

Enhancing the Stability and Biological Functionalities of Quantum Dots via Compact Multifunctional Ligands

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Abstract: We have designed and synthesized a series of modular ligands based on poly(ethylene glycol) (PEG) coupled with functional terminal groups to promote water-solubility and biocompatibility of quantum dots (QDs). Each ligand is comprised of three modules: a PEG single chain to promote hydrophilicity, a dihydrolipoic acid (DHLLA) unit connected to one end of the PEG chain for strong anchoring onto the QD surface, and a potential biological functional group (biotin, carboxyl, and amine) at the other end of the PEG. Water-soluble QDs capped with these functional ligands were prepared via cap exchange with the native hydrophobic caps. Homogeneous QD solutions that are stable over extended periods of time and over a broad pH range were prepared. Surface binding assays and cellular internalization and imaging showed that QDs capped with DHLLA-PEG-biotin strongly interacted with either NeutrAvidin immobilized on surfaces or streptavidin coupled to proteins which were subsequently taken up by live cells. EDC coupling in aqueous buffer solutions was also demonstrated using resonance energy transfer between DHLLA-PEG-COOH-functionalized QDs and an amine-terminated dye. The new functional surface ligands described here provide not only stable and highly water-soluble QDs but also simple and easy access to various biological entities.

Introduction

Semiconductor nanocrystals (quantum dots, QDs) and metallic nanoparticles have attracted a tremendous interest in the past two decades. This interest has been driven by both the potential for use in developing a variety of technological applications, ranging from electronic devices to biotechnology, and a strong desire to better understand the fundamental properties of such materials.^{1–5} Luminescent QDs (such as those made of CdSe–ZnS core–shell nanocrystals), in particular, have generated an increasing interest for use in developing bio-oriented applications. This interest is motivated by the QDs' remarkable photochemical stability and several unique properties, including (i) size-dependent broad absorption, (ii) very high extinction coefficients, (iii) readily size-tunable narrow emission, and (iv) high fluorescence quantum yield.^{1,2,6} Such features offer several advantages for use in biological assays and imaging. Their

successful integration in biotechnology necessitates the preparation of water-soluble QDs that are highly luminescent, stable over a broad range of biological conditions, and compatible with simple conjugation techniques. High-quality QDs (nanocrystals having low size distribution and high fluorescence quantum yields) are synthesized using high-temperature solution reaction from organometallic precursors and are capped with hydrophobic organic ligands made of a mixture of trioctylphosphine/trioctylphosphine oxide (TOP/TOPO) and alkyl amines.^{6–8} Alternative approaches using co-precipitation in aqueous solution (and even in olive oil) have been reported, but additional work is still needed to bring these materials to par with those made using high-temperature reactions.^{9,10} QDs capped with the native ligands are not soluble in aqueous solutions, and thus further surface modification is required to make them biocompatible. Various methods have been reported for achieving water-solubility of such materials,^{3,4} including silica coating,^{11,12} encapsulation of the native TOP/TOPO-capped QDs within

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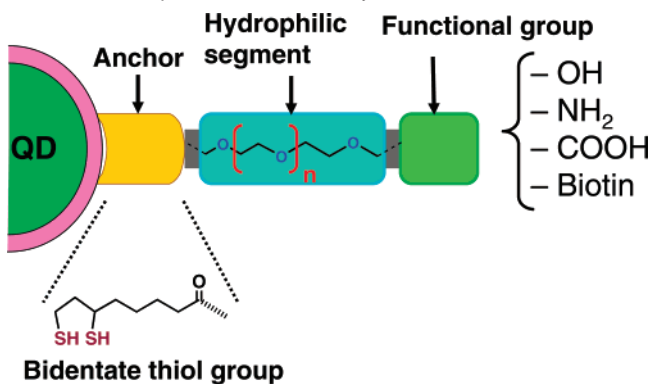
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amphiphilic polymer shells¹³ or lipid micelles,¹⁴ and exchanging the native caps with hydrophilic ligands.^{15–18} Among those reported strategies, cap exchange with bifunctional ligands is relatively simple and has a strong potential for providing QDs that are small in size. Compactness of hydrophilic QDs has distinct advantages in targeted studies, including the ability to realize efficient fluorescence resonance energy transfer (FRET)-based sensing with minimal QD-to-acceptor dye ratios^{19,20} and use in cellular uptake and imaging.^{3,21}

