affinity once the carboxy groups are reacted[3,6]. It does, however, require the use of proteins that have been engineered to express a leucine zipper domain or a polyhistidine tag. An example application is provided for a multiplexed sandwich immunoassay that is capable of detecting four soluble toxins simultaneously, by which the specificity of toxin detection using surface-bound antibodies and the ability to resolve multiple QD emission signals using a single excitation source have been demonstrated.

EXPERIMENTAL DESIGN

This protocol uses high-quality CdSe nanoparticles with crystalline cores and narrow size distributions. These nanoparticles can be prepared by reacting organometallic precursors at high temperatures in a coordinating solvent mixture, following the approaches described previously[10,11]. Subsequent overcoating of the CdSe cores with a relatively thin layer (3–7 monolayers) of ZnS or CdS produces highly luminescent CdSe–ZnS or CdSe–CdS core-shell QDs[12–14]. It is important to note that the more red-emitting QDs tend to be anisotropic in shape compared with their smaller green and yellow-emitting counterparts. This is due to the uniaxial symmetry of the wurtzite crystal structure of the parent bulk CdSe (maintained within QDs), which increasingly affects the overall shape of the nanoparticles when larger sizes (red-emitting) are grown. Aspect ratios of ~1.2–2.0 have been reported for core-shell QDs that emit at wavelengths beyond 600 nm. For additional details on the choice of precursors, growth/coordinating solvent

TABLE 1 | A list of reported conjugation and functionalization methods for colloidal quantum dots. These methods require a passivating ligand bound to the quantum dot surface.

Covalent coupling	Hydrogen bonding	Hydrophobic bonding	Electrostatic non-covalent self-assembly
Carbodiimides (-COOH)	Avidin–biotin	Phospholipids	Charged proteins
NHS esters (-NH_2)	Oligonucleotides	Block co-polymers	Charged peptides
Maleimides (-SH)			DNA
Isocyanates (-OH)			Polyelectrolytes
Metal affinity (His–M^{2+})			

mixtures and general reaction conditions, we refer the reader to previous publications[10–14]. In this report, we provide a brief description of water-soluble CdSe–ZnS QDs that we have developed and refined, and their further use for applications in functional assays. The procedure described here represents one of several variations that has been developed and that is used by various research groups.

The first scheme that we describe is based on electrostatic self-assembly in which biomolecules having either a natural positive surface charge (such as avidin) or engineered positively charged domains interact with negatively charged QDs capped with a DHLA solubilizing layer[6]. We have engineered maltose binding protein (MBP) and protein G (PG) appended with a positively charged leucine zipper attachment domain to form MBP–zb and PG–zb, respectively[6,8]. Adding these proteins to the QDs in combination enables the formation of mixed-surface bioconjugates in which each protein provides a specific functionality. MBP–zb allows purification of the QD bioconjugates over amylose resin, whereas a second protein (e.g., PG–zb or avidin) serves as a bridging molecule for attaching antibodies to the QDs[8,9]. Nearly all immunoassays developed in our laboratory have used mixed-surface QD bioconjugates using MBP.

A general approach for attaching biomolecules to QDs uses avidin as a bridging molecule. In principle, any biotinylated molecule (e.g., biotin-labeled antibody) can be readily attached to an avidin-coated QD; this obviates the need for an engineered bridging protein such as PG–zb. However, there is a limit to the number of avidin molecules that can be self-assembled onto a QD, due to charge-induced agglomeration. The present protocol provides a general description of how to prepare QD–antibody conjugates via an avidin bridge. **Figure 1** shows a schematic of a typical MBP–zb/avidin/antibody mixed-surface conjugate having only a few proteins attached to the surface (usual bioconjugates will have a surface that is saturated with proteins). Alternatively, PG–zb specifically interacts with the Fc domain of immunoglobulin G (IgG) and provides a structured orientation of antibodies on the QD with the binding sites directed outwards into the surrounding solution and away from the nanocrystal surface. This arrangement can potentially decrease heterogeneity and improve conjugate avidity. In addition, it does not require biotinylation of antibodies[8].

The conjugation scheme using electrostatic self-assembly was further expanded to include metal-affinity interactions between polyhistidine-appended protein and DHLA-capped CdSe–ZnS QDs to form functional bioconjugates[15–18]. Many proteins are engineered to express an amino- or carboxy-terminal sequence of repeating His residues (typically five or more) for purification on a Ni–nitrilotriacetic acid (Ni–NTA) column. This approach may prove to be a facile and general route for the creation of QD–protein bioconjugates, because the metal-affinity-driven self-assembly significantly simplifies the bioconjugation procedure and reduces the overall hydrodynamic size of the resulting nanoparticle assembly by eliminating the need for a bridging protein. Self-assembly schemes using His-terminated proteins may be preferable to methods that use an intermediate bridging protein (e.g., avidin or PG–zb), particularly for applications that require more compact bioconjugates. These considerations may be particularly critical for intracellular delivery and imaging or fluorescence resonance energy transfer (FRET) applications[2–5,15,16]. FRET involves the non-radiative transfer of excitation energy from an excited donor (e.g., QD) to a ground-state proximal acceptor (e.g., dye) via dipole–dipole interactions. Its efficiency, E, varies with the donor–acceptor separation distance to the sixth power[19]. Compact assemblies made of QD–bioreceptor-dye conjugates are therefore crucial for designing FRET-based assays[15,16].

