Multifunctional ligands based on dihydrolipoic acid and polyethylene glycol to promote biocompatibility of quantum dots

Kimihiro Susumu[1], Bing C Mei[1,2] & Hedi Mattoussi[1]

[1]Division of Optical Sciences, US Naval Research Laboratory, Washington, District of Columbia 20375, USA. [2]Department of Chemical Engineering, University of Massachusetts, Amherst, Massachusetts 01003, USA. Correspondence should be addressed to H.M. (hedi.mattoussi@nrl.navy.mil).

Published online 5 March 2009; doi:10.1038/nprot.2008.247

One of the common strategies to promote the transfer of quantum dots (QDs) to buffer media and to couple them to biological molecules has relied on cap exchange. We have shown previously that dihydrolipoic acid (DHLA) and polyethylene glycol (PEG)-appended DHLA can effectively replace the native ligands on CdSe-ZnS QDs. Here we explain in detail the synthesis of a series of modular ligands made of the DHLA-PEG motif appended with terminal functional groups. This design allows easy coupling of biomolecules and dyes to the QDs. The ligands are modular and each is comprised of three units: a potential biological functional group (biotin, carboxylic acid and amine) and a DHLA appended at the ends of a short PEG chain, where PEG promotes water solubility and DHLA provides anchoring onto the QD. The resulting QDs are stable over a broad pH range and accessible to simple bioconjugation techniques, such as avidin–biotin binding.

INTRODUCTION

Luminescent QDs (such as those made of CdSe-ZnS core-shell nanocrystals) have generated an increasing interest for use in developing biological applications. QDs possess remarkable photo and chemical stability and exhibit very high extinction coefficient combined with unique spectroscopic properties that include size- and composition-dependent broad absorption and readily tunable narrow photoemission[1–3]. These features make these nanoparticles very promising tools for use in biology. However, to use QDs in a variety of biological application, it is crucial that stable water-soluble QDs be reproducibly prepared through simple techniques, have compatibility with simple conjugation procedures[4] and that the nanocrystals are highly luminescent. These nanocrystals should also have low size distribution to reduce the spectral width of their emission[1]. High-quality semiconductor nanocrystals are commonly synthesized at high temperatures from organometallic precursors and in coordinating solvents[5,6]. As prepared, these QDs are routinely capped with mixtures of trioctylphosphine/trioctylphosphine oxide (TOP/TOPO) and alkyl amines (hydrophobic ligands), and therefore are not dispersible in aqueous media without further modification. Several approaches for achieving water-solubility of such materials have been reported[3]. These methods include silica coating[7,8], encapsulation of the hydrophobic (TOP/TOPO-capped) QDs within amphiphilic polymer shells[9,10] or lipid micelles[11] and exchanging the native caps with hydrophilic ligands[12,13]. Most commercially available hydrophilic QDs use encapsulation methods within polymeric shells or micelles. These nanocrystals have been used in a variety of applications ranging from live (and fixed) cell staining to animal imaging[2,3]. However, these nanoparticles tend to be rather large in size (diameter >15 nm), due to the large molecular weight of the amphiphilic polymers used to make the nanocrystals hydrophilic, which is a drawback in assays and applications requiring stringent constraints on overall nanoparticle dimensions[14]. In comparison, cap exchange with bifunctional ligands is relatively simple and has a strong potential for providing QDs (and other metallic nanoparticles) that are small in size. Preparing small hydrophilic QDs is beneficial to a variety of targeting studies, including use in cellular uptake and imaging[3], renal clearance[15] and the ability to realize efficient fluorescence resonance energy transfer[16,17].

In earlier reports, we described the use of DHLA and PEG-appended DHLA (DHLA–PEG–OH) as surface ligands (through cap exchange) to promote the aqueous dispersion of luminescent QDs[12,18]. Capping QDs with DHLA permitted their dispersion in basic buffer solutions; aggregate-free dispersions that are stable over extended periods of time can be made using this route, a remarkable progress compared with QDs capped with mono-thiol ligands such as mercaptoacetic acid and mercaptoundecanoic acid[12]. However, aggregation of the DHLA-capped QDs occur under acidic conditions[12,18]. The use of the neutral PEG-terminated DHLA (as ligands) permitted us to expand the range of pH stability of the QD to acidic conditions[18,19]. The synthetic scheme we used for making DHLA–PEG–OH is relatively simple. Coupling of readily available thioctic acid and PEGs in simple esterification schemes followed by reduction of the 1,2-dithiolane allowed us to prepare large quantities (several grams) of PEG-terminated DHLA (DHLA–PEG–OH)[18]. These PEG derivatives not only increase the stability of the QDs, but also reduce nonspecific adsorption. The DHLA–PEG ligands were also applied to gold nanocrystals[19]. However, the lack of accessible functional end groups on the DHLA–PEG–OH prevented the implementation of commonly used bioconjugation techniques, such as avidin–biotin binding and covalent conjugation chemistry (through 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) coupling). It is of great practical importance to expand the above synthetic scheme and make DHLA–PEG ligands presenting additional biocompatible functions. These ligands will also promote water solubility and biocompatibility of gold nanoparticles.

In this protocol, we expand on the above progress and describe the synthesis and characterization of a series of modular ligands

having end groups that can be further coupled with biofunctional molecules, such as dyes, peptides and proteins[20]. The functional groups that will be detailed in this report include biotin, amine and carboxylic acid. In this design, each ligand is modular in its structure, comprised of a central PEG segment with tunable length to promote hydrophilicity, a dithiol terminal group for anchoring onto the QD surface at one end, and a lateral functional/reactive group that promotes biological linkage with target biomolecules at the other end (DHLA–PEG–FN, where FN is the functional group; see scheme in **Fig. 1**). Cap exchange reactions were carried out with these new ligands and representative assays using the resulting water-soluble QDs were performed to verify the enhanced biocompatibility of nanoparticles. We should emphasize that even though both thiotic acid (TA)- and DHLA-appended ligands can be used to surface cap Au nanoparticles[19], only the reduced form of the ligand can provide effective cap exchange of semiconducting nanocrystals. Thus, ring opening of the disulfide group in the presence of $NaBH_4$ will be detailed for all compounds described in this protocol.

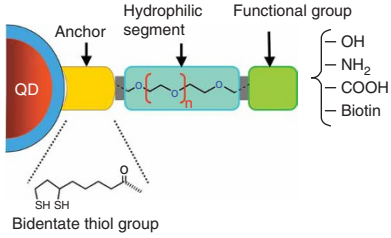

Figure 1 | Modular design of hydrophilic ligands with terminal functional groups used in this study. Reprinted with permission from ref. 20. Copyright 2007 American Chemical Society.

Experimental design

Although this protocol is primarily focused on the use of CdSe-ZnS core-shell QDs, the ligands described here can eventually be applied to other types of nanoparticles (e.g., additional semiconductor nanocrystals and gold nanoparticles). As QDs (and other nanoparticles) present large surface areas, their surface functionalization with molecular-scale ligands and block copolymers can produce high numbers of reactive functional groups per nanocrystal. Changing the density of those groups on the final nanocrystal surface becomes crucial, as it allows control over the nanocrystal 'reactivity'. To achieve this goal in the present strategy, capping QDs with a desired DHLA–PEG–FN ligand is most often carried out using mixtures with an 'inert' ligand (e.g., DHLA–PEG–OH or DHLA–PEG–OCH_3)[18,19]. The previously described synthesis of the end-functionalized TA–PEG and DHLA–PEG ligands focused on using PEG with molecular weight (MW)=400 (PEG400)[20]. More recently, we have extended the synthesis to using longer segments (including PEG600) and simultaneously improved some of the reaction and purification steps; one of the most important modifications pertains to the preparation of the compound NH_2-PEG-N_3. This has resulted in a simpler synthetic scheme and higher reaction yields. In this protocol, we describe the latest development and will be referring to PEG400 throughout the manuscript, unless otherwise noted. All the steps described below to prepare all the precursors and final products also apply to PEG600. It is important and useful to provide a brief description of the synthesis and purification of DHLA–PEG600–OH (PEG with MW ~600), as a representative example of these 'inert' ligands used in the cap exchange procedure (see **Box 1**).

Details on the synthetic procedures, cap exchange using DHLA-PEG-OH and DHLA–PEG–OCH_3 ligands were provided in previous reports[18,19].

