Synthesis of functionalized amphiphilic polymers for coating quantum dots

Dominik Jańczewski[1], Nikodem Tomczak[1], Ming-Yong Han[1,2] & G Julius Vancso[1,3]

[1]Institute of Materials Research and Engineering, A*STAR (Agency for Science, Technology and Research), Singapore. [2]Division of Bioengineering, Faculty of Engineering, National University of Singapore, Singapore. [3]Permanent address: Department of Materials Science and Technology of Polymers, Faculty of Science and Technology, University of Twente, MESA+ Institute for Nanotechnology, Enschede, The Netherlands. Correspondence should be addressed to G.J.V. (g.j.vancso@utwente.nl).

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Quantum dots (QDs) need to be attached to other chemical species if they are to be used as biomarkers, therapeutic agents or sensors. These materials also need to disperse well in water and have well-defined functional groups on their surfaces. QDs are most often synthesized in the presence of ligands such as trioctylphosphine oxide, which render the nanoparticle surfaces hydrophobic. We present a complete protocol for the synthesis and water solubilization of hydrophobic CdSe/ZnS QDs using designer amphiphilic polymeric coatings. The method is based on functionalization of an anhydride polymer backbone with nucleophilic agents. Small functional groups, bulky cyclic compounds and polymeric chains can be integrated into the coating prior to solubilization. We describe the preparation of acetylene- and azide-functionalized QDs for 'click' chemistry. The method is universal and applicable to any type of nanoparticle stabilized with hydrophobic ligands able to interact with the alkyl chains in the coating in water.

INTRODUCTION

We provide a protocol for the synthesis of functionalized water-dispersible semiconductor nanocrystals (quantum dots, QDs), using an amphiphilic copolymer. The copolymer is obtained from the reaction of poly(isobutylene-*alt*-maleic anhydride) with *n*-octylamine. The amphiphilic character of the copolymer stems from the hydrophobic alkyl chains grafted to the polymer backbone in combination with the hydrophilic carboxylic groups[1].

The advent of QDs substantially altered the molecular landscape of bioimaging labels and contributed significantly to advances in biology[2,3], optoelectronics and sensing[4]. Applications for QDs in biology stem from their superior optical properties when compared with organic chromophores[5]. Variation of the synthetic protocol allows one to obtain different batches of QDs made of a single semiconductor material that emit anywhere from the blue to the infrared by tuning simple process parameters such as temperature or reaction time. This is in sharp contrast with traditional chromophores for which the emission properties of a dye are related directly to their molecular structure, and for which unique synthetic protocols are needed for each novel dye family. The narrow emission lines of QDs surpass those of organic chromophores, which usually have long tails toward the lower-energy part of the electromagnetic spectrum. The absorption spectrum of the QDs is broad and the absorption increases toward higher energies. Therefore, large Stokes shifts are possible, and multiplexed detection using QDs is relatively easy. In contrast, multicolor imaging with organic chromophores requires careful selection of different dyes, light sources and optical filters to minimize the overlap between the absorption and emission spectra of different chromophores. Finally, unlike organic chromophores, QDs have low rates of photobleaching, and the emission from QDs can be observed for minutes or hours under constant illumination[6]. High saturation levels also allow the use of high excitation power, a prized property for *in vivo* medical imaging through thick layers of tissues or skin[7].

New generations of QD probes for biological applications require the integration of many different functional groups at the nanoparticles' surfaces. For example, targeting tumors *in vivo* requires biocompatibility, stability under *in vivo* conditions, improved circulation times and the presence of specific molecules that bind to overexpressed biomarkers[8]. Preferably, diagnostic or therapeutic agents should be incorporated into every QD probe.

A stable aqueous dispersion of QDs in water is the primary requirement for the application of QD in the medical and life sciences. Although many methods exist for the synthesis of QDs, high-quality QDs are often obtained through an organometallic route based on the pyrolysis of precursor compounds in the presence of a coordinating solvent. QD solubilization in water can be performed by ligand exchange reactions after[9–11] or during the nanocrystal synthesis[12]. For example, thiols are known to bind to the surface of QDs, and thus ligand exchange with bifunctional thiols having a hydrophilic head group is routinely performed[13].