Currently, most commercially available water-soluble QDs are large in size (due to long-chain amphiphilic polymers used to make the nanocrystals hydrophilic) and cannot allow easy implementation of FRET-based assays and sensor design; useful FRET efficiencies require the use of high acceptor-to-QD ratios to compensate for the large separation distances[3]. DHLA-capped QDs directly coupled with His-tagged proteins can significantly reduce the overall size of bioconjugates, especially when compared with the streptavidin–biotin functionalities that are offered in many commercial conjugation kits. Bioconjugate size can be further reduced by using only relevant fragments of antibodies or short peptides appended with His-tags that adequately mimic full protein behavior[17,18].

When preparing QD bioconjugates, it is important to consider the protein-to-QD ratio. This can be determined from the molar ratio of the solution precursors containing QDs and proteins that

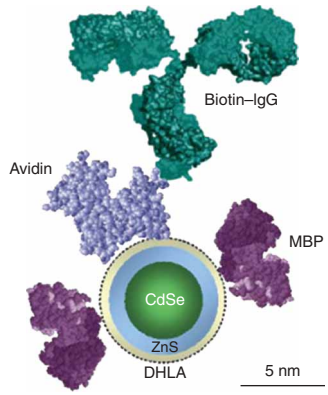

Figure 1 | Schematic representation of a typical QD bioconjugate. In this example, a CdSe–ZnS core-shell quantum dot (QD) is capped with a layer of dihydrolipoic acid (DHLA) and conjugated to maltose binding protein (MBP)–zb and avidin via electrostatic self-assembly. The immunoglobulin G (IgG) is biotinylated and bound to one of four binding sites on the avidin molecule. Typical QD bioconjugates have multiple copies of these surface-bound proteins surrounding the central QD. (Figure is approximately to scale.)

are used in the mixture to prepare self-assembled conjugates. For mixed-surface QD conjugates (with protein A and protein B), the desired protein-to-QD ratios are realized by mixing the QD solution with aliquots containing protein A and protein B at the desired molar concentrations. In the following protocol that we developed in our laboratory, conjugates usually have total protein-to-QD ratios of 10–20 (near saturation).

For conditions in which the total number of proteins initially mixed in solution is lower than the number of available binding sites on all QDs (i.e., binding is not sterically limited), the actual protein-to-QD ratio of individual bioconjugates will be a distribution around the molar average ratio of proteins to QD in the mixture. If the binding is sufficiently random (i.e., well-mixed), the number of proteins per QD will naturally follow a Poisson distribution. For example, if proteins and QDs are mixed in a 1:1 molar ratio, there will be an equal number of conjugates having one protein per QD as there are QDs with no protein at all. The rest of the conjugates would have two or more proteins per QD. These considerations become more important for applications that use FRET as a signal-transduction mechanism for quantifying either the distance between fluorophores or the number of proximally located fluorophores. However, this effect can be largely mitigated by using a larger average ratio of bound protein or peptide per QD (above 4:1).

Tests for proper conjugation should, particularly, be run for any new bioconjugate system (e.g., a new QD sample or new proteins) and periodically checked once in common use. The binding of a biomolecule to a QD sample can be tested in a number of ways. One of the most practical methods is to measure the photoluminescence (PL) signal before and after conjugation. Due to a better passivation of surface charges with attached proteins when self-assembly is used, the bound bioconjugate sample should have a larger PL signal than the unmodified water-soluble QDs. The amount of this change will vary from sample to sample (~20–200%). Enhancement in the QD bioconjugate PL (i.e., quantum yield) following self-assembly is reflective of binding interactions. We have consistently observed PL enhancement with our self-assembled QD–protein conjugates, regardless of the protein and the particular DHLA-capped QD sample used. Using DHLA-capped QDs, the final quantum yield of a bioconjugate will most often be 15–40% depending on the particular sample, biomolecules used and extent of surface coverage.

For QDs that have bound MBP (either via the leucine zipper or His-tag), it is relatively simple to verify binding using an amylose column. MBP–QDs that bind the column strongly and then release following addition of maltose are considered to be ideal. Contrary behavior at either stage (i.e., no initial binding, or lack of release after adding maltose) indicates poor conjugate formation.