Using ligands with a short PEG segment appended onto DHLA, we can render QDs (or Au nanoparticles) water soluble, independent of the presence of charged carboxyl groups, as was the case of DHLA. If specific functional groups (FN) are attached at the end of the PEG segment before or after coupling with TA, a new set of ligands, referred to as DHLA–PEG–FN, can be made (see scheme in **Fig. 1**). These ligands will ultimately allow conjugation of the nanoparticles to target molecules, such as dyes, peptides and proteins. In this protocol, we will detail the synthesis and characterization of some of these ligands, namely DHLA–PEG–NH_2, DHLA–PEG–COOH and DHLA–PEG–biotin (**Fig. 2**). As all of the ligands described here are based on the DHLA motif, they all present a dithiol group for strong anchoring onto the surface of the nanocrystals. It is thus easy to perform mixed-surface ligand exchange of the nanoparticles, where the fraction of a particular ligand presenting a specific terminal function is determined by controlling the molar ratio of that ligand in the mixture used for cap exchange. For example, to prepare a sample of nanocrystals having 10% of their surface ligands made of carboxyl terminal groups, whereas the rest is neutral, a mixture of DHLA–PEG–OH (or DHLA–PEG–OCH_3) and DHLA–PEG–COOH at a molar ratio of 9:1 can be used during the cap exchange step and transfer of the nanocrystals to water. The presence of a small number of terminal functions allows controlled coupling to target molecules (dyes, peptides and proteins). Nanocrystals capped with any mixture

BOX 1 | SYNTHESIS OF OH-TERMINATED TA–PEG AND DHLA–PEG LIGANDS

PEG600 was first appended onto thioctic acid, through *N,N'*-dicyclohexylcarbodiimide, to provide thioctic acid-appended PEG (TA–PEG600–OH), the precursor to DHLA–PEG600–OH. Once the coupling reaction was complete, purification of TA–PEG600–OH was carried out by filtering away the precipitate and evaporating the solvent. Saturated sodium bicarbonate ($NaHCO_3$) solution was added to the residue, and then extraction with ethyl acetate (EtOAc) was performed several times until the transfer of TA–PEG600–OH from the aqueous layer to the organic layer was complete. This step removed excess unreacted PEG600 (which mostly stayed in the saturated $NaHCO_3$ aqueous solution). The combined organic layers were dried over Na_2SO_4, filtered and the solvent evaporated. Such selective extraction of the crude product made the following column chromatography purification much easier compared with the previous procedure[20]. The crude product was then chromatographed on silica gel with $CHCl_3$:MeOH = 15:1 (vol/vol) as the eluent and pale yellow oil was collected, characterized using thin-layer chromatography (TLC) and ^1H NMR. The DHLA and DHLA–PEG600–OH ligands used for cap exchange reaction on the QDs were prepared through reduction of the 1,2-dithiolane groups of thiotic acid (TA) and TA–PEG600–OH, respectively, with $NaBH_4$.

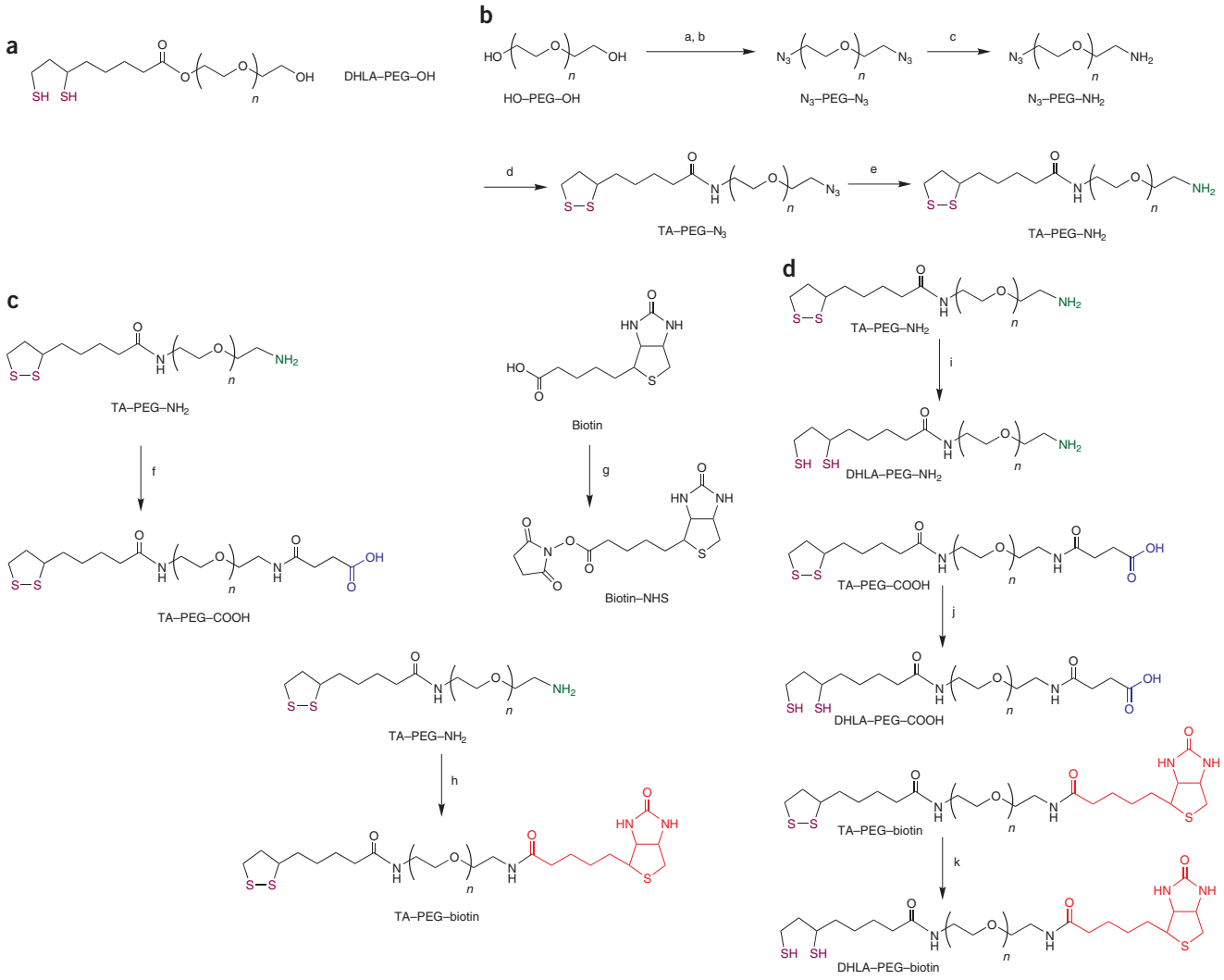

Figure 2 | Chemical structures and synthetic routes of the surface ligands used in this study. (**a**) Hydroxy-terminated DHLA–PEG. (**b**) Synthetic route of TA–PEG–NH_2: (a) MsCl, Et_3N, THF; (b) NaN_3, $NaHCO_3$, H_2O; (c) PPh_3, EtOAc and 1 M HCl; (d) thioctic acid, DCC, DMAP, CH_2Cl_2; (e) PPh_3, H_2O, THF. (**c**) Modification of TA–PEG–NH_2 to TA–PEG–COOH and TA–PEG–biotin: (f) succinic anhydride, CH_2Cl_2 and Et_3N; (g) NHS, DCC, DMF; (h) biotin NHS ester, Et_3N, DMF. (**d**) Ring-opening reactions of 1,2-dithiolane groups: (i) $NaBH_4$, EtOH, H_2O; (j) $NaBH_4$, EtOH, H_2O; (k) $NaBH_4$, EtOH, H_2O.

of DHLA–PEG–OH/DHLA–PEG–FN (or DHLA–PEG–OCH_3/DHLA–PEG–FN) are stable over a relatively broad pH range (pH 4–10), which makes these nanocrystals compatible with coupling reactions that need to be carried out under acidic or basic conditions.

The first conjugation scheme we describe for the newly functionalized nanocrystals uses biotin–avidin interactions starting with QDs capped with a mixture of DHLA–PEG–OH/DHLA–PEG–biotin. This conjugation method is simple, as it involves addition of biotinylated target biomolecules together with streptavidin or NeutrAvidin bridges, and the resulting conjugates are stable, owing to the strong avidin–biotin interactions (dissociation constant $K_D \sim 10^{-15}$ M). The use of avidin (or a streptavidin) could promote cross-linking due to the presence of four binding sites for biotin per avidin, which will ultimately affect the final assay data collection and analysis. However, using only a small molar ratio of biotinylated ligand (10–20%) on the nanocrystal would reduce the chance of aggregate formation.

The second conjugation scheme uses the ubiquitous EDC coupling of any amine-functionalized dye, peptide or protein to carboxylic acid groups present on the nanocrystal cap-exchanged with a mixture of DHLA–PEG–OH/DHLA–PEG–COOH. This reaction can be carried out in buffer solutions and in certain organic solvents. Similar to what was discussed above, to reduce incidence of potential cross-linking or making QD-bioconjugates that have too many copies of the target molecules, nanocrystals with mixed surface ligands (hydroxy-terminated and carboxyl-terminated) could be used.

The third applicable conjugation scheme, not discussed in detail in this protocol, uses coupling of QD surface functionalized with amines (QDs with DHLA–PEG–NH_2) to target molecules presenting isothiocyanate groups. This reaction can be potentially more efficient, as it does not require the use of excess substrate nor any intermediate reaction step, as is the case of EDC coupling.