We have previously used dihydrolipoic acid (DHLA) as surface ligands for cap exchange to prepare water-soluble QDs.¹⁶ The DHLA-capped QDs are easily dispersed in basic buffer solutions and are stable over extended periods of time (a year and longer). The enhanced stability is attributed to the bidentate chelate effect afforded by the dithiol groups at the end of the ligand, as compared to monothiol-terminated ligands.^{3,16} However, while the DHLA-capped QDs are stable in basic buffers, they tend to slowly aggregate under acidic conditions. This feature is attributed to the fact that steric stabilization of these nanoparticles hinges on the deprotonation of the terminal carboxyl groups of DHLA. In order to overcome the pH limitation of DHLA-capped QDs, design of new (bidentate and eventually multidentate) ligands is consequently needed. We have utilized readily available thioctic acid and poly(ethylene glycol)s (PEG) in simple esterification schemes, followed by reduction of the 1,2-dithiolane to produce multigram quantities of PEG-terminated dihydrolipoic acid (DHLA-PEG) capping substrates.²² Promoting dispersion of inorganic nanoparticles in water using PEG has been reported.^{23–26} The cap exchange reactions of TOP/TOPO-capped QDs with these substrates produced water-soluble nanocrystals that are stable over extended periods of time and over a relatively broad pH range, from weakly acidic to strongly basic conditions. Though compact and stable, these ligands still have not allowed easy implementation of common QD bioconjugation techniques, such as avidin–biotin binding and covalent conjugation chemistry (via 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) coupling) due to lack of accessible functionalities at their lateral end.

In this study, we further developed the use of PEG attachment onto thioctic acid (followed by ring opening) to design and

Scheme 1. Modular Design of Hydrophilic Ligands with Terminal Functional Groups Used in This Study



synthesize a series of DHLA–PEG ligands having a variety of functional end groups (biotin, amino, and carboxyl groups), as shown in Scheme 1. Appending functions such as biotin at the end of a PEG chain permits the use of the ubiquitous avidin–biotin binding, whereas availability of amino and carboxyl groups will allow the use of covalent reaction schemes to attach QDs to a variety of bioreceptors.²⁷ In our design, the ligands are modular, and each has a central (tunable length) PEG segment to promote hydrophilicity, a bidentate terminal group for strong anchoring on the QD surface at one end, and a lateral functional group that promotes biological linkage with target biomolecules at the other end. Cap exchange reactions were carried out with these new ligands, and preliminary assays of the water-soluble QDs were performed to verify the enhanced biocompatibility.

Experimental Section

1. Materials. All manipulations were carried out under nitrogen (previously passed through a drying tower filled with anhydrous CaSO₄), unless otherwise stated. Air-sensitive solids were handled in a MBraun Labmaster 130 glovebox (Stratham, NH). Poly(ethylene glycol)s (molecular weight average 400 and 600), triphenylphosphine, thioctic acid, 4-(*N,N*-dimethylamino)pyridine, *N,N'*-dicyclohexylcarbodiimide, and *N*-hydroxysuccinimide were purchased from Acros Organics (Morris Plains, NJ). Sodium azide and biotin were purchased from Alfa Aesar (Ward Hill, MA). Methanesulfonyl chloride was purchased from GFS Chemicals (Powell, OH). Succinic anhydride was purchased from Fluka (Milwaukee, WI). Sodium borohydride was purchased from Strem Chemicals (Newburyport, MA). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and *N*-hydroxysulfosuccinimide (sulfo-NHS) were purchased from Pierce Biotechnology (Rockford, IL) and used as received. Lissamine rhodamine B ethylenediamine dye was purchased from Invitrogen (Carlsbad, CA) and used as received. All the other chemicals (including solvents and phosphate-buffered saline, 0.138 M NaCl, 0.0027 M KCl, PBS) were purchased from Sigma Aldrich and Acros Organics. Tetrahydrofuran (THF) and pyridine were dried over CaH₂. Deuterated solvents employed in all NMR measurements were used as received.