MATERIALS

REAGENTS

- Selenium (99.99%)
- Cadmium acetylacetonate ($Cd(acac)_2$)
- 1,2-Hexadecanediol (HDDO)
- Trioctyl phosphine (TOP; 90–95%)
- Trioctyl phosphine oxide (TOPO)
- Hexadecylamine (HDA; 90%)
- Inert gas (nitrogen or argon)
- Solvents (hexane, toluene, butanol, ethanol, methanol, dimethylformamide)
- Diethylzinc ($ZnEt_2$)
- Hexamethydisilathiane (TMS_2S)
- Thioctic acid (DHLA precursor)
- Sodium borohydride
- DHLA
- Deionized water
- Potassium tert-butoxide (K[t-BuO])
- Millipore hydrophilic and organic filters (single use)
- Scintillation vials for purification of organic and aqueous QD solutions
- Ultra-free centrifugal filters (Millipore)
- Sodium tetraborate buffer (10 mM, pH 9.5, Sigma)
- Amylose affinity resin (New England Biolabs)
- Maltose (Sigma)
- Small columns (e.g., Bio-Spin columns or Micro-Bio-Spin columns; Bio-Rad)
- Eppendorf tubes, 1.5 ml volume (Eppendorf International)
- Phosphate-buffered saline (PBS, pH 7.4; Sigma)
- MBP engineered with a terminal basic leucine zipper attachment domain (MBP–zb)
- MBP engineered with a terminal ploy histidine domain (MBP–His)
- Protein G engineered with a terminal basic leucine zipper attachment (PG–zb)
- Avidin (Sigma), appropriate antibodies (e.g., IgG) and antigens
- Single-chain antibody fragment engineered with a terminal polyhistidine domain (ScFv–His)

EQUIPMENT

- Dual inert/vacuum line
- Inert atmosphere glove box for handling air-sensitive materials
- Rotovap for DHLA reduction and purification
- Centrifuge for solution purification
- UV-vis absorption spectrophotometer
- Fluorescence spectrophotometer
- 96-well white microtiter plates (FluoroNunc Plates MaxiSorp surface; Nalge Nunc International)
- Fluorescence microtiter plate reader
- Hand-held UV lamp (preferably UVA wavelength range)

REAGENT SETUP

MBP and protein G basic zipper fusion proteins Construction and purification of the MBP and protein G appended with a basic leucine zipper domain (MBP–zb and PG–zb) have been detailed previously[6,8,20]. Briefly, PCR was used to introduce a unique Cys upstream of the sequence and to amplify the DNA fragment coding for the basic zipper[21]. The amplified DNA segment was then ligated into the *XmnI*/*XbaI* sites within the polylinker that exists downstream of the *MalE* gene in the commercially available pMal-c2 vector (New England Biolabs). Similarly, the PG–zb fusion protein was constructed by cloning the coding sequence for the β2 IgG binding domain of streptococcal protein G, and the tail including the poly-Asn linker, dimer-promoting cysteine, basic leucine zipper and C-terminal hexa–His tag into the expression vector pBad/HisB (Invitrogen).

For expression, both constructs were transformed into *Escherichia coli* Top 10 (Invitrogen), grown at 37 °C to an optical density at 600 nm of ~0.5 and then induced with a final concentration of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Promega) or 0.002% (w/v) L-(+)arabinose, for MBP–zb and PG–zb, respectively, and grown for an additional 2 h at 37 °C. A denaturing protein purification was necessary to avoid contamination with nucleic acids and proteases. Cells were suspended and stirred for 1 h in buffer containing 6 M guanidine HCl, spun to pellet debris and the supernatant loaded onto a Ni–NTA agarose (Qiagen) column. The loaded sample was then washed extensively with the 6 M guanidine HCl-containing buffer, buffer containing 8 M urea, and finally with PBS to facilitate re-folding of the proteins before elution with 250 mM imidazole. Proteins were dialyzed extensively against PBS, concentrated to ~1–2 mg ml^{-1} and stored over the short term at 4 °C or flash-frozen for storage at –80 °C.

His-tagged MBP and single-chain antibody fragment Construction and purification of the His-tagged MBP and single-chain antibody fragment have been detailed previously[15,17,22]. MBP–His was expressed in the Top 10 strain of *E. coli*, whereas the scFv–His was expressed in the Tuner strain of *E. coli*

(Novagen). Both proteins were extracted from the periplasmic space of *E. coli*, using an osmotic-shock-based protocol followed by purification on a Ni–NTA column. Briefly, the induced cell pellet was resuspended in sucrose solution, followed by lysozyme digestion. EDTA was then added into the mixture, followed by the addition of $MgCl_2$ to saturate EDTA. The spheroplasts were separated from the osmotic shockate by centrifugation and the shockate was purified on Ni–NTA resin. His-tagged protein was eluted by the addition of PBS containing 250 mM imidazole. Purified protein was dialyzed extensively against PBS, concentrated to 1–2 mg ml^{-1} and stored at 4 °C.

Quantum dots Luminescent CdSe–ZnS core-shell QDs capped with hydrophobic ligands (e.g., TOP/TOPO) are prepared step-wise using reaction of organometallic precursors (for cadmium, zinc and sulfur) at high temperature and in coordinating solvent mixtures, followed by size selection to purify the materials and to select samples with a narrow size distribution. It is commonly accepted that QDs that have a relatively thick overcoating layer of ZnS (exceeding three monolayers) should be used, as they provide aqueous dispersions that are more stable and highly luminescent. Removal of the organic solvents (e.g., hexane or toluene) during transfer to buffers is crucial for optimized performance in biological assays. Commercially available QDs may be used; however, this procedure is optimized for laboratory-synthesized materials using the procedure outlined below[6].