The reactions described in this protocol can easily be scaled up. The main limitation of this approach to prepare biocompatible QDs is the loss of the overall photoluminescence quantum yield compared with that measured for nanocrystals capped with the

native TOP/TOPO shell, which is an issue that affects all of the other surface functionalization strategies. The measured yield is usually about 50% of that measured in organic solutions (anywhere between 15 and 30%)[20]. This set of ligands can be applied to various semiconductor QDs (such as PbSe, ZnS- and ZnSe-overcoated nanocrystals) and metallic nanoparticles. However, it will most likely not work with other metal oxide nanocrystals, including those made of Fe_3O_4 and TiO_2.

MATERIALS

REAGENTS

- PEG (average MW: 400 and 600; Acros Organics)
- Methanesulfonyl chloride (GFS Chemicals)
- Tetrahydrofuran (THF; Acros Organics or Sigma-Aldrich)
- Triethylamine (Sigma-Aldrich)
- Deionized water
- Sodium bicarbonate ($NaHCO_3$; Acros Organics)
- Sodium azide (NaN_3; Alfa Aesar)
- Chloroform ($CHCl_3$; Acros Organics or Sigma-Aldrich)
- Magnesium sulfate ($MgSO_4$; Acros Organics or Sigma-Aldrich)
- Methanol (MeOH; Acros Organics or Sigma-Aldrich)
- Deuterated chloroform ($CDCl_3$) with 0.03% (vol/vol) tetramethylsilane (TMS) (Sigma-Aldrich and Cambridge Isotope Laboratories Inc.)
- Triphenylphosphine (PPh_3; Acros Organics)
- Ether (Acros Organics or Sigma-Aldrich)
- Potassium hydroxide (KOH; Acros Organics)
- Methylene chloride (CH_2Cl_2; Acros Organics or Sigma-Aldrich)
- Thioctic acid (Acros Organics or Sigma-Aldrich)
- 4-(*N*,*N*-dimethylamino)pyridine (Acros Organics)
- *N*,*N*′-dicyclohexylcarbodiimide (Acros Organics)
- Ethyl acetate (EtOAc; Acros Organics or Sigma-Aldrich)
- d-Biotin (Alfa Aesar)
- *N*-hydroxysuccinimide (NHS; Acros Organics)
- *N*,*N*-dimethylformamide (DMF; Acros Organics or Sigma-Aldrich)
- Deuterated dimethylsulfoxide (DMSO-d_6; Acros Organics)
- Ethanol (EtOH; Acros Organics or Sigma-Aldrich)
- Sodium borohydride ($NaBH_4$; Strem Chemicals) ▲ **CRITICAL** This reagent is sensitive to moisture. Storage in glove box is recommended.
- Succinic anhydride (Fluka) ▲ **CRITICAL** This reagent is sensitive to moisture. Storage in glove box is recommended.
- Pyridine (Acros Organics or Sigma-Aldrich)
- Hydrochloric acid (HCl; Fisher Scientific)
- Sodium sulfate (Na_2SO_4; Acros Organics or Sigma-Aldrich)
- Celite (Sigma-Aldrich)
- Silica gel (60 Å, 230–400 mesh; Bodman Industries)
- Hexanes (Acros Organics or Sigma-Aldrich)
- Phosphate-buffered saline (PBS; 0.138 M NaCl and 0.0027 M KCl; Sigma-Aldrich and Acros Organics)
- Lissamine rhodamine B ethylenediamine (Invitrogen)
- Sulfo-NHS (Pierce Biotechnology)
- EDC (Pierce Biotechnology) ! **CAUTION** Most of the chemicals are hazardous (e.g., toxic, irritant, corrosive, flammable, lachrymator). Thus, care must be taken when handling them. All solvents (HPLC or reagent-grade quality) were used as received, except THF, which was dried by passing over an aluminum oxide column.

EQUIPMENT

- Round-bottom flasks, one- and two-necked
- Addition funnels
- Keck clips
- Magnetic stirring bars
- Rubber septa (24/40)
- Hotplate magnetic stirrers
- Thermometers
- Distilling head
- Glass syringes and needles
- Separatory funnels
- Filter paper (Whatman qualitative circles)
- Funnels
- Rotary evaporators
- Chromatography columns (glass)
- Thin-layer chromatography (TLC) plates (silica gel matrix with aluminum support; Sigma-Aldrich)
- Iodine chamber to stain samples on TLC
- Vacuum line and nitrogen source
- NMR (Bruker SpectroSpin 400 MHz spectrometer)
- UV-visible spectrophotometer (e.g., HP 8453 diode array spectrophotometer; Agilent Technologies)
- Fluorimeter (e.g., Spex Fluorolog-3 spectrophotometer equipped with a red-sensitive R2658 Hamamatsu PMT detector; Jobin Yvon Inc.)
- Quartz cuvettes/fluorimeter cells (Spectrocell Inc.)
- Fourier transform-infrared (FT-IR) spectrometer (e.g., Nicolet Nexus 870, Thermo Fisher Scientific Inc.)
- Vials
- Plastic syringes and needles
- Millex-LCR filter (0.45-μm-pore size, hydrophilic PTFE, 25 mm diameter, nonsterile; Millipore)
- Amicon Ultra-15 centrifugal filter unit with Ultracel-50 membrane (MW cutoff ~50 kDa; Millipore)
- Centrifuge (IEC Centra CL2 centrifuge; Thermo Scientific)
- NeutrAvidin-covered 96-well microtiter flat-bottomed plates (Pierce Biotechnology)
- Eppendorf tubes (1.5 ml; Eppendorf International)
- Shaker
- PD-10 desalting gel columns (GE Healthcare)
- Fluorescence plate reader (e.g., Tecan Saphire plate reader; Tecan US)

REAGENT SETUP

The QDs described and used in this protocol were mainly CdSe-ZnS core-shell nanocrystals prepared in our laboratory (in two steps) by reacting organometallic precursors at high temperature and in strong coordinating solvents[6,21–24]. The CdSe cores were first grown in mixtures of TOP/TOPO and alkyl amines. Following purification, several monolayers of ZnS were then assembled over the core to make CdSe-ZnS core-shell QDs; ZnS-overcoating was carried out at slightly lower temperature than that used for the CdSe synthesis. This set of ligands applies to other semiconductor nanocrystals and metallic Au nanoparticles (AuNPs). We have, for example, shown that our ligands can be used to cap exchange commercially available citrate-stabilized AuNPs[19].

EQUIPMENT SETUP

Characterization by ^{1}H NMR Chemical shifts for ^{1}H NMR spectra are reported relative to the TMS signal in the deuterated solvent (TMS, $\delta = 0.00$ p.p.m.). All *J*-values are reported in Hertz. The molar amounts of PEG derivatives were calculated by assuming an average repeat number *n* of ethylene glycol monomer units (see **Fig. 1**). For example, a PEG400 segment has $n \sim 8$, whereas for a PEG600 chain, $n \sim 12$.

PROCEDURE

Synthesis of diazide-terminated PEG, N_3–PEG400–N_3 ● TIMING ~1 d

1| Add PEG (average MW ~400) (72.4 g, ~0.175 mol), THF (200 ml) and methanesulfonyl chloride (46.1 g, 0.402 mol) in a 1-liter two-necked round-bottomed flask equipped with an addition funnel, septa and a magnetic stirring bar. Add triethylamine (60 ml, 0.43 mol) to the addition funnel. Purge the reaction vessel with nitrogen and cool the mixture to ~0 °C in an ice bath. **? TROUBLESHOOTING**

2| Add triethylamine dropwise to the reaction mixture through the addition funnel (addition takes ~30 min).

3| Warm up the reaction mixture gradually to room temperature (20–25 °C) and stir for several hours (or overnight). TLC of the intermediate product (MsO–PEG–OMs, precursor to the bis-azide–PEG) can be performed after 2–4 h using PEG as a co-spot to determine if the reaction is complete. Using $CHCl_3$:MeOH = 10:1 (vol/vol) for elution, R_f(MsO–PEG400–OMs) = ~0.5, wheeras R_f(HO–PEG400–OH) = 0.36.
▲ CRITICAL STEP The presence of a band (spot) characteristic of the starting PEG on the TLC plate indicates an incomplete reaction. In this case, allow the reaction to continue longer (additional 1–2 h). Otherwise, proceed to the next step.

4| Dilute the mixture with H_2O (200 ml) and add $NaHCO_3$ (12.5 g, 0.149 mol).

5| Add sodium azide (31.0 g, 0.477 mol) and attach a distilling head with a round-bottomed flask as a solvent trap. Cool the solvent trap with an ice-bath. Heat the biphasic reaction mixture under N_2 to distill off the THF, and then reflux for several hours (6–8 h or overnight).

6| Cool the reaction mixture and transfer to a separatory funnel. Extract the product five times with $CHCl_3$ (100 ml each).
▲ CRITICAL STEP After three or four extractions, check the TLC of the last $CHCl_3$ layer and make sure that there is no product left. If residual materials remain, repeat the extraction procedure 2–3 additional times until the band associated with the product is essentially absent from the last $CHCl_3$ extraction; using $CHCl_3$:MeOH = 10:1 (vol/vol) for elution, R_f(N_3–PEG400–N_3) = 0.75, R_f(HO–PEG400–OH) = 0.36.