Stringent stability of the ligand shell is required, which is important to prevent the possible release of toxic species, such as Cd^{2+}, into the solution[14]. Unstable ligand shells also lead to aggregation and precipitation of the QDs, which makes the interpretation of fluorescence images of labeled cells or tissues difficult and ambiguous. Currently, there are several proven and well-researched protocols available for the preparation of water-soluble QDs based on dihydrolipoic acid[15] or PEG-based bidentate ligands for improved stability in biological media[16]. Other methods for QD solubilization without using ligand exchange have been explored, including the formation of a stable silica shell[17,18] and multidentate polymeric[19] or dendrimeric coatings[20,21].

In particular, promising solubilization methods are based on the encapsulation of hydrophobic QDs by amphiphilic molecules without perturbing the original ligands. The encapsulation is driven by hydrophobic interactions between these ligands, such as octyl chains of trioctylphosphine oxide (TOPO), and the hydrophobic parts of the amphiphile. Encapsulation of QDs using hydrophobic-hydrophobic interaction has been demonstrated with small molecules, such as amphiphilic sugar clusters[22] or phospholipids[23,24], for which there are published encapsulation protocols for use in cellular and *in vivo* imaging[25].

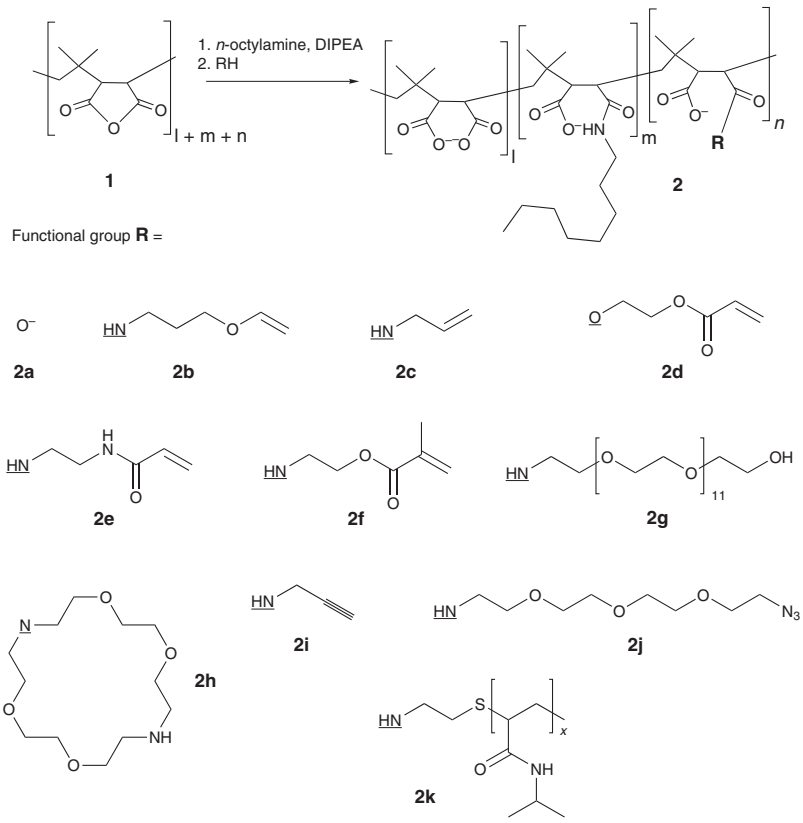

Figure 1 Synthesis of the amphiphilic polymers. The functional groups were attached by the nucleophilic ring opening of the anhydride in the presence of a base catalyst (DIPEA) under mild conditions. The functional groups (R) were attached to the amphiphilic polymer and incorporated in the QD coating. An underline indicates the attachment site. The respective polymers were described in the following publications: **2a**, **2b** in ref. 1; **2c**, **2d**, **2e** in ref. 45; **2f** in ref. 46; **2g**, **2h** in ref. 47; **2i**, **2j** in ref. 43; and **2k** in ref. 42.

