Chapter 5

Encapsulating Quantum Dots in Lipid–PEG Micelles and Subsequent Copper-Free Click Chemistry Bioconjugation

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Abstract

The utility of quantum dots (QDs) for biological applications is predicated on stably dispersing the particles in aqueous media. During transfer from apolar organic solvents to water, the optical properties of the fluorescent nanoparticles must be maintained; additionally, the resulting colloid should be monodisperse and stable against aggregation. Furthermore, the hydrophilic coating should confer functional groups or conjugation handles to the QDs, as biofunctionalization is often critical to biosensing and bioimaging applications. Micelle encapsulation is an excellent technique for conferring hydrophilicity and conjugation handles to QDs. One interesting conjugation handle that can easily be added to the QDs is an azide group, which conjugates to strained alkynes via strain promoted azide–alkyne cycloaddition (SPAAC) reactions. SPAAC, or copper-free click chemistry, utilizes very mild reaction conditions, involves reactive groups that are bio-orthogonal, and is nearly quantitative. Micelle encapsulation is also very mild and preserves the optical properties of the QDs nearly perfectly. The combination of these approaches comprises a mild, effective, and straightforward approach to preparing functionalized QDs for biological applications.

Key words Micelle encapsulation, Strain promoted azide–alkyne cycloaddition, Copper-free click chemistry, Biofunctionalization, Hydrophilic, DSPE-PEG

1 Introduction

Semiconductor quantum dots (QDs) are fantastic fluorophores for a number of biological applications but cannot be used directly as-synthesized, as most QD synthesis reactions are carried out in organic solvents, producing nanoparticles with hydrophobic surface coatings [1–4]. There are two predominant pathways to water solubilizing QDs: copolymer/micelle encapsulation and ligand exchange [1, 5–8]. Encapsulation relies on the self-assembly of hydrophobic domains with the QD's surface-bound, hydrocarbon-based ligands coupled with hydrophilic domains that interact with the solvating water shell [1, 6, 9, 10]. This results in the preservation of the native organic surface ligands and therefore

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largely preserves the QD optical properties, including quantum yield. Ligand exchange involves the displacement of native ligands by new ligands that are capable of binding metal ions on the surface of the QD (e.g., thiols or imidazole rings), while presenting a hydrophilic group to interface with the aqueous solvation layer [5, 11–14]. Ligand exchange tends to reduce the QD quantum yield as the semiconductor surface is disturbed; however, ligand exchange also typically yields particles with smaller hydrodynamic radii than encapsulation [5, 11–16]. In this context, the ideal QD coating is application-dependent. For applications where size is a critical factor, such as Förster resonance energy transfer (FRET)-based sensing or imaging vesicle transport through exceptionally small (~30 nm) synaptic clefts, ligand exchange is typically most appropriate. For many optical imaging applications and ensemble measurements, however, QD brightness and colloidal stability are prioritized over minimizing the hydrodynamic size; in these cases, encapsulation-based transfer into water is preferred [8–18].

One of the most straightforward and gentle encapsulation strategies involves the use of amphiphilic lipid–polyethylene glycol (lipid-PEG) moieties to encapsulate the QD in a micelle. The hydrophobic tail of the lipid interdigitates with the native ligands on the QD surface via van der Waals interactions, while the lipid polar head group and PEG chain orient themselves toward the aqueous media (Fig. 1) [1]. In this way, a micelle self-assembles around the QD, conferring hydrophilicity without affecting the QD surface. The micelle-encapsulated QDs are colloidally stable over a wide range of pH and salt conditions and extended periods of time, so long as the underlying QD itself is stable, that is, not susceptible to dissolution in water [1, 6, 9, 10].

The use of PEG in generating the QD-encapsulating micelle also confers biological "stealth," whereby the QD is shielded from opsonins that would trigger rapid blood clearance through the