7| Dry the combined organic layers over Na_2SO_4 (~50 g, for ~30 min) while stirring, filter through a filter paper and evaporate the solvent using a rotary evaporator; a pale brown oil is obtained. In all steps that require Na_2SO_4 within this protocol, it can be replaced with $MgSO_4$.
? TROUBLESHOOTING

8| Chromatograph the crude product on a silica gel (7.5 cm i.d. × 20 cm length) with 20:1 (vol/vol) $CHCl_3$:MeOH as the eluent. Collect 50–100 ml fractions of the eluting volume.

9| Check TLC for each fraction and combine fractions with the pure product. Evaporate the solvent and further dry the product using a vacuum pump.
■ PAUSE POINT The product can be stored at room temperature for at least several months. However, storage in a refrigerator is recommended if possible, as this can extend the product shelf life.

Amine transformation of one terminal azide group, N_3–PEG400–NH_2 ● TIMING ~1 d
10| Add N_3–PEG400–N_3 (12.0 g, ~2.58 × 10^{-2} mol), EtOAc (150 ml) and 1 M HCl (60 ml, 6 × 10^{-2} mol) in a 500-ml two-necked round-bottomed flask equipped with an addition funnel, septa and a magnetic stirring bar. Add triphenylphosphine (7.42 g, 2.83 × 10^{-2} mol) and 100 ml of EtOAc into the addition funnel. Purge the reaction vessel with N_2 and cool the mixture to 0 °C in an ice-bath while stirring.
▲ CRITICAL STEP The use of a biphasic reaction is rather critical. This promotes selective monoazide transformation (versus the two amines) because a single amine transformation changes the product solubility and results in its transfer to the aqueous phase, suppressing the transformation of both azide groups.

11| Add a solution of triphenylphosphine dropwise through the addition funnel under N_2. Maintain the temperature below 5 °C during the addition.

12| Once the addition is complete, let the reaction mixture gradually warm up to room temperature and stir for 6–8 h (or overnight) under N_2.

13| Transfer the reaction mixture to a separatory funnel and separate the biphasic solution. Collect the aqueous layer and wash it with EtOAc (100 ml, two times).

14| Transfer the aqueous layer to a round-bottomed flask equipped with a magnetic stirring bar. Cool the solution in an ice bath. Add KOH (30.0 g, 0.535 mol) slowly to the aqueous solution and stir the mixture until the KOH is dissolved.

15| Transfer the aqueous solution into a separatory funnel and extract the product repeatedly with EtOAc (~5 times).
▲ CRITICAL STEP After three or four extractions, check the TLC of the last EtOAc layer and make sure that there is no product left. If residual materials remain, repeat the extraction procedure 2–3 additional times until the TLC band associated with the product disappears from the last EtOAc extraction; with $CHCl_3$:MeOH = 5:1 (vol/vol) as the eluting solvent, R_f(N_3–PEG400–NH_2) = 0.09, R_f(N_3–PEG400–N_3) = 0.82.

16| Dry the combined organic layers over Na_2SO_4 (50 g for ~20 min) with stirring, and filter off Na_2SO_4 through filter paper.

17| Evaporate the solvent using a rotary evaporator and dry the product under vacuum.
■ **PAUSE POINT** Storage of the product in a refrigerator is recommended, with a shelf life of at least several months.

Coupling to thioctic acid, TA–PEG400–N_3 ● **TIMING ~1 d**
18| Add N_3–PEG400–NH_2 (3.00 g, ~6.84×10^{-3} mol), 4-(*N,N*-dimethylamino)pyridine (0.168 g, 1.38×10^{-3} mol), *N,N'*-dicyclohexylcarbodiimide (1.45 g, 7.03×10^{-3} mol) and CH_2Cl_2 (40 ml) in a 250-ml round-bottomed flask equipped with a magnetic stirring bar and an addition funnel. The mixture should be kept at ~0 °C in an ice bath.

19| Add thioctic acid (1.41 g, 6.83×10^{-3} mol) and 25 ml of CH_2Cl_2 into the addition funnel.

20| Add thioctic acid solution dropwise over 30 min under N_2 while stirring.

21| Once the addition is complete, let the reaction mixture gradually warm up to room temperature and further leave it stirring for 6–8 h (or overnight).

22| Filter the mixture through celite and rinse the celite plug with CH_2Cl_2. Evaporate the solvent using a rotary evaporator.

23| Add H_2O to the residue. Wash the aqueous mixture with hexanes (100 ml, two times). Saturate the aqueous layer with $NaHCO_3$. Extract the product with CH_2Cl_2 (100 ml, three times).
▲ **CRITICAL STEP** When using PEG600 as the precursor, saturated aqueous $NaHCO_3$ solution can be added to the residue and hexanes can be replaced with ether.

24| Dry the combined organic layers over Na_2SO_4 (for 20–30 min). Filter off Na_2SO_4 (using a paper filter) and evaporate the solvent using a rotary evaporator.

25| Chromatograph the residue on silica gel (5.0 cm i.d. × 20 cm length) with 20:1 (vol/vol) $CHCl_3$:MeOH as the eluent to collect the product. Collect 50–100 ml fractions of the eluting solution.

26| Check TLC for each fraction and combine those with the pure product ($CHCl_3$:MeOH = 10:1 (vol/vol), R_f(TA–PEG400–N_3) = 0.62, R_f(TA) = 0.54, R_f(N_3–PEG400–NH_2) = 0.04). Evaporate the solvent and dry the product under vacuum.
■ **PAUSE POINT** The product can be stored at room temperature for several months. However, storage in a refrigerator is recommended, as this will improve its shelf life.

Transformation of TA–PEG–N_3 to amine derivatives, TA–PEG400–NH_2 ● **TIMING ~1 d**
27| Dissolve TA–PEG400–N_3 (2.75 g, ~4.39×10^{-3} mol) in dry THF (50 ml) in a 250-ml round-bottomed flask equipped with a magnetic stirring bar.

28| Add triphenylphosphine (1.73 g, 6.60×10^{-3} mol) and stir the reaction mixture at room temperature for 30 min under N_2.

29| Add H_2O (0.80 ml, 4.4×10^{-2} mol) and further stir the reaction mixture at room temperature for ~8 h (or overnight) under N_2.
▲ **CRITICAL STEP** TLC of the product can be performed using TA–PEG400–N_3 as a cospot to determine if the reaction is complete after 8 h (i.e., all of TA–PEG400–N_3 is consumed). (With $CHCl_3$:MeOH = 10:1 (vol/vol), R_f(TA–PEG400–NH_2) = 0.1, R_f(TA–PEG400–N_3) = 0.62, R_f(PPh_3) = 0.89.) If the TLC plate shows that there is a significant amount of TA–PEG400–N_3 left in the solution, allow the reaction to continue longer as needed. Otherwise, proceed to Steps 30–34 for purification.

30| Evaporate the solvent using a rotary evaporator. Add EtOAc into the residue and transfer to a separatory funnel.

31| Add 1 M HCl solution (100 ml) slowly and remove the organic layer. Add EtOAc into the aqueous layer again and remove the organic layer. This removes unreacted triphenylphosphine and residual TA–PEG–N_3 and triphenylphosphine oxide.

32| Add Na_2CO_3 slowly into the aqueous layer until rendered basic (pH ~9).

33| Extract the product with $CHCl_3$ (100 ml, three times) and dry the combined organic layers over Na_2SO_4 (50 g for ~20 min).

34| Filter off Na_2SO_4 through filter paper, evaporate the solvent using a rotary evaporator and dry the product under vacuum.

■ **PAUSE POINT** The neat product may be transformed into a gel that is difficult to redissolve even in organic solvents (e.g., CH_2Cl_2). Storage as a solution (by dissolving in small amount of CH_2Cl_2) in a freezer is recommended, as this helps to avoid gel transformation.

35| Derivatives of TA–PEG can now be prepared using option A to prepare carboxylic acid derivatives or option B to prepare biotin derivatives.

(A) Synthesis of TA–PEG400–COOH: transformation of TA–PEG400–NH_2 to carboxyl derivatives ● **TIMING** ~**1 d**

(i) Add TA–PEG400–NH_2 (0.748 g, $\sim 1.24 \times 10^{-3}$ mol), succinic anhydride (0.25 g, 2.50×10^{-3} mol), 10 ml of dry CH_2Cl_2 and triethylamine (0.45 ml, 3.2×10^{-3} mol) in a 100-ml round-bottomed flask equipped with a magnetic stirring bar. Stir the mixture at room temperature for 6–8 h (or overnight) under N_2.

(ii) Pour the reaction mixture into 1 M HCl solution (60 ml).

(iii) Extract the product with $CHCl_3$ (100 ml, three times). TLC can be performed to determine if the product is pure ($CHCl_3$:MeOH = 10:1 (vol/vol), R_f(TA–PEG400–COOH) = 0.25).