Chemical shifts for ¹H NMR spectra are reported relative to tetramethylsilane (TMS) signal in the deuterated solvent (TMS, $\delta = 0.00$ ppm). All *J* values are reported in hertz. Column chromatography was performed using silica gel (Bodman Industries, Aston, PA; 60 Å, 230–400 mesh). The molar amounts of PEG400 derivatives were calculated by assuming that the repeated number of PEG chain (*n* on the chemical structures) is ~8.

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2. Instrumentation. ^1H NMR spectra were recorded on a Bruker SpectroSpin 400 MHz spectrometer. Electronic absorption spectra were recorded using an HP 8453 diode array spectrophotometer (Agilent Technologies, Santa Clara, CA). Fluorescence spectra were collected using a Spex Fluorolog-3 spectrophotometer (Jobin Yvon Inc, Edison, NJ) equipped with a red-sensitive R2658 Hamamatsu PMT detector. The obtained fluorescence spectra were corrected (to account for the wavelength dependence of the detection efficiency of the PMT) using the spectral output of a calibrated light source supplied by the National Bureau of Standards. FT-IR spectra were measured using a Nicolet Nexus 870 FT-IR spectrometer (Thermo Fisher Scientific, Inc., Waltham, MA).

3. Synthesis and Design. DHLA (1) and DHLA-PEG600 (2). These compounds were synthesized by reduction of 1,2-dithiolane groups of thiotic acid (TA) and TA-PEG600, respectively, with NaBH_4 as previously reported.²² Even though the synthesis of TA-PEG600 was detailed in ref 22, the modified purification procedure we recently developed expanded that scheme and significantly simplified the column chromatography step. Briefly, for workup after DCC coupling, the precipitate was filtered and the solvent evaporated. The residue was mixed with saturated sodium bicarbonate (NaHCO_3) solution and extracted with ethyl acetate (EtOAc) until complete extraction of TA-PEG600 from the aqueous layer. Importantly, excess unreacted PEG600 mostly stays in the saturated NaHCO_3 solution, and TA-PEG600 and TA-PEG600-TA (bis-TA-substituted PEG) are extracted with EtOAc. Such selective extraction of the crude product makes the following column chromatography purification much easier compared with the previous procedure.²² The combined organic layers were dried over Na_2SO_4 and filtered, and the solvent was evaporated. The crude product was chromatographed on silica gel with CHCl_3 :MeOH (15:1), and a pale yellow oil was obtained.

N_3 -PEG400- N_3 . Poly(ethylene glycol) (average MW 400) (72.4 g, ~ 0.175 mol), THF (200 mL), and methanesulfonyl chloride (46.1 g, 0.402 mol) were placed in a 1-L round-bottom flask and cooled to $\sim 0^\circ\text{C}$. Triethylamine (60.0 mL, 0.430 mol) was added dropwise over 30 min. The reaction mixture was gradually warmed to room temperature and stirred overnight. The mixture was then diluted with H_2O (200 mL) and NaHCO_3 (12.5 g, 0.149 mol). Sodium azide (31.0 g, 0.477 mol) was added, and the biphasic reaction mixture was heated to distill off the THF and then refluxed overnight. After cooling, the reaction mixture was extracted five times with CHCl_3 . The combined organic layers were dried over MgSO_4 , filtered, and evaporated to give a pale brown oil. The crude product was chromatographed on silica gel with 20:1 CHCl_3 :MeOH as the eluent; the reaction yielded 73.13 g ($\sim 90\%$). ^1H NMR (400 MHz, CDCl_3): δ 3.62–3.71 (m), 3.40 (t, 4H, $J = 5.0$ Hz). IR (neat): 2868, 2103, 1453, 1115 cm^{-1} .