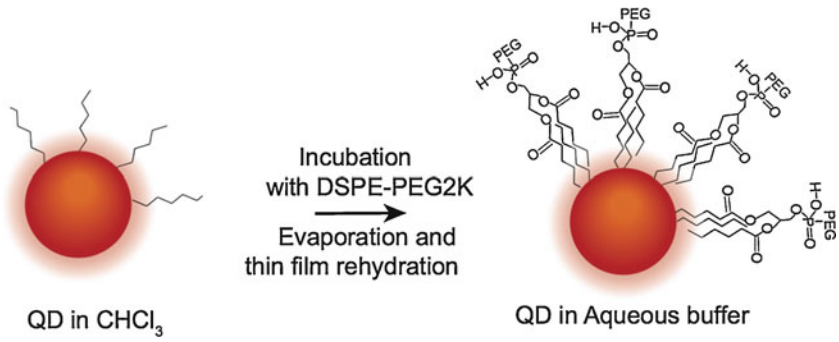

Fig. 1 The nonpolar fatty acid chains interdigitate with native surface ligands present on the QDs through van der Waals interactions. This hydrophobic interaction gives rise to the self-assembly of lipid-PEG with the QDs resulting in micelle formation. The chemical structure of each of the lipid–PEG components used in this system is shown below

mononuclear phagocytic system (MPS). This allows for extended circulation times that are critical to targeted molecular imaging [19–22]. The size of the micelle-encapsulated QDs precludes renal excretion, so most circulating particles are eventually sequestered by uptake in the MPS, mainly in the liver [22]. For specific targeting, derivatization of the PEG terminus imparts chemical handles such as azides, malemides, dibenzocyclooctyne (DBCO), and amines for subsequent bioconjugation reactions. A number of prefunctionalized lipid–PEG moieties are commercially available. The diversity of available chemical handles enables a wide array of conjugation schemes to attach targeting molecules and functional groups to the QDs.

Strain-promoted azide–alkyne cycloaddition (SPAAC; aka copper-free click chemistry) is ideal for QD conjugation due to the biological and chemical orthogonality of azides and strained alkynes as well as the mild conjugation conditions. The reaction is pH agnostic and runs to completion in any azide-free buffer at room temperature (ca. 22 °C) over several hours or at 4 °C over a few days [23]. This ensures that the QD properties are not adversely affected by the conjugation reaction. Furthermore, the high specificity and reactivity of azides with strained alkynes affords high conjugation yields and enables excellent control over the stoichiometry of the functional agent being conjugated (Fig. 2) [24].

In concert, micelle encapsulation and SPAAC chemistry allow for the generation of water-soluble, functionalized QDs that retain their optical properties. These particles also exhibit excellent

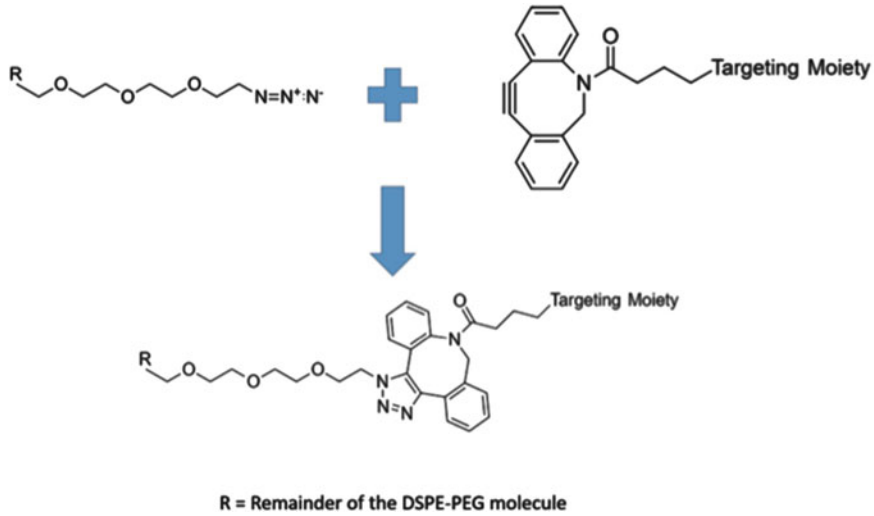

Fig. 2 SPAAC reaction schematic between the azide group at the distal end of the PEG chain and the strained alkyne group conjugated to a targeting moiety resulting in the functionalization of the micelle encapsulated QD

coating biocompatibility with long circulation times that are critical for effective targeting [1, 2, 9, 10, 20].

2 Materials

Prepare and store all reagents at room temperature (ca. 22 °C) unless indicated otherwise. Diligently follow all relevant waste disposal regulations when disposing waste materials.

2.1 Quantum Dot Precipitation

1. Quantum dots (*see* **Note 1**).
2. Microbalance with 0.1 mg precision or better.
3. Microcentrifuge and 2 mL microcentrifuge tubes.