(iv) Wash the combined organic layers with brine and dry over Na_2SO_4 (for 20–30 min).

(v) Filter off Na_2SO_4 through filter paper, evaporate the solvent using a rotary evaporator and dry the product under vacuum. **? TROUBLESHOOTING**

(B) Synthesis of NHS–biotin and TA–PEG–biotin ● **TIMING 2 d**

(i) To synthesize biotinyl–NHS (biotin–NHS) dissolve d-biotin (5.00 g, 2.05×10^{-2} mol) and NHS (2.36 g, 2.05×10^{-2} mol) in DMF (150 ml) in a 500-ml round-bottomed flask, while stirring.

(ii) Add *N,N'*-dicyclohexylcarbodiimide (5.50 g, 2.67×10^{-2} mol) to the mixture and let the solution stir for ~8 h (or overnight) at room temperature under N_2. A white precipitate is formed during this period.

(iii) Filter the reaction mixture; evaporate the solvent from the filtrate (using a rotary evaporator) and tritulate with ether.

(iv) Filter the white precipitate and wash with ether to give a white powder containing biotinyl-NHS. Dry the white powder under vacuum.

(v) To synthesize the TA–PEG400–biotin, mix TA–PEG400–NH_2 (0.22 g, 3.7×10^{-4} mol), biotinyl-NHS (0.122 g, 3.6×10^{-4} mol) and DMF (8.0 ml) in a round-bottomed flask equipped with a magnetic stirring bar and a septum.

(vi) Add triethylamine (0.24 ml, 1.7×10^{-3} mol) dropwise to the mixture with a syringe through the septum under N_2, and let the reaction mixture stir at room temperature for 6–8 h (or overnight).

(vii) Filter the reaction mixture through a paper filter and remove DMF from the filtrate under vacuum.

(viii) Chromatograph the residue on silica gel (4.0 cm i.d. × 20 cm length) with 8:1 (vol/vol) CH_2Cl_2:MeOH as the eluent. Collect approximately 10–20 ml fractions of the eluted solution.

(ix) Check TLC for each fraction (using $CHCl_3$:MeOH = 5:1 (vol/vol), R_f(TA–PEG400–biotin) = 0.68) and combine into product. Evaporate the solvent and dry the product under vacuum.

36| The procedure for the reduction of the dithiolane ring is specific to different end groups and can be carried out using synthesis of DHLA–PEG400–NH_2 (option A), synthesis of DHLA–PEG400–COOH (option B) or synthesis of DHLA–PEG400–biotin (option C).

(A) Synthesis of DHLA–PEG400–NH_2 ● **TIMING 12 h**

(i) Place TA–PEG400–NH_2 (0.435 g, $\sim 7.24 \times 10^{-4}$ mol) and EtOH (5 ml) in a round-bottomed flask equipped with a magnetic stirring bar.

(ii) Cool the solution in an ice bath, and add $NaBH_4$ (0.11 g, 2.9×10^{-3} mol) in 2.0 ml of H_2O dropwise while stirring.

(iii) Warm the reaction mixture gradually to room temperature and stir for 3–4 h under N_2.

(iv) Evaporate EtOH using a rotary evaporator and dilute the reaction mixture with 20 ml of brine.

(v) Extract with $CHCl_3$ (50 ml, four times) and dry the combined organic layers over Na_2SO_4 (for 20–30 min). Filter off Na_2SO_4 and evaporate the solvent.

(B) Synthesis of DHLA–PEG400–COOH ● **TIMING 12 h**

(i) Place TA–PEG400–COOH (0.364 g, $\sim 5.19 \times 10^{-4}$ mol) and EtOH (5.0 ml) in a round-bottomed flask equipped with a magnetic stirring bar.

(ii) Cool the solution in an ice bath, and add $NaBH_4$ (0.10 g, 2.6×10^{-3} mol) in 2 ml of H_2O dropwise while stirring.

(iii) Warm the reaction mixture gradually to room temperature and stir for 3–4 h under N_2.

(iv) Add 1 M HCl solution until the pH of the reaction mixture reaches ~ 2, and extract the reaction mixture with $CHCl_3$ (50 ml, four times).

(v) Dry the combined organic layers over Na_2SO_4 with stirring; filter off Na_2SO_4 and evaporate the solvent.

(C) Synthesis of DHLA–PEG400–biotin ● **TIMING 12 h**

(i) Place TA–PEG400–biotin (0.56 g, $\sim 6.77 \times 10^{-4}$ mol), EtOH (12 ml) and H_2O (6.0 ml) in a round-bottomed flask equipped with a magnetic stirring bar.

(ii) Add $NaBH_4$ (0.132 g, 3.5×10^{-3} mol) in small portions to the solution while stirring. Once complete, let the reaction mixture stir at room temperature for 30 min under N_2.
(iii) Dilute the reaction mixture with H_2O, and extract the product with $CHCl_3$ (100 ml, four times).
(iv) Dry the combined organic layers over $MgSO_4$, filter and evaporate the solvent.
(v) Chromatograph the residue on silica gel with 10:1 (vol/vol) CH_2Cl_2:MeOH as the eluent.
(vi) Check TLC for each fraction and combine all the product containing fractions. Evaporate the solvent and dry the product under vacuum.

▲ **CRITICAL STEP** TA and DHLA derivatives have similar R_f values, but the latter show elution spots with long tailing on the TLC plates. It is thus difficult to confirm whether the ring-opening reaction is complete or not based only on the TLC analysis. Color change (from yellowish to clear solution) provides additional and strong indication of disulfide reduction. In addition, the DHLA unit tends to reform the disulfide bond, which essentially entails progressive reversal to the thioctic acid unit, if the product is left in air over extended periods of time (days).

■ **PAUSE POINT** Store the sample under N_2 in a sealed container in a freezer to ensure long shelf life.

? TROUBLESHOOTING

Cap exchange of TOP/TOPO-capped QDs with DHLA–PEG-based ligands ● TIMING ~18 h

37| Transfer ~500 µl of TOP/TOPO-capped QDs in toluene (i.e., stock solution, concentration of ~10–50 µM) to a vial.

38| Add EtOH to precipitate the TOP/TOPO-capped QDs.

39| Centrifuge the turbid solution (1,900*g*, 3,400 r.p.m. on IEC Centra CL2, 5 min at room temperature) and discard the supernatant.

? TROUBLESHOOTING

40| Add ~0.5 ml of pure or mixed ligands and ~0.5 ml of EtOH to the precipitate. If you would like to perform EDC coupling of COOH-functionalized QDs to amine-appended dyes (Steps 54–58), then prepare QDs capped with DHLA–PEG600–OH:DHLA–PEG400–COOH (19:1) mixture. **Box 1** provides a brief summary for the synthesis of OH-terminated TA–PEG and DHLA–PEG ligands[18]. These amounts guarantee a large excess of ligands in the solution (approximately 25,000–50,000 excess), a necessary condition because cap exchange is a mass-driven process.

41| Seal the vial and purge with nitrogen for 5–10 min.

42| Heat the mixture to 60–80 °C while stirring for several hours.

? TROUBLESHOOTING

43| Once homogenized, precipitate out the sample by adding a mixture of hexane, EtOH and $CHCl_3$, then centrifuge the sample at 1,900*g* for 5–10 min at room temperature. This will allow separation of the newly capped QDs.

▲ **CRITICAL STEP** First, add ~2 ml of EtOH into the homogeneous reaction solution. Add hexane slowly until the solution becomes turbid. Add $CHCl_3$ dropwise until the solution becomes clear again. Finally, add hexane dropwise again until the solution becomes turbid. Overall, the ratio of hexane, EtOH and $CHCl_3$ is approximately 11:10:1 and may slightly vary from batch to batch (total volume should be ~5 ml).

44| Discard the supernatant and disperse the precipitate in water (2–5 ml).

45| Filter through Millex-LCR filter.

46| Transfer the filtrate to Amicon Ultra-15 centrifugal filter unit with Ultracel-50 membrane.

47| Add deionized water to fill the filter unit (~15 ml total) and centrifuge at 1,900*g* for 5 min at room temperature. Discard the filtered solution.

48| Perform two more cycles of Step 47.

49| Redisperse in deionized water or buffer (1–2 ml) and measure the absorption spectra of the QD samples. Determine the sample concentration as reported previously[22,25].

Surface-binding assay using biotin-labeled QDs ● TIMING ~18 h

50| Add 100 pmol of QDs capped with the desired ligand mixtures (e.g., DHLA–PEG600–OH:DHLA-PEG400–biotin (4:1)) in 100 µl solutions to the NeutrAvidin-covered well and incubate overnight[20]. The QD with 100% DHLA–PEG600–OH can be used as control.

51| Measure the fluorescence signal of the plate. Fluorescence can be collected as a spectrum, an integrated intensity, or intensity at the peak emission.

52| Wash the plate three times with PBS buffer solution and measure the fluorescence signal again.

53| The remaining fluorescence intensities could be normalized using 100% for the highest value[20].