PROCEDURE

Reagent preparation and purification: synthesis and purification of CdSe–ZnS core-shell QDs

1| Prepare a 1 M stock solution of trioctylphosphine selenide (TOP:Se) by dissolving 7.9 g of Se (99.99%) into 100 ml of TOP (90–95%). A 2 M solution of TOP:Se could be used, but this higher concentration must be taken into account when adding Se precursors to the reaction mixture.

2| Fill a 100 ml three-neck round-bottomed flask, fitted with a thermocouple temperature sensor, condenser and a nitrogen/vacuum inlet adapter, with TOPO (20 g), HDA (10 g) and TOP (5 ml) and heat to 120–140 °C under vacuum for 1–2 h.

3| Switch to nitrogen atmosphere and raise the temperature to 340–350 °C.

4| In a separate vial, mix $Cd(acac)_2$ (620 mg), HDDO (1.2 g) and TOP (10 mL) and heat under vacuum to 100 °C; the solution should become homogeneous. This protocol is optimized for the above conditions. However, other comparable precursors, including CdO and cadmium acetate, can be used as Cd precursors.

5| Let the mixture cool to approximately 80 °C and add 10 ml of a 1 M TOP:Se (or 5 ml of a 2 M TOP:Se) and mix thoroughly.

6| Rapidly inject the solution mixture of cadmium and selenium precursors into the hot flask containing the coordinating solvent, and then quickly cool to a temperature of ~100–200 °C (to prevent further growth of the nanoparticles).

7| The resulting nanoparticles can either be collected for subsequent processing and use or further grown to larger sizes with additional heating to 250–280 °C for several minutes.

■ **PAUSE POINT** Nanoparticles collected immediately following injection and without additional growth are usually small in size and have a first absorption peak at around 470–490 nm. Submitting the solution to additional heating allows further growth of the nanocrystals (to a larger size) and shifts the location of the first absorption peak and emission maximum to the green, yellow, orange and red regions of the optical spectrum. Collecting absorption spectra from aliquots retrieved from the solution provides information on when to stop the growth once the desired size (emission color) is reached.

8| The solution is then cooled to 60–80 °C, mixed with toluene (or hexane) and butanol, and centrifuged to remove any unreacted metal salts and other impurities.

9| Purification is carried out using a solution mixture with excess methanol or ethanol, followed by centrifugation and removal of the supernatant. Steps 10–18 below describe ZnS overcoating of the resulting core CdSe QDs after purification of the above materials.

10| Mount a round-bottomed flask (100 ml or larger) with a pressure-equalizing addition funnel.

11| Load 20–30 g of TOPO into the round-bottomed flask and dry/de-gas at 120–140 °C for 2–3 h under vacuum. This permits removal of adsorbed water and reduces the presence of oxygen, as performed above in Step 2.

12| Add purified CdSe QD solution (dispersed in hexane or toluene) at 70–80 °C to a final Cd concentration of ~0.1–1 mM, and remove the solvent by evaporation under vacuum. Complete removal of the solvent is desirable for effective growth of the overcoating ZnS layer.

13| Increase the temperature of the QD/TOPO solution to between 140 °C and 180 °C, depending on the initial core radius.

▲ **CRITICAL STEP** The range of temperatures used for ZnS overcoating should be lower than those used for growing the core (see Step 7 above). The above temperature window applies, for overcoating, the full range of QD sizes throughout the spectrum. Lower temperatures are used for smaller core sizes, e.g., 140 °C is appropriate for overcoating a CdSe QD sample that has a first absorption peak at ~490–500 nm. If the temperature used is high, broadening in the size distribution quickly takes place, reducing the sample quality.

14| Separately, to a vial containing 4–5 ml of TOP, add equimolar amounts of diethylzinc ($ZnEt_2$) and hexamethyldisilathiane (TMS_2S) precursor that correspond to the desired overcoating layer for the appropriate CdSe nanocrystal radius. Use inert atmosphere (e.g., glove box) to carry out this step, as the $ZnEt_2$ is pyrophoric.

15| Load the Zn and S precursor solution from Step 14 into a syringe (inside the glove box) and transfer the contents to the addition funnel.

16| Slowly add the Zn and S precursor solution to the QD/TOPO solution at a rate of about 0.3–0.5 ml min^{-1}.

17| Once the addition is complete, lower the solution temperature to 80 °C and leave the mixture stirring for several hours.
■ **PAUSE POINT** This extra annealing step can result in a substantial improvement in the quality (in terms of stability and photoluminescence quantum yield) of the core-shell QDs.

18| Add about 10 ml of butanol and hexanes and precipitate the ZnS-overcoated QDs with methanol to recover the QD product by centrifugation.

Reagent preparation and purification: water solubilization of QDs

19| Preparation of DHLA. Prepare an aqueous solution of 0.25 M sodium bicarbonate.

20| Add 6 g of thiotic acid to 117 ml of the sodium bicarbonate solution (0.25 M) mounted in a cold bath (~0–5 °C). The steps described here are for the preparation of 5–6 g of reduced thiotic acid (DHLA)[6,23].

21| Slowly add a total of 1.2 g of sodium borohydride ($NaBH_4$) (in aliquots of 10–20 mg).

22| Let the mixture stir for ~30 min. The resulting solution should be clear.
■ **PAUSE POINT** Letting the reaction mixture stir for a longer time (1–2 h) can further improve the quality of the final product.