N_3 -PEG400- NH_2 . N_3 -PEG400- N_3 (32.02 g, $\sim 6.89 \times 10^{-2}$ mol) and 0.7 M H_3PO_4 (150 mL, 0.105 mol) were placed in a 500-mL round-bottom flask and cooled to 0°C in an ice-bath with stirring. A solution of triphenylphosphine (18.18 g, 6.93×10^{-2} mol) in ether (150 mL) was added dropwise under N_2 while temperature was maintained below 5°C . The reaction mixture was then warmed to room temperature and stirred under N_2 for 20 h. The biphasic solution was separated, and the aqueous layer was washed with ether (3×100 mL). KOH (30.0 g, 0.535 mol) was slowly added to the aqueous layer, which was cooled to 0°C with stirring overnight. After filtration, the filtrate was basified with an additional 30.0 g of KOH and cooled using an ice-bath. The mixture was extracted with CHCl_3 (4×100 mL), the combined organic layers were dried over MgSO_4 and filtered, and the solvent was evaporated. The crude product was chromatographed on silica gel with 5:1 CH_2Cl_2 :MeOH as the eluent; 15.51 g of product was obtained (a yield of $\sim 51\%$). ^1H 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): 3379, 2868, 2107, 1598, 1455, 1119 cm^{-1} .

TA-PEG400- N_3 (3). Thioctic acid (2.48 g, 1.2×10^{-2} mol), N_3 -PEG400- NH_2 (5.0 g, $\sim 1.1 \times 10^{-2}$ mol), 4-(*N,N*-dimethylamino)-

pyridine (0.31 g, 2.5×10^{-3} mol), and CH_2Cl_2 (100 mL) were placed in a 250-mL round-bottom flask, cooled to 0°C . *N,N'*-Dicyclohexylcarbodiimide (2.52 g, 1.2×10^{-2} mol) was slowly added under N_2 with stirring. The reaction mixture was stirred at 0°C for 2 h, warmed to room temperature, and further stirred overnight. The mixture was filtered through Celite and rinsed with ethyl acetate. The filtrate was evaporated, and the residue was chromatographed on silica gel with CHCl_3 and then 10:1 CH_2Cl_2 :MeOH as the eluents; 6.37 g of material was obtained (a yield of $\sim 92\%$). ^1H 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): 3328, 3075, 2918, 2867, 2105, 1670, 1540, 1456, 1119 cm^{-1} .

TA-PEG400- NH_2 (4). TA-PEG400- N_3 (3.40 g, $\sim 5.4 \times 10^{-3}$ mol) was dissolved in THF (60 mL) in a 250-mL round-bottom flask. Triphenylphosphine (2.00 g, 7.6×10^{-3} mol) was added, and the reaction mixture was stirred at room temperature for 2 h under N_2 . After H_2O (3.0 mL, 0.17 mol) was added, the reaction mixture was further stirred at room temperature for 3 days under N_2 . The solvent was evaporated, and the residue was chromatographed on silica gel with 10:1 CH_2Cl_2 :MeOH and then 100:20:1 CH_2Cl_2 :MeOH: Et_3N as the eluents; 2.46 g of product was measured (a yield of $\sim 76\%$). ^1H 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): 3298, 3053, 2866, 1666, 1545, 1456, 1109 cm^{-1} .

Biotinyl-*N*-hydroxysuccinimide. *d*-Biotin (5.00 g, 2.05×10^{-2} mol) and *N*-hydroxysuccinimide (2.36 g, 2.05×10^{-2} mol) were dissolved in hot DMF (150 mL) in a 500-mL round-bottom flask with stirring. *N,N'*-Dicyclohexylcarbodiimide (5.50 g, 2.67×10^{-2} mol) was added, and the solution was stirred overnight at room temperature, during which time a white precipitate was formed. The reaction mixture was filtered, and the filtrate was evaporated and triturated with ether. The white precipitate was filtered and washed with ether to give a white powder. The yield was 6.99 g ($\sim 100\%$). ^1H NMR (400 MHz, 1 drop of $\text{DMSO-}d_6$ 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): 3226, 2941, 2918, 2876, 1820, 1789, 1746, 1731, 1708, 1470, 1370, 1217, 1072 cm^{-1} .