Coating of QDs with amphiphilic polymers via hydrophobic interactions is a relatively easy and robust method for rendering the QDs water soluble[8,26–31]. In some cases, shell cross-linking with diamines is performed to increase QD stability in aqueous buffers[26]. The hydrophobic shell around the QDs formed by binding of the hydrophobic parts of the polymer to the hydrophobic QD ligands resist hydrolysis and enzymatic degradation, as shown by many research groups during *in vivo* imaging of cells and animal models[8,32–34]. Octylamide-modified poly(acrylic acid)-coated QDs were also used for labeling of subcellular components[8], angiography[33], *in vivo* imaging in mice[32] and cancer targeting and imaging[30].

Specific functionalization of the polymeric shell is often performed after transferring the QDs into water. The available protocols include, for example, coupling amine-containing biomolecules to COOH groups on the surface of the QDs using a water-soluble activator 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC)[8]. Although this method is very popular, it is expensive and suffers from problems related to the excess of EDC required for good yields and EDC-induced precipitation of QDs[35]. These drawbacks stimulated the exploration of new activating agents based on noncharged and nonpolar carbodiimides[36].

It should be noted that the colloidal stability of the bioconjugated QDs might change after functionalization because the physicochemical properties of the surface are altered. An alternative approach is to integrate the required functionalities directly into the multifunctional amphiphilic polymeric coating before solubilization[37–40].

In this report, we present a simple protocol for the synthesis of hydrophobically capped CdSe/ZnS nanocrystals and their transfer to water using designer amphiphilic polymers[41]. The major advantages of the presented method include the wide availability of the robust, commercially available and cheap polymeric precursors, as well as the simple functionalization of the polymer backbone based on anhydride ring opening with any nucleophilic agent. We also show that the desired functionality can be integrated into the polymeric shell at the time of the coating synthesis. Therefore, there is no need for functionalization reactions after solubilization in water. In addition, no cross-linking is needed to achieve highly stable QD dispersions. The robustness of the amphiphilic coating is demonstrated by hydrophilization of the QDs with amphiphilic polymers bearing attached polymeric chains such as PEG[1], PNIPAM[42] and highly hydrophobic functional ligands[43–45]. The latter feature is unique to this method, as it allows one to obtain hydrophilic nanoparticles functionalized with inherently hydrophobic molecules that are not soluble in water, therefore precluding their attachment after the solubilization. The presented method can also be applied to other types of hydrophobic nanoparticles.

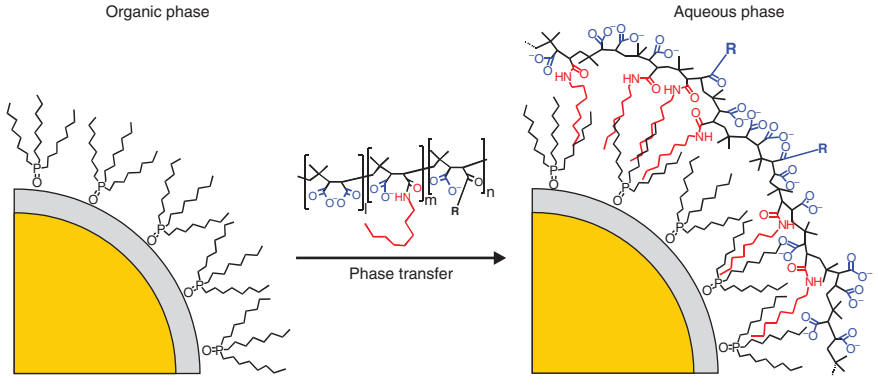

Figure 2 | Scheme of the phase-transfer procedure. The hydrophobic parts of the amphiphile interact with the TOPO coating on the QD surface. The hydrophilic parts, in turn, are directed toward water and induce colloidal stability.