23| Add 100 ml of toluene; a two-phase solution will result.

24| Acidify the mixture to ~ pH 1.

25| The reduced thiotic acid will transfer fully into the organic phase, resulting in a whitish milky appearance.

26| Separate the two phases using a separatory funnel and collect the organic phase containing the product (reduced thiotic acid).

27| Add magnesium sulfate drying agent to remove excess water. The solution should become clear.

28| Filter the solution.

29| Remove the solvent under vacuum to produce pure DHLA.

30| An additional distillation step may be used to further purify the prepared DHLA. This could be carried out under modest vacuum (~0.3–0.5 atm) and at 140–150 °C.
▲ **CRITICAL STEP** Pure DHLA is a transparent, colorless liquid that is made when thioctic acid precursor (yellow powder) is fully reduced[6]. High purity (nearly colorless liquid) should be used for cap-exchange and transfer into buffer solutions.

Transfer of TOP/TOPO-capped CdSe–ZnS QDs to water

31| Disperse 100–500 mg of purified native hydrophobic QDs (e.g., TOP/TOPO-capped) in 0.3–1.0 ml of freshly prepared DHLA.

32| Heat the mixture to 60–80 °C for ~30–120 min while stirring. This accelerates cap exchange and homogenization of the dispersion.
▲ **CRITICAL STEP** Longer incubation (for even several hours) of TOP/TOPO-capped QDs with DHLA can be beneficial as this may improve solubility and the efficiency of the cap-exchange process.

33| Dilute the QD solution in 3–5 ml dimethylformamide (DMF) or methanol.

34| Slowly add excess potassium-tert-butoxide (K[t-BuO]). This allows deprotonation of the terminal carboxyl groups on the dihydrolipoic acid. A precipitate is formed consisting of DHLA-capped nanoparticles and free TOP/TOPO ligands.

35| Sediment the precipitate by centrifugation and discard the supernatant.

36| Add deionized water to the precipitate. The new surface-functionalized (or capped) QDs with DHLA should disperse readily in water, and the pH of the resulting solution should be high (~12–13).
? TROUBLESHOOTING

37| An additional purification step is required to remove excess K[t-BuO] and residual DMF, as well as solubilized TOP/TOPO. For this, use an ultra-free centrifugal filter (M_W cut off ~50–100 kDa). This should provide an aqueous solution of DHLA-capped QDs.

38| Repeat the centrifugation cycle using the centrifugal filtration device three to four times and resuspend the QD solution in deionized water.

▲ **CRITICAL STEP** Dispersions of water-soluble QDs typically have stock concentrations ranging from 5 to 50 μM using this approach. The final concentration is usually determined by a mass balance calculation (aided by UV-vis absorption) to estimate the number of QDs (of a given size) generated following reaction of the precursors[6]. Skipping the purification step using the centrifugation device could provide well-dispersed QDs in solution. However, this will also leave remnant free/solubilized TOP/TOPO, DHLA, K[t-BuO] and DMF present in the solution, which could significantly interfere with the subsequent conjugation steps.

39| An additional optional filtration of the dispersion (using a 0.45 μm disposable filter) allows removal of residual solubilized TOP/TOPO and provides a clear dispersion of the alkyl-COOH capped nanocrystals.

■ **PAUSE POINT** The resulting dispersions of DHLA-capped QDs are stable for several months (6–24 months) in basic buffer solutions (pH >7).

? TROUBLESHOOTING

40| Conjugate the DHLA-capped QDs with mixtures of MBP–zb, PG–zb and antibody (A); MBP–zb, avidin and antibody (B); or His-tagged proteins (C). Option C describes a procedure for preparing QDs that are conjugated to dye-labeled proteins for use in assays and sensor design, based on FRET. The self-assembled QD–protein-dye conjugates use a fixed total number of proteins per conjugates, but the fraction of dye-labeled to unlabeled protein is varied. This obviates the need to account for changes in the photoluminescence quantum yield of the conjugates compared with the starting DHLA-capped QDs alone[6,15,16]. This protocol allows the preparation of QD conjugates with a total of 15 proteins per QD; 5 out of these are dye-labeled[15,16].

(A) MBP–zb/PG–zb/antibody mixed-surface conjugate formation

(i) Add MBP–zb (0.25 nmol), PG–zb (0.22 nmol) and QDs (0.10 nmol) to 200 μL borate buffer (10 mM sodium borate, pH 9.5), in order.

(ii) Gently mix and incubate at room temperature for about 15 min.

(iii) Add a second aliquot of MBP–zb (0.33 nmol) to the QD–protein preparation.

(iv) Mix gently and incubate for an additional 5 min at room temperature.

(v) Add about 35 μg IgG to the QD–protein conjugate solution.

(vi) Incubate the mixture at 4 °C for 1 h. If fewer antibodies are added per available PG–zb, generic IgG (i.e., goat IgG) can be added to block free PG–zb on the QD surface.