EDC coupling of COOH-functionalized QDs to amine-appended dyes ● TIMING ~5 h

54| Mix QDs capped with DHLA–PEG600–OH:DHLA–PEG400–COOH (19:1 ratio) (1 nmol), lissamine rhodamine B ethylenediamine (15 nmol), sulfo-NHS (2.5 μmol) and different amounts of EDC (0, 5, 20 and 50 μmol) with 0.1 M PBS solution (pH ~ 6.5) in Eppendorf tubes (0.5 ml in total). Using different amounts of EDC allows one to test the effects of the coupling agents on the labeling efficiencies.

55| Shake each reaction mixture for 2 h at room temperature.

56| Load the reaction mixture on a pre-equilibrated disposable PD-10 desalting gel column and elute with the same PBS solution to remove excess unreacted dyes and reagents. This allows separation of the QD–dye conjugates from free reagents and unbound dyes.

57| Collect fractions (~1 ml of each) of the eluted solution and measure the absorption spectrum for each.

58| Also load these solutions or fractions onto a microtiter well plate and collect the fluorescence spectra using a Tecan Safire plate reader.

● **TIMING**

Steps 1–9, synthesis of N_3–PEG400–N_3: ~1 d
Steps 10–17, synthesis of N_3–PEG400–NH_2: ~1 d
Steps 18–26, synthesis of TA–PEG400–N_3: ~1 d
Steps 27–34, synthesis of TA–PEG400–NH_2: ~1 d
Steps 35A(i–v), synthesis of TA–PEG400–COOH: ~1 d
Steps 35B(i–iv), synthesis of NHS–biotin: ~18 h
Steps 35B(v–ix), synthesis of TA–PEG400–biotin: ~1 d
Steps 36A(i–v), synthesis of DHLA–PEG400–NH_2: ~12 h
Steps 36B(i–v), synthesis of DHLA–PEG400–COOH: ~12 h
Steps 36C(i–vi), synthesis of DHLA–PEG400–biotin: ~12 h
Steps 37–49, cap exchange of TOP/TOPO–QDs with DHLA–PEG-based ligands: ~18 h
Steps 50–53, surface-binding assays: ~18 h
Steps 54–58, EDC coupling to amine functionalized dyes: ~5 h

? TROUBLESHOOTING

Step 1

The molar ratio of methanesulfonyl chloride to PEG used was ~2:1, which should result in full conversion of the OH groups to OMs. However, if an old stock of methanesulfonyl chloride is used, the conversion can be incomplete. Increasing the molar ratio to ~4:1 may speed the reaction and achieve a more efficient conversion.

Step 7

If the solution still looks cloudy and/or does not easily go through the paper filter, it is not dry enough; repeat the drying step one more time.

Step 35A(v)

Simple extraction should provide a pure compound. However, if the product is still not pure (based on NMR data), chromatograph the solution on silica gel (4.0 cm i.d. × 20 cm length) with 10:1 (vol/vol) CH_2Cl_2:MeOH as the eluent. Collect approximately 10–20 ml fractions of the eluted solution. Check TLC for each fraction with the condition above and combine the product. Evaporate the solvent and dry the product under vacuum.

Step 36C(vi)

These ligands have an amide bond between TA and PEG. A molar excess of $NaBH_4$ (2–4 times) ensures a complete reduction of all material in the solution. If ring opening is still incomplete, replace $NaBH_4$ with a fresh batch. $NaBH_4$ is best stored in an inert environment, in a glove box, for example.

Step 39

Centrifuging the solutions was performed at 3,400 r.p.m. using an IEC Centra CL2 centrifuge, which is approximately equal to 1,900*g*. In general, the rotation speed needed to achieve the same relative centrifugal force varies depending on the model and configuration used.

Step 42
Stirring for 3–4 h should suffice to produce effective cap exchange. However, longer mixing time (~overnight) could improve the effectiveness of the cap exchange.

ANTICIPATED RESULTS

The first anticipated set of products of the various synthetic steps detailed above is the ligands. The second set is the surface functionalized nanocrystals. The third set of results is implementation of specific functional assays with direct relevance to biology using the newly prepared hydrophilic QDs. In the following paragraphs, we provide characterization of the various ligands using ^{1}H NMR and IR spectroscopy. We will also describe the results collected from specific assays (namely avidin–biotin binding and coupling to functional dyes) using the newly designed nanocrystals.

Characterization of the prepared ligands using ^{1}H NMR and IR spectroscopy

Only data for the PEG400 series are shown here; similar spectra were collected for the PEG600 series.

N_3–PEG400–N_3
^{1}H NMR (400 MHz, $CDCl_3$): δ 3.62–3.71 (m), 3.40 (t, 4H, $J = 5.0$ Hz).
IR (neat): 2,868; 2,103; 1,453; 1,115 cm^{-1}.
The typical yield of this reaction is approximately 70–90%.

N_3–PEG400–NH_2
^{1}H NMR (400 MHz, $CDCl_3$): δ 3.6–3.9 (m), 3.52 (t, 2H, $J = 5.2$ Hz), 3.40 (t, 2H, $J = 4.8$ Hz), 2.87 (t, 2H, $J = 5.0$ Hz).
IR (neat): 3,379; 2,868; 2,107; 1,598; 1,455; 1,119 cm^{-1}.
The typical yield of this reaction is approximately 70–80%.

TA–PEG400–N_3
^{1}H NMR (400 MHz, $CDCl_3$): δ 6.42 (br s, 1H), 3.6–3.9 (m), 3.56 (t, 2H, $J = 5.0$ Hz), 3.46 (t, 2H, $J = 5.0$ Hz), 3.40 (t, 2H, $J = 5.2$ Hz), 3.08–3.23 (m, 2H), 2.46 (m, 1H), 2.20 (t, 2H, $J = 7.4$ Hz), 1.91 (m, 1H), 1.7 (m, 4H), 1.47 (m, 2H).
IR (neat): 3,328; 3,075; 2,918; 2,867; 2,105; 1,670; 1,540; 1,456; 1,119 cm^{-1}.
The typical yield is approximately 75–95%.

TA–PEG400–NH_2
^{1}H NMR (400 MHz, $CDCl_3$): δ 6.4–6.8 (m, 1H), 3.4–3.8 (m), 3.4–3.5 (m, 2H), 3.06–3.22 (m, 2H), 2.87–2.99 (m, 2H), 2.5–2.8 (br s, 2H), 2.42–2.51 (m, 1H), 2.17–2.27 (m, 2H), 1.86–1.96 (m, 1H), 1.59–1.78 (m, 4H), 1.39–1.55 (m, 2H).
IR (neat): 3,298; 3,053; 2,866; 1,666; 1,545; 1,456; 1,109 cm^{-1}.
The typical yield of this reaction is approximately 70–90%.

TA–PEG400–COOH
^{1}H NMR (400 MHz, $CDCl_3$): δ 6.78–6.98 (br s, 1H), 6.20–6.52 (m, 1H), 3.60–3.74 (m), 3.52–3.60 (m, 5H), 3.45 (m, 4H), 3.08–3.22 (m, 2H), 2.67 (m, 2H), 2.55 (m, 2H), 2.42–2.51 (m, 1H), 2.21 (m, 2H), 1.86–1.96 (m, 1H), 1.60–1.78 (m, 4H), 1.39–1.55 (m, 2H).
IR (neat): 3,319; 3,084; 2,922; 2,870; 1,732; 1,633; 1,549; 1,454; 1,109 cm^{-1}.
The typical yield of this transformation is approximately 70–85%.

Biotinyl-NHS
^{1}H NMR (400 MHz, one drop of DMSO-d_6 in $CDCl_3$): δ 5.23 (s, 1H), 4.96 (s, 1H), 4.52 (m, 1H), 4.33 (m, 1H), 3.16 (m, 1H), 2.87–2.97 (m, 1H), 2.86 (s, 4H), 2.75 (d, 1H, $J = 12.8$ Hz), 2.58–2.70 (m, 2H), 1.6–1.9 (m, 4H), 1.5–1.6 (m, 2H).
IR (neat): 3,226; 2,941; 2,918; 2,876; 1,820; 1,789; 1,746; 1,731; 1,708; 1,470; 1,370; 1,217; 1,072 cm^{-1}.
The typical yield of this reaction exceeds 90%.

TA–PEG400–biotin
^{1}H NMR (400 MHz, $CDCl_3$): δ 6.85–7.05 (m, 1H), 6.4–6.8 (m, 1H), 6.2–6.4 (m, 1H), 5.3–5.5 (m, 1H), 4.51 (m, 1H), 4.33 (m, 1H), 3.5–3.9 (m), 3.4–3.5 (m, 2H), 3.08–3.23 (m, 3H), 2.88–2.96 (m, 1H), 2.75 (d, 1H, $J = 12.8$ Hz), 2.42–2.51 (m, 1H), 2.16–2.3 (m, 4H), 1.87–1.96 (m, 1H), 1.6–1.8 (m, 8H), 1.4–1.6 (m, 4H).
IR (neat): 3,295; 3,083; 2,928; 2,865; 1,707; 1,645; 1,550; 1,461; 1,108 cm^{-1}.
The yield is approximately 50–90%.