TA-PEG400-Biotin (5). To a mixture of TA-PEG400- NH_2 (0.22 g, 3.7×10^{-4} mol), biotinyl-*N*-hydroxysuccinimide (0.122 g, 3.6×10^{-4} mol), and DMF (8.0 mL) was added triethylamine (0.24 mL, 1.7×10^{-3} mol) dropwise, and the reaction mixture was stirred at room temperature for 18 h under N_2 . The reaction mixture was filtered, and DMF was removed in vacuo. The residue was chromatographed on silica gel with 8:1 CH_2Cl_2 :MeOH as the eluent, to yield 0.264 g ($\sim 89\%$). ^1H 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): 3295, 3083, 2928, 2865, 1707, 1645, 1550, 1461, 1108 cm^{-1} .

DHLA-PEG400-Biotin (6). To a solution of TA-PEG400-Biotin (0.56 g, $\sim 6.77 \times 10^{-4}$ mol), EtOH (12 mL), and H_2O (6.0 mL) was added NaBH_4 (0.132 g, 3.5×10^{-3} mol) in small portions, and the reaction mixture was stirred at room temperature for 30 min under N_2 . The reaction mixture was diluted with H_2O and extracted with CHCl_3 (four times). The combined organic layers were dried over MgSO_4 and filtered, and the solvent was evaporated. The residue was chromatographed on silica gel with 10:1 CH_2Cl_2 :MeOH as the eluent; 0.45 g of product was collected (a yield of $\sim 80\%$). ^1H 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): 3296, 3084, 2928, 2865, 2549, 1704, 1688, 1645, 1549, 1461, 1108 cm^{-1} .

TA-PEG400-COOH (7). A solution of TA-PEG400-NH₂ (1.102 g, $\sim 1.83 \times 10^{-3}$ mol) and succinic anhydride (0.313 g, 3.1×10^{-3} mol) in 15 mL of dry pyridine was stirred at room temperature for 26 h under N₂. After the solvent was removed in vacuo, the residue was chromatographed on silica gel with 10:1 CHCl₃:MeOH as the eluent; the reaction provided 0.718 g (a yield of $\sim 56\%$) of product. ¹H NMR (400 MHz, CDCl₃): 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): 3319, 3084, 2922, 2870, 1732, 1633, 1549, 1454, 1109 cm^{-1} .

DHLA-PEG400-COOH (8). A solution of TA-PEG400-COOH (0.502 g, $\sim 7.16 \times 10^{-4}$ mol) in MeOH (10 mL) was cooled in an ice-bath, and NaBH₄ (0.132 g, 3.5×10^{-3} mol) in 4.0 mL of H₂O was added dropwise with stirring. The reaction mixture was gradually warmed to room temperature and stirred for 18 h under N₂. A 1 M HCl solution was added until the reaction mixture reached pH ~ 2 , and the reaction mixture was extracted with CHCl₃ (four times). The combined organic layers were dried over Na₂SO₄, filtered, and evaporated. The residue was chromatographed on silica gel with 8:1 CHCl₃:MeOH as the eluent; 0.38 g (a yield of $\sim 75\%$) of pure compound **8** was collected. ¹H NMR (400 MHz, CDCl₃): 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): 3319, 3082, 2924, 2854, 2553, 1734, 1649, 1547, 1462, 1111 cm^{-1} .