▲ **CRITICAL STEP** Conjugation of DHLA-capped QDs to protein using this process is usually accompanied by an increase in the sample photoluminescence quantum yield. This can be used as an initial indicator of conjugate formation before proceeding to the purification step on an amylose-filled column. If the protein samples have lost their biological functionality (e.g., due to aging) or if the QD solution is no longer at basic pH, conjugate formation may not proceed or is poorly formed, with the resulting conjugates losing their biological activity (i.e., binding to amylose and release by maltose).

(vii) In parallel, prepare an amylose column by pouring 0.5 ml of suspended amylose resin per column. Wash the column with at least 1 ml buffer (borate buffer or PBS).

(viii) Load the solution of QD/MBP–zb/PG–zb/IgG conjugate (QDs conjugated to a mixture of MBP–zb plus PG–zb followed by interaction with the antibody) at the top of the column with a pipette.

(ix) Once the conjugate solution has entirely penetrated the column resin at the top, immediately wash the column by adding 2 ml buffer. This step separates the QD conjugate from free antibody.

? TROUBLESHOOTING

(x) Elute the QD/MBP–zb/PG–zb/IgG conjugate solution with 1 ml of 10 mM maltose in PBS.

▲ **CRITICAL STEP** A hand-held UV lamp can be used to monitor the column purification process and to confirm whether the QDs are bound to the column and are not being eluted with the initial wash.

■ **PAUSE POINT** The prepared reagents should be stored at low temperature (4 °C). They can be used as long as the bioreceptors are still active. Use within 1–2 weeks of preparation. Freshly made reagents are preferable, however, to guarantee reliable assays results.

(B) Preparation of MBP–zb/avidin/antibody mixed-surface conjugates

(i) Add MBP–zb (0.43 nmol), avidin (15 μg, about 0.22 nmol) and QDs (0.10 nmol) to 200 μl borate buffer, in order.

(ii) Mix gently and incubate at room temperature for about 15 min.

(iii) Add a second aliquot of MBP–zb (0.22 nmol) to the QD–protein mixture, mix gently and incubate for another 5 min at room temperature.

(iv) Prepare an amylose column by pouring 0.5 ml of suspended amylose resin per column. Wash the column with at least 1 ml buffer (borate buffer or PBS).
(v) Add the MBP–zb/avidin-coated QDs to the top of the column, and wash with 1 ml buffer (borate or PBS).
(vi) Add biotinylated antibody (20 μg) to the top of the column.
(vii) Allow the solution to penetrate into the resin and cap column to stop the flow.
▲ CRITICAL STEP Attachment of biotinylated antibody to QDs conjugated to avidin is carried out on the column to prevent the formation of cross-linked aggregates, which could occur if conjugation occurs in solution.
(viii) Add 50 μL buffer to the top of the resin and allow the biotinylated antibody to react with the QD–avidin on the column for about 1 h.
(ix) Remove the cap and let the PBS run into the column. Wash the resin with 1 ml PBS.
(x) Elute the MBP–zb/avidin/IgG-conjugated QDs with 1 ml 10 mM maltose in PBS. The elution can be monitored with a hand-held UV lamp.
▲ CRITICAL STEP Deprotonated DHLA on the QD surface (negatively charged) provides water solubility and a favorable surface for positively charged biological molecules. In forming these mixed-surface QDs, it is important to optimize the ratio of each protein to QD. We have found that a ratio exceeding two avidin molecules per QD leads to visible aggregation. Too few MBP–zb per QD can cause problems with the column purification. Some experimentation may be required to find the optimal ratios for a particular combination of proteins.
■ PAUSE POINT As above, the prepared reagents should be stored at low temperature (4 °C). They can be used as long as the bioreceptors are still active. Use within 1–2 weeks of preparation. Freshly made reagents are preferable, however, to guarantee reliable assay results.
? TROUBLESHOOTING

(C) Mixed-surface self-assembly of DHLA-capped QDs with His-terminated proteins

(i) Add the labeled and unlabeled proteins (0.30 and 0.60 nmol, respectively) to 100 μL borate buffer. Gently mix the solution to distribute the proteins uniformly throughout the buffer.
(ii) Add QDs (0.06 nmol) and mix the sample thoroughly (~10 s).
(iii) Incubate the sample for 15 min at room temperature and add buffer to reach the desired concentration of QDs.
(iv) Once prepared, use the conjugate sample within a few hours.

Fluorescence-based immunoassay studies

41| Conjugated QDs can be used in fluorescence-based immunoassay studies (A) or competition immunoassays (B).