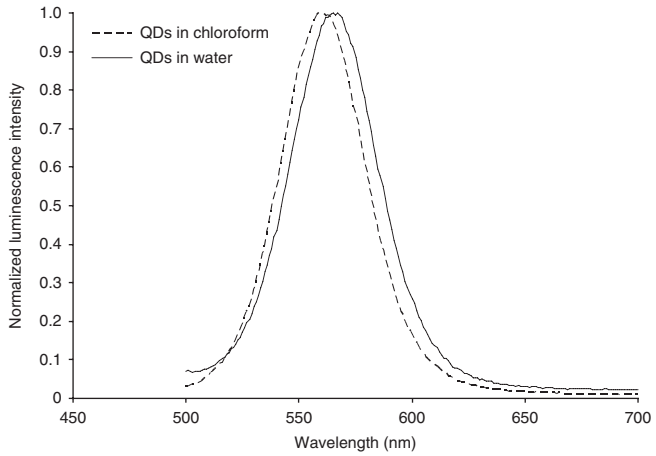

Figure 3 | Emission spectra of the initial QDs in chloroform and in water after the phase transfer using polymer **2i**.

Experimental design

CdSe/ZnS QDs were prepared by pyrolysis of organometallic compounds in the presence of coordinating solvents (TOPO and *n*-hexadecylamine (HDA)). For the QD core synthesis, cadmium stearate and pure selenium powder in trioctylphosphine (TOP) were used as starting materials. The ZnS shell is synthesized immediately after the end of the synthesis of the core using diethyl zinc in TOP as the zinc source, and sulfur in TOP as the sulfur source. This procedure results in hydrophobic QDs covered with TOPO and hexadecylamine ligands. Double purification by centrifugation of the precipitated solution results in clear and transparent QD solutions. An additional centrifugation should be performed before transferring the QDs to water. The amphiphilic polymer was synthesized by the reaction of the poly(isobutylene-*alt*-maleic anhydride) backbone (M_w = 6,000 g mol^{-1}) with *n*-octylamine. The number of hydrophobic *n*-octylamide groups can be tuned to achieve a desired ratio between the hydrophobic and hydrophilic units[1]. A typical amphiphilic polymer described here consists of 40% of repeat units bearing *n*-octylamide groups (index m in **Fig. 1**). Virtually any functional molecule having a nucleophilic anchor for reaction with the anhydride, e.g., amine $-NH_2$ or hydroxyl $-OH$, can be introduced at this stage of the protocol. Attachment of large hydrophobic domains is eventually limited, as the size of the attached molecules may perturb proper folding of the amphiphilic coating around the nanoparticle.

The requirements for the hydrophilic groups are not as stringent. For example, long hydrophilic polymeric chains of M_w = 25,000 g mol^{-1} attached to an amphiphilic backbone were also successfully used to transfer the QDs into water[42].

The water transfer of the QDs was performed by mixing a water solution of the amphiphilic polymer and a tetrahydrofuran (THF) suspension of the purified nanocrystals. Wrapping of the QDs with the polymeric micelles was induced by evaporation of THF and later by slowly evaporating water at temperatures below 10 °C (**Fig. 2**). Formation of QD/polymer assemblies can be clearly observed by monitoring the turbidity of the solution, as the aqueous phase becomes clear, transparent and luminescent under UV excitation. The resulting assemblies have a narrow size distribution, and the luminescent properties of the original nanocrystals are largely preserved. (**Figs. 3** and **4**)

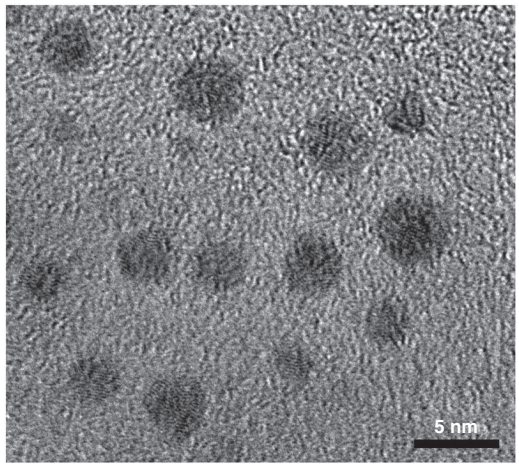

Figure 4 | Transmission electron microscope image of the QDs transferred into water and encapsulated by the amphiphilic polymeric micelle.