3. Quantum Dot Synthesis and Cap Exchange. CdSe–ZnS core–shell QDs were synthesized using high-temperature reaction of organometallic precursors (e.g., trioctylphosphine selenium (TOP:Se) and cadmium acetylacetonate) in a mixture of TOP/TOPO and hexadecylamine, as described in the literature.^{6,28–30} The QDs are capped primarily with TOP/TOPO ligands and, therefore, are not soluble in aqueous media. Cap exchange of the TOP/TOPO-capped QDs with the newly synthesized ligands was carried out following the procedures described previously.²² Briefly, ~ 50 – 300 mg of TOP/TOPO-capped QDs were precipitated using EtOH or a mixture of methanol/butanol. After centrifugation, the supernatant was discarded. To the precipitate were added ~ 0.5 mL in total of pure or mixed ligands and ~ 0.5 mL of EtOH. The mixture was then heated to 60–80 °C with stirring for several hours. Once homogenized, the sample was then precipitated out with hexane, EtOH, and CHCl₃ mixtures and centrifuged. The supernatant was discarded, and the precipitate was redispersed in water. A few additional cleaning steps using an ultracentrifugal device were used to remove excess ligands for the final dispersion. Concentrations of QD solutions were estimated from size-dependent absorption cross sections as previously described.³¹

4. Surface Binding Assay. The binding capacity of NeutrAvidin-covered 96-well microtiter flat-bottom plates (Pierce Biotechnology) is 60 pmol of biotin per well. A total of 100 pmol of QDs capped with the desired ligand mixtures in 100 μL solutions were added to the well and incubated overnight. After the initial fluorescence signal was measured, the plates were washed three times with PBS buffer solution, and the fluorescence was measured again. The remaining fluorescence intensities following rinsing were compared (normalized) to the highest

value measured, and data were reported as percentage binding of biotin-capped QDs onto NeutrAvidin-functionalized wells.

5. Cellular Uptake of Quantum Dot–Bioconjugates and Imaging. QDs emitting at 540 nm and capped with a mixture of DHLA–PEG600: DHLA–PEG400–biotin (9:1 molar ratio) were utilized for these experiments. One-to-one b-phycoerythrin–streptavidin conjugates (b-PE, 1 mg/mL), Alexa Fluor 647 dye-labeled transferrin (AF647-TF), and DAPI nuclear stain were obtained from Invitrogen (Carlsbad, CA). COS-1 cell lines (ATCC, Manassas, VA) were cultured as previously described.²¹ Cellular internalization experiments were performed in sterile Lab-Tek chambered coverglass wells (Nunc, Rochester, NY). Chambers were coated with 50 $\mu\text{g}/\text{mL}$ fibronectin (Sigma-Aldrich) in sodium bicarbonate buffer pH 8.5, and approximately 2×10^4 cells were seeded into the wells and cultured overnight.

The mixed-surface QD–b-PE conjugates used consist of QDs assembled with b-PE (at 1:1 ratio on average) together with cell-penetrating peptides (CPP) added at a ratio of 25 peptides per QD. The CPP sequence (His₈WGLA(Aib)SGR₈, where Aib is α -aminoisobutyric acid) was appended with a terminal polyhistidine (His₈) tag to facilitate self-assembly on the QDs.²¹ QD–b-PE–CPP bioconjugates were diluted to 75 nM concentration in complete culture medium containing the cells and incubated at 37 °C for 1 h as previously described.²¹ AF647-TF endosome marker (at 40 $\mu\text{g}/\text{mL}$) was also added during incubation. Excess unbound QD conjugates were removed by washing with PBS. Cells were then fixed in 3.7% paraformaldehyde at room temperature for 10 min, washed twice with PBS, and mounted in ProLong Antifade mounting media containing DAPI dye to allow for staining of the cell nuclei.²¹ Cultures were imaged using a total internal reflection fluorescence microscope (Olympus IX-71), and when necessary, split fluorescence images were collected and quantitated with a DualView system (Optical Insights, Tucson, AZ) equipped with a 565-nm dichroic filter. QDs and b-PE were excited at 488 nm, separated with the 565 nm dichroic filter, and then deconvoluted. DAPI fluorescence was detected using a Xe lamp and a DAPI cube (D350/50x for excitation, dichroic 400DCLP, D460/50m for detection). Finally, AF647-TF was excited using the Xe lamp and fluorescence detected using a Cy5 cube (excitation HQ620/60x, dichroic Q660LP, HQ700/75m for detection). Cubes were purchased from Chroma Technology (Rockingham, VT). Differential interference contrast (DIC) images were collected using a bright light source.