(A) Single and multiple analyte sandwich immunoassays

(i) Coat the multi-well plate with 2.5–10 μg ml^{-1} each of appropriate capture antibodies diluted into 0.1 M sodium bicarbonate at pH 8.6 using 100 μl capture antibody mix per well.
▲ CRITICAL STEP It is important to include control wells with PBS only and no antigen. Experimental and control wells should be plated, at a minimum, in triplicate.
(ii) Let the plate incubate overnight at 4 °C.
(iii) Remove excess free capture antibody and block plates with 4% (w/v) powdered non-fat milk in PBS for 1–2 h at room temperature.
(iv) After blocking, wash the plate twice with PBS plus 0.1% Tween-20 (PBST).
(v) Add 100 μl antigen solution(s) (diluted in PBS) to the wells. Antigen solution can consist of single antigen solutions or in mixes.
(vi) Rock the plate gently at room temperature for 1 h.
(vii) Wash the plate twice with PBST.
(viii) Add 100 μl antibody-conjugated QD reagents (in PBS). Usually, the QDs that are eluted in Step 40 (A), (B) or (C) are diluted to 3–5 times the elution volume.
▲ CRITICAL STEP Although the conjugation strategy based on metal–His interaction has proven successful with a variety of His-appended proteins[15–18], it is important that the His-tag be in an extended conformation away from the protein structure to allow formation of stable conjugates.
(ix) Rock the plate gently at room temperature for 1 h.
(x) Wash the plate 2–4 times with borate buffer (pH 9.5) containing 1% bovine serum albumin. Remove all liquid from the wells.
? TROUBLESHOOTING
(xi) Measure the fluorescence in a fluorescent plate reader.
▲ CRITICAL STEP We used a Tecan Safire plate reader for data collection (Tecan US) and an excitation of 300–400 nm. However, the broad absorption spectra of the QDs allow a broad choice of excitation lines. Choice of emission setting/filter depends on the emission spectra of the QDs used in the assay.

(B) Competition immunoassay

(i) Coat wells of opaque white microtiter plates overnight (at 4 °C) with the target analog (usually conjugated to a protein) at 10 μg ml^{-1} dissolved in 0.1 M $NaHCO_3$ (pH 8.6).
(ii) Discard the coating solution and block the wells with 4% w/v non-fat powdered milk in PBS for 1–2 h at room temperature.
(iii) Add 50 μl aliquots of analyte dilutions in PBS buffer (and analyte-free controls) to appropriate test and control wells.
(iv) Immediately add 50 μl of QD–antibody conjugate to each well. Allow the contents of the wells to come to equilibrium by incubation at room temperature with gentle shaking for about 1 h.
(v) Wash the wells with borate buffer.
(vi) Measure the fluorescence with a fluorescent plate reader.
? TROUBLESHOOTING

? TROUBLESHOOTING

Step 36

If the precipitated QDs, following reaction with K[t-BuO], do not readily disperse in water, this may be due to incomplete deprotonation of the carboxylic-acid end groups. Addition of a small amount of K[t-BuO] should produce a clear solution in water.

Step 39

Some minor aggregation may form in buffer solutions over time. Additionally, the pH of the solution may decrease as the sample ages. Adding a small amount of K[t-BuO] can redisperse the QDs and result in a clear solution. Water-soluble QD solutions having concentrations below 1 μM (10–100 nM) can be affected by irreversible precipitation after several weeks of storage and should therefore be avoided for long-term storage.

Steps 40A(ix) and B(x)

Although the number of antibodies per QD can be tuned by varying the amount of PG–zb (or avidin) per QD, problems may arise with the amylose purification if there are too few MBP–zb per QD or if the buffer used turns slightly acidic. A low MBP–zb valency on the QD surface can affect purification of the conjugate reagent over an amylose column. To remediate this problem, slightly increase the ratio of MBP–zb-to-QD in the conjugate.

Steps 41 A(x) and B(vi)

If the control wells have a high non-specific signal from the QDs, wells may be washed several more times with the borate buffer to further reduce non-specific signal.

ANTICIPATED RESULTS

QD bioconjugates can be used for a variety of molecular sensing applications in aqueous solution[8,9,15–18]. Specific biomolecules on the surface of the QD determine the affinity for target molecules. Initial versions of fluorescence-based immunoassays targeted only one type of molecule in solution[9]. However, the narrow emission bandwidth of QDs can be used to spectrally encode these specific interactions by emission color and enable simultaneous signal multiplexing. In an expanded assay, we used these QD–antibody conjugates to detect up to four toxins simultaneously in the same solution[24]. Four populations of mixed-surface QD bioconjugates having MBP–zb and PG–zb–IgG proteins were prepared as previously described to target binding with four different toxins: cholera toxin (CT; Calbiochem), ricin (Sigma), shiga-like toxin (SLT; Toxin Technology, Inc.) and staphylococcal enterotoxin B (SEB; Toxin Technology, Inc.). **Figure 2a** shows schematic depictions of the four QD bioconjugates that are specific for each toxin. Sample wells with a high capacity for protein binding are incubated overnight with antibodies that are specific for each of the four targets. A solution containing the various toxins is added to the wells and allowed to incubate for 1 h, after which the wells are rinsed with buffer. The wells are then incubated with the QD bioconjugate tags and rinsed again. A monochromatic source ranging from 300 to 400 nm is used to simultaneously excite all four QD populations.