MATERIALS

REAGENTS

- Cadmium oxide (CdO; Aldrich, cat. no. 202894)
- Selenium (Aldrich, cat. no. 229865)
- Sulfur (Aldrich, cat. no. 344621)
- Diethyl zinc (Et_2Zn) 1M/heptane (Aldrich, cat. no. 406023)
- *n*-Hexadecylamine (HDA; Aldrich, cat. no. H7408)
- Trioctylphosphine (TOP; Aldrich, cat. no. 117854)
- Trioctylphosphine oxide (TOPO; Aldrich, cat. no. 223301)
- Stearic acid (Aldrich, cat. no. S4751)
- Chloroform ($CHCl_3$; Fluka, cat. no. 25693)
- Methanol (CH_3OH; Aldrich, cat. no. 646377)
- Poly(isobutylene-*alt*-maleic anhydride) (M_w = 6,000, Aldrich, cat. no. 531278)
- THF (TEDIA, cat. no. TS 2123-001)
- *n*-Octylamine (Aldrich, cat. no. O5802)
- Diisopropyl ethyl amine (DIPEA; Aldrich, cat. no. 38320)
- 2-Aminoethyl methacrylate hydrochloride (Aldrich, cat. no. 516155)
- Allylamine (Aldrich, cat. no. 145831)
- Propargylamine (Aldrich, cat. no. P50900)
- 11-Azido-3,6,9-trioxaundecan-1-amine (Aldrich, cat. no. 17758)
- Millipore purified water (e.g., Milli-Q)
- Sodium hydroxide (NaOH)

EQUIPMENT

- A glove box with O_2 and H_2O levels maintained below 1 p.p.m.
- Metal-heating bath created by melting Woods metal alloy (Aldrich, cat. no. 244104) in a stainless steel pot. **! CAUTION** Woods metal alloy contains lead and cadmium. Appropriate safety procedures should be followed.
- Schlenk line
- Rotary evaporator equipped with a diaphragm pump and a condenser capable of working at −10 °C
- Centrifuge
- Freeze dryer
- Dialysis membrane with a molar mass cutoff of 6,000–8,000 kDa (Fisher, cat. no. 21-152-4)

• Disposable syringes
• Millex PES membrane filter (Millipore)

REAGENT SETUP

S/TOP stock solution In a glove box, prepare a 1 M stock solution of S in TOP (S/TOP) by dissolving 0.321 g of S in 10 ml of TOP, and a 1 M stock solution of Se in TOP (Se/TOP) by dissolving 0.790 g of Se in 10 ml of TOP. TOP solutions of reagents can be stored for 12 months at room temperature (glove box) with no degradation.

EQUIPMENT SETUP

QD synthesis setup This consists of a three-neck 100-ml round-bottomed flask equipped with an air condenser, inert gas/vacuum adaptor, septum and a temperature probe.

Polymer synthesis setup This consists of a three-neck 1,000-ml round-bottomed flask equipped with a water condenser, inert gas/vacuum adaptor, septum and a temperature probe.

PROCEDURE

Reagent preparation and purification: synthesis of CdSe/ZnS core shell QDs ● TIMING 5 h

1| At room temperature (25 °C), charge a 100-ml flask fitted with a thermocouple temperature sensor, air condenser, a vacuum adaptor and septum with CdO (0.105 g) and stearic acid (1 g; **Supplementary Figs. 1** and **2**). Dry the reagents under vacuum (0.01 mbar) for 15 min.