2.2 Quantum Dot Encapsulation in Lipid PEG Micelles

1. Lipid-PEG: 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000] (DSPE-PEG_{2K}) (*see* **Note 2**).
2. Lipid-PEG-azide: 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[azido(polyethylene glycol)-2000] (DSPE-PEG_{2K}-azide) (*see* **Note 2**).
3. Two glass marbles: glass only, no paint or enamel. To prepare marbles, rinse with the following solvent in succession: hexanes, isopropyl alcohol, ethanol, acetone. Rinse well with deionized (DI) water (18.2 MΩ·cm) and place in a 50 mL conical tube. Add 10 mL of DI water to the same 50 mL conical tube (*see* **Note 3**).
4. Rotary evaporator (RotoVap) with vacuum and warming water bath and 100 mL single neck evaporating flask.
5. 0.1 or 0.2 μm pore size syringe filters—polyvinylidene difluoride (PVDF) and polyethersulfone (PES) are acceptable—10- to 30 mm diameter filters are sufficient (*see* **Note 4**).
6. 50 or 100 kDa molecular weight cutoff (MWCO) centrifugal filters sized for either 4 or 15 mL of sample, which fit into 15 and 50 mL centrifuge tubes, respectively.
7. Centrifuge.
8. Ultracentrifuge and appropriate tubes (i.e., ones designed to fit the available rotor, can withstand high g-forces, and are sized to hold ~5 mL).

2.3 Quantum Dot Labeling with Peptide

1. Custom peptide designed for the specific application of interest; peptide must contain a cysteine for labeling (*see* **Note 5**).
2. Dibenzocyclooctyne-maleimide (DBCO-maleimide); maleimides are sensitive to hydrolysis and are thus shipped and stored frozen and/or under inert atmosphere. Purchase

aliquots so the entire container is used immediately once the bottle is opened.

3. Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (*see* **Note 6**).
4. A typical biologically compatible solution such as 0.9% NaCl (saline; prepared by dissolving 0.9 g NaCl in 100 mL deionized water) or buffer like 25 mM HEPES (prepared by dissolving 6.5 g 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid in ~900 mL deionized (DI) water, adjusting pH to 7 with NaOH, and raising volume to 1 L with DI water).
5. Microcentrifuge, 2 mL microcentrifuge tubes, and 1.5 mL siliconized microcentrifuge tubes.
6. 12 and 3 mL polypropylene syringes.
7. UV-Vis spectrophotometer.

3 Methods

3.1 Quantum Dot Precipitation

In order for encapsulation to be successful, QDs must be purified from the raw reaction solution and transferred to chloroform. This step removes high boiling point solvents and organic ligands that would otherwise prevent the formation of a thin film during rotary evaporation. Complete removal of high boiling point contaminants requires two subsequent precipitations and resuspensions. During this step, the mass of the QDs to be encapsulated is also carefully measured.

1. Preweigh and label a 2 mL microcentrifuge tube on microbalance.
2. To a different 2 mL microcentrifuge tube, add 100–200 μL of the QD solution (*see* **Note 1**).
3. Centrifuge at 21,000 × *g* for 5 min to remove aggregates from solution.
4. Decant supernatant into the preweighed 2 mL microcentrifuge tube and add 200 μL of hexanes followed by 800–1600 μL of ethanol (*see* **Notes 7** and **8**).
5. Centrifuge the solution at 21,000 × *g* for 5 min to pellet QDs.
6. Discard the supernatant and invert the microcentrifuge tube on a clean lab tissue to facilitate the removal of solvent (*see* **Note 9**).
7. Redisperse pelleted QDs by adding 200 μL of hexanes to the microcentrifuge tube and shaking or vortexing, as needed.
8. Add sufficient ethanol to cause the solution to become turbid (~800–1600 μL). Repeat **steps 5** and **6**.

9. Weigh the microcentrifuge tube again and calculate the weight of the QD pellet.
10. Add 400 μL of chloroform to the tube containing the QD pellet and vortex to redisperse the QDs.