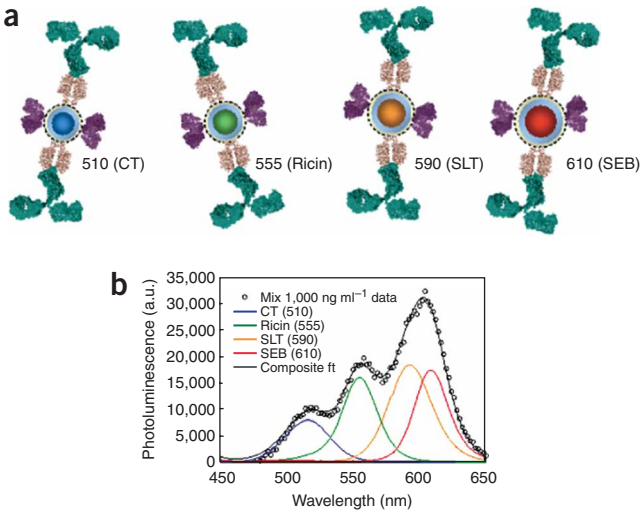

Figure 2 | Multiplexed toxin analysis using QD–antibody conjugates. (**a**) Schematic of the four quantum dot (QD) bioconjugates used in the multiplexed sandwich assay targeting cholera toxin (CT), ricin, shiga-like toxin (SLT) and staphylococcal enterotoxin B (SEB). Bioconjugates contain MBP–zb, PG–zb adapters and immunoglobulin G antibodies specific for one of the four toxins. Populations with emission maxima of 510, 555, 590 and 610 nm were used in the assay. (**b**) Multiplexed assay results for 1 μg ml^{-1} mixtures of the four toxins. Circles show the measured spectrum for the mixed sample. The composite fit is shown as a black line with individual QD contributions to the fit as colored lines. Adapted partially from ref. 24 with permission from the American Chemical Society.

Detection of fluorescence emission at a certain color (i.e., wavelength range) following signal deconvolution indicates the presence of a sandwich-binding interaction and is a positive indicator for that particular toxin in solution. **Figure 2b** shows data from a multiplexed sandwich immunoassay for CT, SEB, SLT and ricin. Goat anti-cholera toxin (Biogenesis), monoclonal anti-SEB 6B ascites (BioVeris) further purified by MEP Hypercel affinity chromotography (Ciphergen), a pool of monoclonal anti-SLT antibodies (9C9, 3C10, BB12; Toxin Technology, Inc.) and monoclonal anti-ricin RIC-03-A-G1 (a gift from the Naval Medical Research Center) were used as the capture antibodies adsorbed onto the wells of plates. Rabbit anti-CT antibody (Biogenesis) was coupled to 510 nm emitting QDs, polyclonal rabbit anti-ricin (a gift from the Naval Medical Research Center) was conjugated to 550 nm emitting QDs, the pool of monoclonal anti-SLT antibodies (9C9, 3C10, BB12; Toxin Technology, Inc.) was coupled to 590 nm emitting QDs, and polyclonal rabbit anti-SEB antibody (Toxin Technology, Inc.) was coupled to 610 nm emitting QDs. The PG–zb conjugation strategy detailed in Step 41 (A) and (B) was used to prepare the various QD–antibody conjugates used in these experiments.

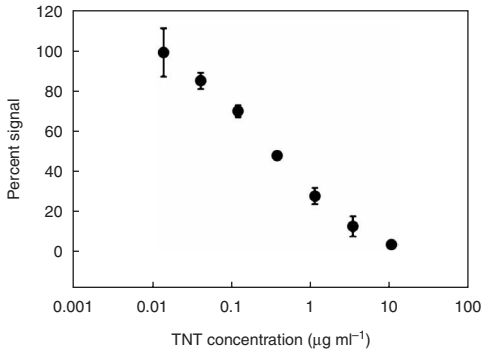

Figure 3 | Results from a competition assay using QD–anti-TNT scFv conjugate to detect soluble TNT. Alone, quantum dot (QD) bioconjugates with attached anti-TNT (4,6-trinitrotoluene) scFv bind to surface-immobilized trinitro-benzenesulfonic acid (TNB). As the concentration of free TNT added to the solution increases, fewer QD bioconjugates bind to the surface-immobilized TNB (due to competition with free TNT) and the measured fluorescence signal decreases. Adapted partially from ref. 17 with permission from Elsevier.

A competition assay specific for soluble 2,4,6-trinitrotoluene (TNT) is constructed using biomolecules that have been engineered to express terminal polyhistidine that binds to QDs. Competition assays are frequently used in the detection of small molecules. **Figure 3** shows data from a competition immunoassay for TNT using QDs coated with both scFv and MBP–zb[17]. Ovalbumin derivatized by modification with trinitro-benzenesulfonic acid (TNB–ovalbumin) was used to coat wells of a fluorescent microtiter plate. Varying amounts of soluble TNT were added to the wells, followed by the addition of anti-TNT scFv/MBP–zb QD bioconjugates. The wells were incubated for 1 h to achieve equilibrium and washed prior to fluorescence measurement in the plate reader.

ACKNOWLEDGMENTS This work was supported by grants from the Office of Naval Research (ONR) and DARPA. A.R.C. was supported by a National Research Council fellowship.

COMPETING INTERESTS STATEMENT The authors declare that they have no competing financial interests.

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Erratum: Capping of CdSe–ZnS quantum dots with DHLA and subsequent conjugation with proteins

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Nat. Protocols doi:10.1038/nprot.2006.184; published online 28 September; corrected online 30 November 2006.

In the version of this article initially published online, the article's page numbers should have been 1258–1266. This error has been corrected in the PDF version of the article.