2| Switch to a N_2 atmosphere and increase the temperature to 240 °C by immersing the flask in a preheated Woods metal bath with an immersed thermocouple for bath temperature readings. Maintain the reagents at this temperature until all dark brown CdO is dissolved and a transparent colorless Cd stearate is obtained.
? TROUBLESHOOTING

3| Cool the reaction mixture down to room temperature by removing the metal bath, and subsequently add TOPO (12 g) and HDA (7 g).

4| Degas the reaction mixture at room temperature under vacuum (0.01 mbar) for 1 h.

5| Switch to an N_2 atmosphere and raise the temperature to 195 °C. Using a Woods metal bath helps maintain stable temperature in the flask.

6| In a glove box, dilute 0.8 ml of the 1 M Et_2Zn solution with 0.8 ml TOP and transfer the solution to a 5-ml disposable syringe. Subsequently, fill a 1-ml disposable syringe with 0.8 ml of the 1 M S stock solution and a 2-ml disposable syringe with 0.8 ml of the 1 M Se stock solution.

7| Inject all of the Se/TOP solution into the reaction flask in a single fast stroke and wait for 60 s. At this stage, tuning the temperature (180–230 °C) and reaction time (5–60 s) allows one to obtain QDs with luminescence emission from 520 to 640 nm.

8| Inject 0.2 ml of S/TOP, wait for 5 s and subsequently inject 0.4 ml of Et_2Zn/TOP; then wait for 5 s and repeat the two steps three times (or until the solutions are depleted).

9| Cool the solution down to room temperature, divide it into four portions of equal volume and transfer them into four 50-ml centrifuge tubes.

10| Add 15 ml of $CHCl_3$ to each of the tubes and dissolve all contents by warming up to ~40 °C using a water bath or a hair dryer.

11| Add 15 ml of methanol to each tube and centrifuge the content at 9,000*g* for 30 min at room temperature (25 °C).
? TROUBLESHOOTING

12| Discard the supernatant and repeat Steps 10 and 11 using the same amounts of fresh solvents.

13| Dissolve the CdSe/ZnS nanoparticles in 20 ml of fresh $CHCl_3$.
■ **PAUSE POINT** A solution of QDs in $CHCl_3$ in a flask flushed with N_2 can be stored in the refrigerator (dark, 4 °C) for 12 months without marked loss of properties.

Reagent preparation and purification: synthesis of amphiphilic polymer for coating of QDs ● TIMING 16 h

14| Add 2 g of poly(isobutylene-*alt*-maleic anhydride; M_w = 6,000 g mol^{-1}) to a 1,000-ml three-neck flask fitted with a thermocouple temperature sensor, air condenser, vacuum adaptor and septum.

15| Degas the polymer under vacuum for 1 h and switch to an N_2 atmosphere.

16| Through the septum, add 500 ml of dry THF, 1.7 ml of DIPEA and 0.8 ml of *n*-octylamine.

17| Increase the temperature to 60 °C using an oil heating bath and stir vigorously for 1 h.

18| This step can be performed using option A or option B. Option A is specific to the example synthesis (polymer **2i**, bearing acetylene functional groups). Option B is a general procedure.

(A) Specific synthesis

(i) Lower the solution temperature to 30 °C and add 0.23 ml of propargylamine.

(B) General synthesis

(i) Lower the solution temperature to 30 °C and add any functional RH component (as listed in **Fig. 1**). The typical amount of the functional unit added at this step is not higher than 25 mol% with respect to the repeating unit of the polymeric anhydride used in Step 14. For details regarding polymers with functional groups, please see corresponding references listed in **Figure 1**.

19| Continue to stir at 30 °C for the next 12 h.

20| Evaporate THF and DIPEA using a rotavap for ~1 h. Do not exceed 30 °C in the heating bath. The following stage of polymer purification was carried out without storing the material.

Reagent preparation and purification: purification of polymer for coating of QDs ● TIMING 1 week

21| Dissolve the solid residue in 40 ml of water and add 13 ml of 1 M NaOH.

22| Evaporate the sample until dry using a rotavap. Do not exceed 30 °C in the heating bath.