3.2 Quantum Dot Encapsulation in Lipid–PEG Micelles

Sufficient amounts of lipid-PEG must be added to the QDs to ensure that encapsulation does not result in multiple QDs per micelle. In addition, the ratio of functionalized to nonfunctionalized lipid-PEG dictates the extent of functionalization possible on the final particles; sufficient functionalized lipid-PEG should be used for successful subsequent conjugation to the functionalized QDs, but excesses can be counterproductive and unnecessarily expensive. The amounts of these components used in the coating reaction is determined using weight ratios; specifically, the summed weight of the lipid–PEG moieties (functionalized and nonfunctionalized) should be at least 4.5-fold the weight of the QD pellet. The specific ratio of DSPE-PEG/DSPE-PEG-azide can be tailored based on the degree of functionalization desired. A mass ratio of 1:4:0.5 for QD:DSPE-PEG:DSPE-PEG-azide yields ~50 azide groups per coated QD (after removal of empty micelles) for QDs approximately 5 nm in diameter.

1. To two separate 2 mL microcentrifuge tubes, add the desired weight of DSPE-PEG and the DSPE-PEG-azide, taking care to ensure that the total mass of lipid-PEG is at least 4.5-fold the mass of the QD pellet (*see* **Note 10**).
2. Add 1.5 mL of chloroform to each of the DSPE–PEG and DSPE–PEG–azide powders and vortex until fully dissolved.
3. Add the QDs in chloroform, lipid–PEG/lipid–PEG–azide solutions, and an additional 2 mL of chloroform to a 100 mL single neck evaporating flask, bringing the total volume of chloroform to approximately 5.5 mL. Let sit for 5 min.
4. Place tube with two clean glass marbles and 10 mL DI water in the RotoVap water bath to warm to 70 °C.
5. Connect the 100 mL single neck evaporating flask containing the QD and lipid–PEG solution to the RotoVap and set it to rotate at 180 rpm or faster. Lower into water bath warmed to 70 °C.
6. Dry the solution by pulling vacuum for 20 min, ensuring that all chloroform is removed and the flask is warmed to 70 °C (*see* **Note 11**).
7. Release the vacuum, unmount the 100 mL single neck evaporating flask, and very quickly add the marbles and DI water to the flask. Starting immediately, swirl the flask vigorously, rapidly, and continuously for 5 min, taking care to expose as much of the flask surface to the marbles as possible.

8. Using a 12 mL syringe with a long needle, withdraw the aqueous QD solution from the flask.
9. Carefully remove the needle from the syringe and syringe filter the aqueous QD solution into a 50 or 100 kDa MWCO centrifugal filter device.
10. Using the centrifugal filter, exchange the full volume of DI water with fresh DI water by centrifuging at the manufacturer recommended $\times g$ to remove the supernatant water; discard the filtrate and add 5 mL of DI water to the top of the filter. Repeat the exchange. After the second filtration step, collect the QDs from the top of the filter in 5 mL or less of DI water.
11. Transfer the aqueous QD solution to an appropriate tube for the ultracentrifuge (*see* **Note 12**). Using an ultracentrifuge, spin the aqueous QD solution for 80 h or more at 159,372 $\times g$ and 4 °C.
12. Extract the centrifuge tube from the rotor and carefully discard the supernatant using a pipette (*see* **Note 13**).
13. Once the supernatant has been removed, gently add 1 mL of DI water using a pipette. Taking care not to touch the pellet with the pipette tip, gently wash the pellet by pipetting the water up and down. Take care to not disrupt the pellet. Remove and discard the water used to wash the pellet (*see* **Note 14**).
14. Add 1 mL of DI water to the centrifuge tube and repeat the washing procedure from **step 13** with slightly more vigor. Repeat the washing three more times, for a total of five times, or until the pellet starts to liquefy, increasing the vigor of wash each time.
15. Once the pellet is washed, add fresh DI water to a volume of ~2 mL and vigorously agitate the pellet by pipetting forcefully in close vicinity to the pellet (*see* **Note 15**).
16. Using a 3 mL syringe with a long needle, draw the aqueous QD solution into the syringe.
17. Carefully remove the needle from the syringe and affix a syringe filter. Filter the QD solution into a 100 kDa MWCO centrifugal filter and exchange the DI water for the desired buffer solution.