6. EDC Coupling. QDs capped with a DHLA–PEG600:DHLA–PEG400–COOH (19:1) mixture (1 nmol), Lissamine rhodamine B ethylenediamine (15 nmol), sulfo-NHS (2.5 μmol), and different amounts of EDC (0, 5, 20, and 50 μmol) were mixed with 0.1 M PBS solution (pH ~ 6.5) in Eppendorf tubes (0.5 mL in total), and each reaction mixture was shaken for 2 h at room temperature. The reaction mixture was loaded on a pre-equilibrated disposable PD-10 desalting gel column (GE Healthcare, Piscataway, NJ) and eluted with the same PBS solution to remove excess unreacted dyes and reagents. Fractions of eluted solution were collected (~ 1 mL each), and the absorption spectrum was measured for each. These solutions/fractions were also loaded onto a microtiter well plate, and the fluorescence spectra were collected using a Tecan Safire plate reader (Tecan US, Research Triangle Park, NC).

Results and Discussion

Synthesis and Characterization. The chemical structures and synthetic schemes of a series of DHLA and DHLA–PEG capping substrates are summarized in Figure 1. The synthesis of our modular DHLA–PEG–FN ligands (where FN designates the terminal function of interest) was carried out following a stepwise approach. Poly(ethylene glycol) of the desired length (in our case here PEG400) was first transformed into diazide-terminated PEG using a two-step reaction with methanesulfonyl chloride and sodium azide. Monosubstitution of one azide into

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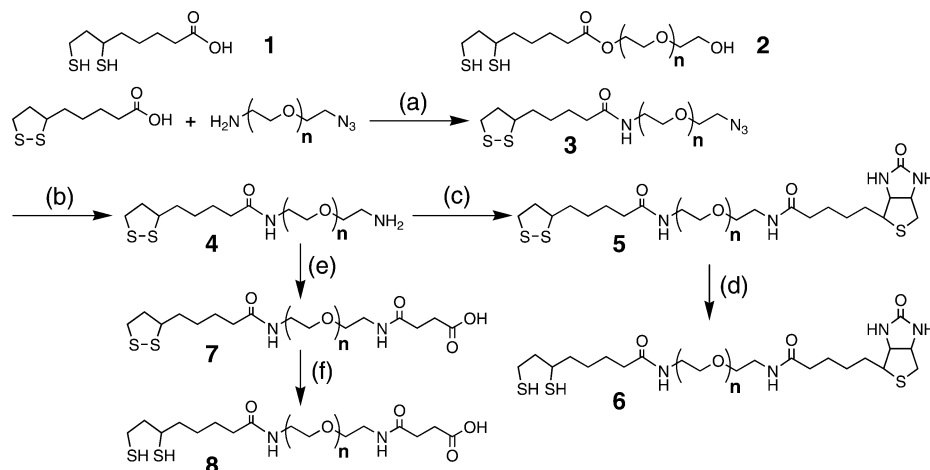


Figure 1. Chemical structures and synthetic routes of the surface ligands used in this study: (a) DCC, DMAP, CH_2Cl_2 ; (b) PPh_3 , H_2O , THF; (c) biotin *N*-hydroxysuccinimide ester, Et_3N , DMF; (d) NaBH_4 , EtOH, H_2O ; (e) succinic anhydride, pyridine; (f) NaBH_4 , EtOH, H_2O .