23| Dissolve the residue in 40 ml of water and transfer the solution into dialysis tubes (6,000–8,000 kDa).

24| Immerse the dialysis tubes in 2 liters of deionized water and 1 ml of 1 M NaOH and dialyze for 12 h.

25| Dialyze three times against a diluted NaOH solution and three additional times against clean water, replacing the solution each time.

26| Freeze-dry the solution from the dialysis tubes in a freeze dryer.

■ **PAUSE POINT** The polymeric backbone and *n*-octylamide groups are highly stable. The overall polymer stability is limited only by the functional groups introduced. The polymer described in Steps 18–26, featuring acetylene functional groups, can be stored as a dry crystalline powder in a closed vial flushed with nitrogen at 4 °C for 12 months without visible changes in the NMR spectrum.

Suspension of hydrophobic QDs in water with an amphiphilic functional polymer ● TIMING 6 h

27| Prepare an aqueous solution of the polymer obtained in Step 26 by dissolving 20 mg of dry polymer in 10 ml of water.

28| In a centrifuge tube, place 1 ml of the solution obtained in Step 13 (~10 mg of QDs) and add 1 ml of CH_3OH.

29| Centrifuge the content at 9,000*g* for 60 min at room temperature (25 °C).

30| Discard the supernatant and dissolve the remaining solid in 20 ml of THF.

31| Transfer the solution to a 100-ml round-bottomed flask and add the solution prepared in Step 27.

32| Use a rotary evaporator to evaporate the THF at room temperature. Do not immerse the flask in the water bath.

33| After the evaporation of ~80% of the initial THF amount, the remaining solution becomes turbid. Stop the evaporation and add 30 ml of water.

? TROUBLESHOOTING

34| Continue with a slow evaporation of the water from the flask until the solution becomes clear and transparent (usually 2–3 h). Do not immerse the flask in the water bath; allow the flask to cool down to ~5 °C (vacuum ~0.5 mbar).
? TROUBLESHOOTING

▲ **CRITICAL STEP** During this operation, the residues of THF are evaporated and the amphiphilic polymer is tightly wrapped around the hydrophobic QD. A good indicator of the progress is the solution turbidity, which disappears when larger aggregates are well suspended.

35| Filter the solution obtained in Step 34 through a 0.22-µm Millex PES membrane filter and subsequently through a 0.10-µm PVDF hydrophilic filter.
■ **PAUSE POINT** In this form, the solution can be stored at 4 °C for 6 months without losing colloidal stability and fluorescent properties.

36| (Optional) The solution can be concentrated by removing water with a rotavap. Concentrating this solution to a volume of 1 ml (~20 mg ml^{-1}) will not affect the stability.

Solution purification, removal of excess polymer (optional) ● **TIMING 2 h**
37| Place 1 ml of the solution obtained in Steps 35 or 36 in a 1.5-ml centrifuge tube. Subsequently, add 20 µl of 1 M NaOH.

38| Centrifuge the contents at 25,000*g* for 120 min at room temperature.

39| Discard the supernatant and resuspend the solid residue in pure water by shaking.
■ **PAUSE POINT** The solution in this form can be stored at 4 °C for 1–2 months without losing colloidal stability or fluorescent properties.

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
2	The resulting Cd stearate is yellowish	Contaminated stearic acid	Use fresh stearic acid of high purity
11	White powder forms during first centrifugation	Precipitation of capping agents	Use higher amount of $CHCl_3$ versus methanol or carry out centrifugation at higher temperature (use 20% more $CHCl_3$; or centrifuge at 30–40 °C instead of room temperature)
33	The QD luminescence is partially quenched	THF influence	All operations using THF must be carried out quickly. (Steps 30–33 should be done within a total of 5–10 min)
34	Polymer is not effective in suspending the nanoparticles in water (only for the custom-made polymers as described in Step 18B)	Proper hydrophobic/hydrophilic balance of the amphiphilic polymer is not maintained	In the case of hydrophobic functional groups, their total molar ratio versus the number of repeat units of the anhydride backbone should not exceed 65% (m + n / l + m + n). This can vary depending on the character and size of the functional group