3.3 Quantum Dot Labeling with Targeting Peptide

Terminal azides confer bio-orthogonal, stoichiometric functionalization handles to micelle encapsulated QDs. These handles can be used to conjugate any moiety with a strained alkyne to the azides in the QD coating utilizing strain-promoted azide–alkyne cycloaddition (SPAAC), also known as copper-free click chemistry. The versatility of this functionalization technique allows for the conjugation of everything from antibodies and peptides to small molecules and even DNA to the nanoparticle surface [25–31]. The

degree of functionalization can be determined using UV-Vis spectroscopy and analysis of the resulting absorbance plots. The protocol below outlines how a small peptide with a terminal cysteine is conjugated to QDs using SPAAC and thiol-Michael addition reaction chemistry (i.e., a maleimide conjugation reaction).

1. Determine the QD concentration using UV-Vis spectroscopy and the molar extinction coefficient equations appropriate to the composition of QD being used (*see* **Notes 16** and **17**). Use the sample volume and QD concentration to determine the moles of QDs to be used in the reaction. Aliquot these QDs from the lipid-PEG coated QD stock solution into a microcentrifuge tube. Adjust the pH of the sample to 6.5 by adding small volumes (1–10 μL) of low molarity (<1 M) HCl or NaOH solutions to the sample. Alternatively, pH can be adjusted by buffer exchanging with a solution at pH 6.5 using centrifugal filtration devices.
2. Plan the reaction: for this application, how many peptides are desired per QD? The reaction requires multiple reagents in particular molar ratios to one another. Specifically, if an average labeling of n peptides per QD is desired, the reaction will use a $1{:}n{:}3n{:}45n$ molar ratio of QD:DBCO-maleimide:peptide:TCEP. For example, if 0.1 nmol of QDs will be labeled with ~10 peptides per QD, the reaction will require 0.1 nmol QD, 1 nmol DBCO-maleimide, 3 nmol peptide, and 90 nmol TCEP (*see* **Notes 18** and **19**). We will use this example reaction for the rest of the protocol, but the total amount of QDs reacted as well as the peptide ratio can be adjusted as needed.
3. Prepare a solution of 0.3 nmol peptides in 200–450 μL aqueous solution in a 1.5 mL siliconized microcentrifuge tube. Adjust to pH 6.5 (*see* **Note 20**).
4. To the peptide solution, add a 30-fold molar excess (i.e., 9 nmol) of TCEP. Mix for 20 min at room temperature (ca. 22 °C).
5. Dissolve 0.1 nmol of DBCO-maleimide in 5–10 μL DMSO.
6. Add the QDs to the peptide in the 1.5 mL siliconized centrifugal tube along with a micro stir bar. Ensure that the volume of the aqueous solution of QD + peptide is at least 50× the volume of the DBCO–maleimide solution in DMSO. It is important to ensure that the DMSO content of the solution is ≤2% by volume (*see* **Note 21**); add more aqueous buffer if needed. Add the DBCO–maleimide solution in DMSO to the QD+ peptide reaction solution.

7. Stir the reaction solution for 48–72 h at 4 °C if there is a concern regarding peptide stability, or 4–6 h at room temperature (ca. 22 °C).
8. Transfer the reaction solution to a 100 kDa MWCO centrifugal filter and buffer exchange 3–5 times with the desired buffer (e.g., 0.9% saline or 1× HEPES buffer).
9. Peptide loading can be determined by analyzing blank-corrected UV-Vis absorbance spectra of the QD, peptide, DBCO, and the QD–peptide conjugate. Once the concentrations of the individual components are known, the ratio of the peptide and QD concentrations indicates the average number of peptides per QD (*see* **Note 21**). This value is not constant for all individual QDs, but rather is assumed to be the center of a Poissonian distribution of the number of peptides per QD.
10. Sterile filter using a 0.1 or 0.2 μm syringe filter prior to biological applications.

4 Notes

1. This protocol can be used with QDs from a wide variety of sources including in-house, lab-made QDs of any number of compositions (CdSe, InP, etc.) capped with a ZnS shell or commercially available QDs sold in organic solvents. The key QD characteristics critical to successful encapsulation are (1) a ZnS capping shell, as this passivation layer ensures that they are still photoluminescent once transferred into water, (2) hydrophobic QDs soluble only in apolar organic solvents, as the hydrocarbon-based ligands on the surface of these QDs are integral to successful lipid–PEG encapsulation, and (3) sufficient surface coverage of organic ligands on the QD surface. In the event that an encapsulation is not successful, incubate QDs in oleic acid for 30 minutes and reprecipitate the QDs prior to encapsulation.
2. There are multiple commercial sources of the lipid-PEG used for the micelle encapsulation available, comprising a number of different PEG chain lengths and functional handles. We have tested several suppliers and specific products; our experience with impure or less charaterized lipid-PEG and the consequent negative results is not exhaustive but demonstrates the importance of high quality/high purity lipid-PEG for the success of the encapsulation procedure. Multiple DSPE-PEG products from one supplier did not come with a stated or guaranteed purity, appeared less pure (clumpy off-white powder), and proved inadequate for the QD coating: QDs were successfully transferred into the aqueous phase, but appeared