DHLA–PEG400–NH_2

^{1}H NMR (400 MHz, $CDCl_3$): δ 6.42 (br s, 1H), 3.58–3.72 (m), 3.49–3.58 (m, 4H), 3.41–3.49 (m, 2H), 2.92 (m, 1H), 2.88 (t, 2H, $J = 5.0$ Hz), 2.62–2.78 (m, 2H), 2.20 (t, 2H, $J = 7.4$ Hz), 1.86–1.96 (m, 1H), 1.38–1.79 (m, 7H).
IR (neat): 3,196; 3,061; 2,866; 2,509; 1,662; 1,543; 1,456; 1,099 cm^{-1}.
The typical yield is approximately 80–90%.

DHLA–PEG400–COOH

^{1}H NMR (400 MHz, $CDCl_3$): δ 6.8–7.0 (br s, 1H), 6.2–6.5 (m, 1H), 3.60–3.72 (m), 3.56 (t, 4H, $J = 4.8$ Hz), 3.45 (m, 4H), 2.87–2.97 (m, 1H), 2.61–2.79 (m, 4H), 2.55 (m, 2H), 2.21 (m, 2H), 1.86–1.96 (m, 1H), 1.40–1.80 (m, 7H), 1.36 (t, 1H, $J = 8.0$ Hz), 1.31 (d, 1H, $J = 7.6$ Hz).
IR (neat): 3,319; 3,082; 2,924; 2,854; 2,553; 1,734; 1,649; 1,547; 1,462; 1,111 cm^{-1}.
The typical yield is approximately 75–90%.

DHLA–PEG400–biotin

^{1}H NMR (400 MHz, $CDCl_3$): δ 6.6–6.8 (m, 1H), 6.3–6.6 (m, 1H), 5.52–5.7 (m, 1H), 4.8–4.9 (m, 1H), 4.51 (m, 1H), 4.33 (m, 1H), 3.6–3.7 (m), 3.56 (t, 4H, $J = 4.6$ Hz), 3.45 (m, 4H), 3.16 (m, 1H), 2.87–2.97 (m, 2H), 2.62–2.79 (m, 3H), 2.16–2.28 (m, 4H), 1.86–1.96 (m, 1H), 1.4–1.8 (m, 13H), 1.36 (t, 1H, $J = 8.0$ Hz), 1.32 (t, 1H, $J = 7.6$ Hz).
IR (neat): 3,296; 3,084; 2,928; 2,865; 2,549; 1,704; 1,688; 1,645; 1,549; 1,461; 1,108 cm^{-1}.
The typical yield is ~80%.

Characterization of end functionalized DHLA-PEG-capped QDs

There are a few simple characterization tools that can be applied to verify the quality of newly capped nanocrystals. The first clear indication of whether or not an effective cap exchange has taken place is the ability to disperse the nanocrystals in aqueous solutions. QDs capped with mixtures of amine-, carboxyl- and biotin-functionalized DHLA–PEG400 and DHLA–PEG600–OH (at various molar ratios) are dispersible in buffer solutions at pH ranging from 5–11. This is the range of pHs explored in our study. A broader range (below 5 and beyond 11) may be accessible using our ligands as well. The absorption and fluorescence spectra are essentially unaffected by the cap exchange and transfer to aqueous media. There is, however, a consistent loss in the fluorescence quantum yield measured for water-dispersed nanocrystals compared with those TOP/TOPO-capped and dispersed in organic solutions. In general, the quantum yields are on average about 50% of the values measured for TOP/TOPO-capped nanocrystals in toluene and hexane, for example.

The second characterization tool relies on the use of FT-IR spectroscopy. FT-IR spectra collected from neat ligands and ligand-capped QDs showed that the main features of the neat ligands are maintained after cap exchange and immobilization on the nanocrystals. In particular, the spectra shown in **Figure 3** indicate that the sharp peak at 1,734 cm^{-1}, ascribed to C=O stretch mode of the ester group for the neat DHLA–PEG600–OH, and the two additional distinct bands measured at 1,666 and 1,541 cm^{-1} for DHLA–PEG400–biotin are maintained in the sample of QDs capped with a mixture of DHLA–PEG600–OH and DHLA–PEG400–biotin (4:1). These bands can be assigned to amide I (C=O stretch) and amide II (N–H bending) bands in biotin-appended DHLA–PEG400 ligand. Similar results were also measured for QDs capped with mixtures of DHLA–PEG600–OH/DHLA–PEG400–COOH[20]. In the design described here, we have used DHLA–PEG600–OH as the major ligand.

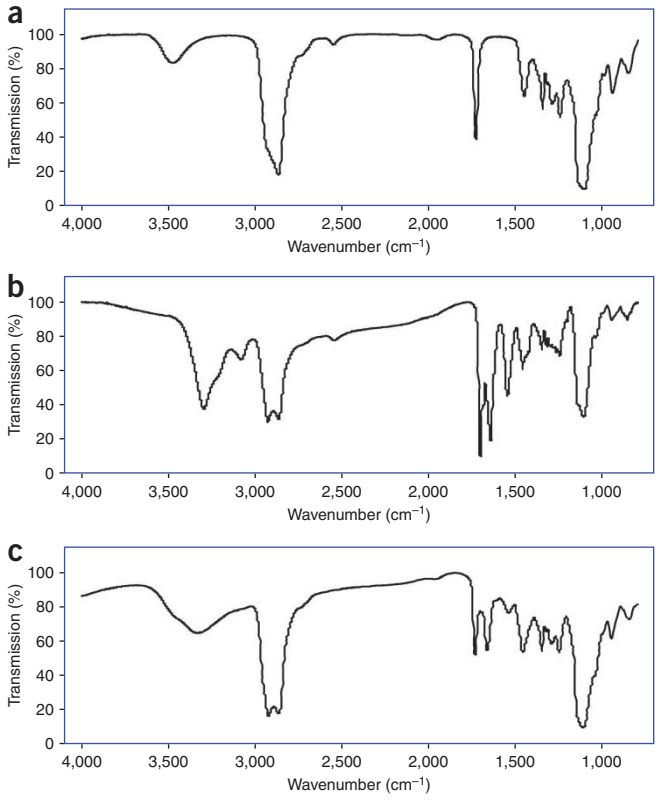

Figure 3 | FT-IR spectra for ligands produced in this study. (**a**) Neat DHLA–PEG600–OH, (**b**) neat DHLA–PEG400–biotin and (**c**) QD capped with a mixture of DHLA–PEG600–OH and DHLA–PEG400–Biotin (4:1 molar ratio). Figure modified and reprinted with permission from ref. 20. Copyright 2007 American Chemical Society.

Avidin–biotin binding assay

For nanocrystals capped with DHLA–PEG–biotin ligands, a simple and easy-to-implement assay involves binding of DHLA–PEG–biotin–QDs to a Streptavidin or NeutrAvidin

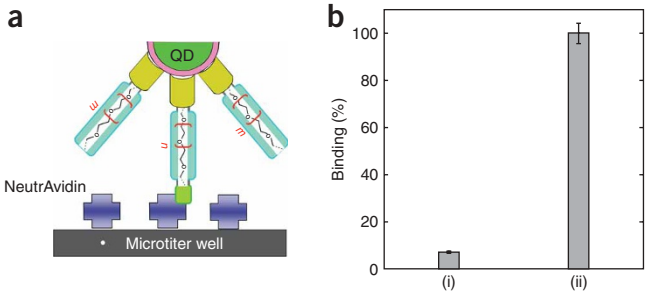

Figure 4 | Surface-binding assays of biotin-modified QDs to NeutrAvidin-functionalized surfaces. Schematic representation of the binding assay used; *m* and *n* designate the number of ethylene oxide repeats, where *n* can be ~8 (PEG400) or ~12 (PEG600) used for making biotin, COOH- and NH_2-terminated PEG ligands; *m* ~12 (PEG600) for the OH-terminated ligand. These numbers can be varied if desired. (**a**) Binding (%) of QDs with (**b**) different surface ligands: (i) DHLA–PEG600–OH, (ii) DHLA–PEG600–OH: DHLA–PEG400–biotin (4:1 molar ratio). Reprinted with permission from ref. 20. Copyright 2007 American Chemical Society.