● **TIMING**
Steps 1–13, Reagent preparation and purification—synthesis of CdSe/ZnS core shell QDs: 5 h
Steps 14–20, Reagent preparation and purification—synthesis of amphiphilic polymer for coating of QDs: 16 h
Steps 21–26, Reagent preparation and purification—purification of polymer for coating of QDs: 1 week
Steps 27–36, Suspension of hydrophobic QDs in water with an amphiphilic functional polymer: 6 h
Steps 37–39, (Optional) Solution purification, removal of excess polymer: 2 h

ANTICIPATED RESULTS
This protocol describes the synthesis of hydrophobic CdSe/ZnS QDs and their solubilization in water using an amphiphilic polymeric coating bearing acetylene functional groups. Such acetylene groups are one of the basic reactants in click

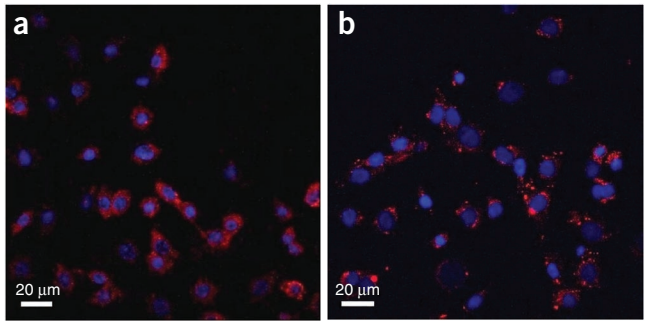

Figure 5 | Fixed and live samples of mammalian cancer cells C-6 imaged with red light–emitting QDs coated with polymer **2a**; the cell nucleus was stained blue with 4,6-diamidino-2-phenylindole. (**a**,**b**) The fixed cells are shown in **a** and the live cells are shown in **b**. Cells were incubated with QDs for 1 h and then washed to remove the excess of free nanocrystals. Images revealed that QD-polymer assemblies were internalized by endocytosis. Adapted from reference 1 with permission from Elsevier.

chemistry. We also expanded the protocol from this particular case to a general fabrication method of functional QD/polymer assemblies for many other chemical functional groups, as shown in **Figure 1** and its legend.

The procedure listed in Steps 1–13 results in bright QDs emitting at 560 nm and having a narrow emission spectrum with a full-width at half-maximum of 26 nm. Changing the temperature and/or time of the synthesis, as described in this protocol, allows one to obtain nanocrystals with emission maxima ranging from 520 to 640 nm.

The QDs/polymer assemblies resulting from this protocol largely maintain the optical properties of the hydrophobic nanocrystals upon transfer into water. Transmission electron microscopy provides evidence that there is no aggregation present. Following the particular conditions described in this protocol (560 nm QDs and an acetylene-functionalized polymer) results in almost quantitative transfer of hydrophobic QDs into water.

The effective procedures described herein allow one to introduce a wide range of functional groups at the stage of the polymer synthesis. For example, we introduced a class of polymerizable groups at the surface of the QDs, which are of interest in the field of materials science[4]. In principle, any water-stable chemical entity with nucleophilic character can be introduced by its reaction with the polymeric anhydride.

The important advantages of the presented protocol are: the ability to introduce hydrophobic functional groups onto the surface of water-soluble QDs; the lack of a cross-linking step at the end of the procedure; easy control over the number of functional units introduced into the polymeric coating; control over the number of hydrophobic *n*-octyl chains, and hence over the hydrophilic/hydrophobic balance of the polymeric coating; and the lack of a carbodiimide (dicyclohexylcarbodiimide (DCC) or EDC) coupling step.

The polymer-coated QDs display good colloidal stability in water, which is sufficient for many proposed applications, such as in cell imaging. We carried out successful cell imaging for the mammalian cancer cells C-6 (**Fig. 5**; ref. 1).

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COMPETING FINANCIAL INTERESTS The authors declare no competing financial interests.

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