cloudy and became trapped on the syringe filters, indicating that a stable colloidal solution had not been formed. In contrast, multiple discrete DSPE-PEG products from Avanti Polar Lipids have been used with consistent success in our lab. Notably, the shipped product is a fine white powder and the specifications sheet indicates >99% purity. DSPE-PEG from multiple suppliers should work well in this procedure, but when problems arise, the source and purity of the DSPE-PEG should be considered during troubleshooting.

3. When cleaning the marbles, it is important that there are no residual organic solvents on the marbles.
4. The size and type of the syringe filter can be varied depending on the cloudiness (turbidity) of the resulting solution. Since the lipid-to-QD ratio is weight-based, there are occasionally significant amounts of unused lipid present, facilitating the formation of large empty micelles that scatter light. In these instances, it is best to use a 45 mm filter to prevent significant backpressure when filtering.
5. The custom designed peptide should only include a single cysteine (Cys) group at the end of the amino acid chain away from the targeting region for uniform peptide conjugation and full accessibility of the peptide on the QD-conjugate surface. To ensure that the conjugation does not affect the function of the peptide, a spacer group of two to four amino acids, for example glycine-glycine-serine (Gly-Gly-Ser), designed for peptide flexibility and solubility, should be inserted between the cysteine group and functional peptide sequence. Inclusion of a tryptophan (Trp) or tyrosine (Tyr) is extremely useful as the native absorptivity of these amino acids can be used for UV-Vis absorption-based concentration determinations. Trp is preferred as it exhibits a higher molar extinction coefficient ($\varepsilon_{280\text{nm}} = 5690\ \text{M}^{-1}\ \text{cm}^{-1}$) than Tyr ($\varepsilon_{280\text{nm}} = 1280\ \text{M}^{-1}\ \text{cm}^{-1}$). Peptides are sensitive to repeated freeze thaw cycles and should be either purchased in aliquots or immediately dissolved in the manufacturer recommended solvent, aliquoted in the smallest useful volume, and frozen until needed.
6. TCEP cleaves disulfide bonds that form between cysteines in the peptide so the thiols are available for maleimide conjugation; while many chemicals can be used to reduce disulfide bonds, most are themselves thiols and would thus have to be removed prior to the maleimide reaction to avoid cross-reactivity. TCEP is preferred, as it is sulfur-free and thus does not have to be removed prior to the downstream steps. TCEP can be added directly to the peptide solution if it is already in

aqueous form. Once opened, TCEP must be stored under inert atmosphere and frozen to avoid oxidation.

7. Although many labs, including ours, have wash bottles of commonly used solvents available for convenience, we find that using ethanol freshly aliquoted from the reagent bottle has a significant effect on maintaining the brightness of the final QD product. Ethanol is hygroscopic and will draw water out of the air, gradually contaminating the solvent.
8. When adding ethanol to precipitate QDs, ensure that enough ethanol is added for the solution to become turbid: this indicates that QDs are flocculating out of solution. If the sample remains transparent following the addition of a large excess of ethanol, add a drop or two of methanol. The addition of the more polar alcohol reduces the solubility of the QDs, facilitating flocculation.
9. Blotting tubes containing precipitated QD pellets upside down on lab tissues longer than 5 min is not recommended as it hinders resuspension; proceeding in a timely fashion is important.
10. For example, if the QD pellet (after subtracting the weight of the tube) is 4.1 mg, then one would use 2.05 mg DSPE-PEG-azide and 16.4 mg DSPE-PEG. When weighing is challenging, err on the side of using a little extra lipid-PEG rather than not enough.
11. A film should form on the inside of the evaporating flask. If the film appears oily, the oily solvents and coordinating ligands were not completely removed from the QD solution in the precipitation step; this will result in aggregated particles that will not pass through the syringe filter in subsequent steps and requires starting the procedure again. Although the chloroform will evaporate below 70 °C in this low-pressure condition, warming plays a helpful role in arranging the lipid–PEG chains around the QDs and is important to the success of the coating procedure.
12. Ultracentrifuges are very sensitive to weight and require training to use. Please take care to undergo training prior to use and ensure that weights of all tubes and balances used are measured and very nearly equal. When loading the ultracentrifuge tubes, it is important to ensure that there is no empty head space in the centrifugal tube, as this can compromise the integrity of the tube under high g-forces.
13. After ultracentrifugation, it is important that the QDs have formed a solid and resilient pellet at the bottom of the tube. The pellet should not be moving as the tube is manipulated and should remain solid as you pipet out the supernatant. If the pellet is not solid, or appears to mix with the water as you pipet