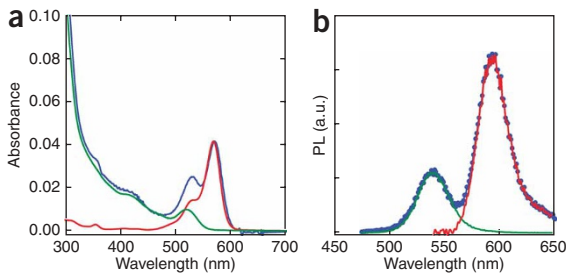

Figure 5 | Absorption and emission spectra of EDC-coupled QD-dye conjugates. (**a**) Absorption spectrum of a sample (with 0.1 M EDC used) after gel filtration (blue line) together with those of pure QDs (green line) and dye in H_2O (red line), (**b**) Composite emission spectrum of a sample (with 0.1 M EDC used) after gel filtration (blue dot/line), together with the deconvoluted contributions of the QDs (green line) and dye (red line). Photoluminescence (PL) spectra were generated using 300-nm excitation. Reprinted with permission from ref. 20. Copyright 2007 American Chemical Society.

functionalized substrate (**Fig. 4a**). This can allow verification of whether the biotin groups on the nanocrystal are available for interaction with avidin. We tested the binding of QDs capped with a mixture of DHLA–PEG600–OH and DHLA–PEG400–biotin to NeutrAvidin-functionalized substrates using a microtiter plate. The surface of the micotiter plate wells were prepared using published procedures[26]. Following incubation with the QD solutions and rinsing with PBS buffer, the fluorescence signals were measured. **Figure 4b** shows the fluorescence intensities for two different QD solutions: one capped with DHLA–PEG600–OH and the other capped with a mixture of DHLA–PEG600–OH and DHLA–PEG400–biotin (4:1). Only QDs presenting terminal biotin could bind to NeutrAvidin-coated wells, clearly showing that specific capture of the DHLA–PEG–biotin–QDs by NeutrAvidin takes place.

Coupling to dyes through EDC coupling

One of the major goals of our work is to provide the research community interested in using QDs with the means of achieving simple and reproducible conjugation strategies that allow the preparation of robust and functional QDs and QD bioconjugates. A simple test of this goal is coupling of COOH-functionalized QDs to amine-terminated dyes using EDC. EDC coupling is a widely used approach, based on targeting amine or carboxyl groups present on peptides and proteins[4]. When Lissamine rhodamine B ethylenediamine dye in PBS buffer was reacted with QDs capped with a mixture of DHLA–PEG600–OH (95%) and DHLA–PEG400–COOH (5%) in the presence of EDC and sulfo-NHS, samples showed no sign of aggregation or precipitation. Following removal of excess unreacted dyes and reagents from the reaction mixture by size exclusion gel filtration, absorption and fluorescence spectra were measured from the eluted solutions. We verified that the first major fractions collected from the gel filtration column contained QDs, even though not necessarily the same reagent concentration. Furthermore, the spectroscopic feature(s) for reactions carried out in the presence of EDC indicated significant contributions from the dye. In particular, there are two new bands (namely one at 571 nm for the absorption and the other at 592 nm for the fluorescence) in addition to the contribution from the QDs. The additional peaks were identical to the absorption and fluorescence maxima of the rhodamine dye alone (**Fig. 5**). In contrast, when the reaction was carried out without EDC (control experiment), the first major fractions collected from the gel column contained only unreacted QDs. We should also emphasize that the rhodamine fluorescence is mostly due to fluorescence resonance energy transfer interactions with the proximal QD in the conjugate; direct excitation contribution is small, since excitation at 300 nm is at the absorption valley of the dye. Although only EDC coupling was discussed, we have also carried out coupling reactions between amine-functionalized QDs and isothiocyanate as well as succinimidyl ester functionalized dyes[4]. The purification and characterization procedures of the formed conjugates follow the same rationales, but those coupling reactions have the added advantage of not requiring additional reagents.

ACKNOWLEDGMENTS We acknowledge NRL, Office of Naval Research (ONR) and the Army Research Office for financial support. We thank Drs Horn-Bond Lin and Igor Medintz for assistance.

Published online at http://www.natureprotocols.com/
Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions/

1. Murray, C.B., Kagan, C.R. & Bawendi, M.G. Synthesis and characterization of monodisperse nanocrystals and close-packed nanocrystal assemblies. *Annu. Rev. Mater. Sci.* **30**, 545–610 (2000).
2. Michalet, X. *et al.* Quantum dots for live cells, *in vivo* imaging, and diagnostics. *Science* **307**, 538–544 (2005).
3. Medintz, I.L., Uyeda, H.T., Goldman, E.R. & Mattoussi, H. Quantum dot bioconjugates for imaging, labelling and sensing. *Nat. Mater.* **4**, 435–446 (2005).

4. Hermanson, G.T. *Bioconjugate Techniques* (Academic Press, San Diego, 2008).
5. Murray, C.B., Norris, D.J. & Bawendi, M.G. Synthesis and characterization of nearly monodisperse CdE (E=S, Se, Te) semiconductor nanocrystallites. *J. Am. Chem. Soc.* **115**, 8706–8715 (1993).
6. Peng, Z.A. & Peng, X. Formation of high-quality CdTe, CdSe, and CdS nanocrystals using CdO as precursor. *J. Am. Chem. Soc.* **123**, 183–184 (2001).
7. Bruchez, M. Jr., Moronne, M., Gin, P., Weiss, S. & Alivisatos, A.P. Semiconductor nanocrystals as fluorescent biological labels. *Science* **281**, 2013–2016 (1998).
8. Gerion, D. *et al.* Synthesis and properties of biocompatible water-soluble silica-coated CdSe/ZnS semiconductor quantum dots. *J. Phys. Chem. B* **105**, 8861–8871 (2001).
9. Wu, X. *et al.* Immunofluorescent labeling of cancer marker Her2 and other cellular targets with semiconductor quantum dots. *Nature Biotechnol.* **21**, 41–46 (2003).
10. Yu, W.W. *et al.* Forming biocompatible and nonaggregated nanocrystals in water using amphiphilic polymers. *J. Am. Chem. Soc.* **129**, 2871–2879 (2007).
11. Dubertret, B. *et al. In vivo* imaging of quantum dots encapsulated in phospholipid micelles. *Science* **298**, 1759–1762 (2002).
12. Mattoussi, H. *et al.* Self-assembly of CdSe–ZnS quantum dot bioconjugates using an engineered recombinant protein. *J. Am. Chem. Soc.* **122**, 12142–12150 (2000).
13. Wang, Y.A., Li, J.J., Chen, H. & Peng, X. Stabilization of inorganic nanocrystals by organic dendrons. *J. Am. Chem. Soc.* **124**, 2293–2298 (2002).
14. Pons, T., Uyeda, H.T., Medintz, I.L. & Mattoussi, H. Hydrodynamic dimensions, electrophoretic mobility, and stability of hydrophilic quantum dots. *J. Phys. Chem. B* **110**, 20308–20316 (2006).
15. Choi, H.S. *et al.* Renal clearance of quantum dots. *Nat. Biotechnol.* **25**, 1165–1170 (2007).
16. Clapp, A.R. *et al.* Fluorescence resonance energy transfer between quantum dot donors and dye-labeled protein acceptors. *J. Am. Chem. Soc.* **126**, 301–310 (2004).
17. Clapp, A.R., Medintz, I.L. & Mattoussi, H. Förster resonance energy transfer investigations using quantum-dot fluorophores. *Chem. Phys. Chem.* **7**, 47–57 (2006).
18. Uyeda, H.T., Medintz, I.L., Jaiswal, J.K., Simon, S.M. & Mattoussi, H. Synthesis of compact mutidentate ligands to prepare stable hydrophilic quantum dot fluorophores. *J. Am. Chem. Soc.* **127**, 3870–3878 (2005).
19. Mei, B.C. *et al.* Modular poly(ethylene glycol) ligands for biocompatible semiconductor and gold nanocrystals with extended pH and ionic stability. *J. Mater. Chem.* **18**, 4949–4958 (2008).
20. Susumu, K. *et al.* Enhancing the stability and biological functionalities of quantum dots via compact multifunctional ligands. *J. Am. Chem. Soc.* **129**, 13987–13996 (2007).
21. Hines, M.A. & Guyot-Sionnest, P. Synthesis and characterization of strongly luminescing ZnS-Capped CdSe nanocrystals. *J. Phys. Chem.* **100**, 468–471 (1996).
22. Dabbousi, B.O. *et al.* (CdSe)ZnS Core-Shell quantum dots: synthesis and characterization of a size series of highly luminescent nanocrystallites. *J. Phys. Chem. B* **101**, 9463–9475 (1997).
23. Reiss, P., Bleuse, J. & Pron, A. Highly luminescent CdSe/ZnSe core/shell nanocrystals of low size dispersion. *Nano Lett.* **2**, 781–784 (2002).
24. Clapp, A.R., Goldman, E.R. & Mattoussi, H. Capping of CdSe-ZnS quantum dots with DHLA and subsequent conjugation with proteins. *Nat. Protoc.* **1**, 1258–1267 (2006).
25. Leatherdale, C.A., Woo, W.-K., Mikulec, F.V. & Bawendi, M.G. On the absorption cross section of CdSe nanocrystal quantum dots. *J. Phys. Chem. B* **106**, 7619–7622 (2002).
26. Sapsford, K.E. *et al.* Surface-immobilized self-assembled protein-based quantum dot nanoassemblies. *Langmuir* **20**, 7720–7728 (2004).