out the supernatant, place it back in the ultracentrifuge and spin for an additional 36 h. If the tube has to be recentrifuged due to pellet instability after some water has been removed from the tube, be sure to gently add enough water back to fill the tube and reweigh/rebalance the tubes before recommencing centrifugation.

14. Ultracentrifugation is used to separate QD-containing micelles from empty micelles. Most empty micelles will remain in solution after centrifugation, but a small portion forms a dispersible film on the surface of the QD pellet. When washing the film of empty micelles off the surface of the QD pellet, the pellet should remain solid. Some minor mixing is tolerable.
15. Resuspending the QD pellet after pelleting and washing requires vigorous pipetting for extended durations of time, normally on the order of 10–15 min. However, with time the pellet will resuspend without any stability issues.
16. This reaction works for a wide range of QD concentrations; however, the more concentrated the QDs, the more rapid the conjugation. Starting QD concentrations of hundreds of nanomolar to micromolar concentrations tend to yield good results.
17. The referenced review paper from the Reiss group has several pertinent equations for determining the concentration and size from absorbance measurements for common QD systems [32]. To determine concentration from absorbance, the spectral position of the band edge absorbance feature (i.e., 1S peak) is first used to determine the diameter of the QD using the equations referenced. This diameter is then used to determine the molar extinction coefficient using another set of equations also found in the Reiss review. Finally, the Beer–Lambert Law is used to determine the concentration of the QDs using their size- and composition-specific molar extinction coefficient.
18. The QDs can be designed to exhibit an excess of azides on the surface, and the DBCO–azide reaction is nearly quantitative, eliminating the need for excess DBCO in the reaction. The maleimide coupling, however, is nonstoichiometric, so an excess of peptide is used to drive this reaction toward coupling over maleimide hydrolysis. Maintaining a slightly acidic to neutral pH is also critical to ensuring that coupling is preferred over hydrolysis. Following the reaction, excess peptide is easily removed from the QD–peptide conjugates via centrifugal filtration using 100 kDa MWCO filters. TCEP reduces the cysteine thiol group on the peptide in preparation for the thiol-Michael addition reaction. The excess ensures complete reduction and high yield in the maleimide coupling reaction. TCEP does not need to be removed from the conjugation solution.

19. A single-step conjugation reaction has the advantage of not requiring a separate purification of the DBCO–peptide complex, as this can be difficult for small peptides.
20. Any azide-free buffer, including plain DI water, can be used for this reaction. Common options include Tris or HEPES buffers, pH 6.5–7.5.
21. In our experience, the addition of up to 2% DMSO (by volume) to a QD sample does not affect the QD optical properties. It is best not to exceed this percentage of DMSO without first ascertaining the impact on QD photoluminescence.
22. First subtract the contribution of the QD from the QD-conjugate spectrum: normalize two spectra to 400 nm (i.e., a wavelength where the functional groups, namely DBCO and peptide, do not absorb) then subtract the normalized QD spectrum from the normalized QD–conjugate spectrum. The result comprises the absorption contributions of DBCO and peptide to the QD–conjugate spectrum. Fit this spectrum to the sum of the individual spectra of the DBCO and peptide, multiplied by constants to adjust their relative intensities, to determine their respective contributions. Use the extracted spectra to determine the concentrations of the QD (*see* **Note 17**), peptide (*see* **Note 5**), and DBCO ($\varepsilon_{309\text{nm}} = 12{,}000\ \text{M}^{-1}\ \text{cm}^{-1}$) in the QD–conjugate solution.

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