an amine group in biphasic acidic solution³² was followed by coupling to thioctic acid to provide azide-terminated TA-PEG400 (compound **3**). An additional modification of the azide group into amine provided reactive amine-terminated TA-PEG400 (compound **4**), to which additional functions (including biotin and carboxyl) can be attached. Biotin-terminated TA-PEG (compound **5**) was synthesized by coupling between **4** and biotin *N*-hydroxysuccinimide ester. Alternatively, coupling between **4** and succinic anhydride in pyridine provided the carboxyl-terminated ligand (compound **7**). As prepared, the TA-terminated ligands do not interact with the QD surfaces and thus cannot be used in cap exchange with TOP/TOPO-capped QDs. A necessary transformation of the above TA-PEG-FN into DHLA-PEG-FN can be carried out using simple ring-opening of the terminal 1,2-dithiolane ring in the presence of sodium borohydride (NaBH_4). We limited our description to using PEG with MW ~ 400 (PEG400), but the method described can be applied to shorter or longer segments. All the reaction steps had relatively high yields, and each compound was simply purified with silica gel column chromatography. All the compounds were characterized by thin layer chromatography (TLC) and ^1H NMR, and when necessary, the new ligands were further characterized by FT-IR.

Figure 2 shows ^1H NMR spectra of five representative compounds used in this study. The spectra of each PEG derivative were basically superimposed of each component. All these PEG compounds have large peaks around 3.6–3.7 ppm, which are ascribed to CH_2 groups of the repeated PEG chains. For TA-PEG- NH_2 (compound **4**), most of the multiplet peaks between 1.4 and 3.5 ppm are from thioctic acid. After coupling between **4** and biotin *N*-hydroxysuccinimide ester, new distinct multiplet peaks (at 2.75, ~ 2.9 , 4.33, and 4.51 ppm) appeared in the NMR spectrum of **5**, which were ascribed to biotin ring protons. In addition, following reduction of the 1,2-dithiolane group with NaBH_4 , new doublet and triplet peaks appeared at 1.32 and 1.36 ppm, respectively. These two peaks were assigned as thiol protons of the DHLA unit (open dithiol).²² Though easily reproducible for DHLA and DHLA-PEG molecules, the

successful ring-opening reaction in the presence of additional end functions (e.g., biotin) was crucial to the use of these ligands with QDs.

Cap Exchange. Water-soluble QDs functionalized with one type or a mixture of the synthesized ligands was prepared via cap exchange with the native caps (TOP/TOPO). QDs capped with any of the DHLA-PEG400, DHLA-PEG600, DHLA-PEG1000 ligands were dispersed well in aqueous buffers with pH ranging between 5 and 10.²² When using the biotin- and COOH-terminated ligands, cap exchange was carried out using a mixture with either DHLA (**1**) or DHLA-PEG600 (**2**). This is done to ensure that a controlled density of biotin or COOH groups is available on each QD, which allows manipulation of the reactivity of the QD and eventually control of the number of biological receptors coupled to its surface. In this particular study, we used a mixture of compounds **1** and **6** (4:1), mixtures of compounds **2** and **6** (4:1 and 9:1), and a mixture of compounds **2** and **8** (19:1) for the cap exchange. Ligand mixtures are designated by their molar ratios. As expected, QDs cap-exchanged with the new ligands were stable and aggregate-free over extended periods of time (months) and over a relatively broad pH range. Figure 3 shows solutions of QDs capped with a mixture of compounds **2** and **8** (19:1) in water over the pH range 5–11. The new water-soluble QDs with carboxyl groups were stable and well-dispersed in a wide pH range, including acidic conditions. This feature is quite different from what we have observed for solutions of QDs capped with either DHLA or mercaptoundecanoic acid (MUA). The DHLA-capped QD solutions were well-dispersed in aqueous basic media but showed progressive aggregation and eventual precipitation at pH lower than 7 (Figure 3), indicating that deprotonation of carboxyl groups is a crucial factor to solubilize those QDs in aqueous solutions.³ In comparison, MUA-capped QDs were also stable in basic buffer solution but only for a limited time (less than one week), then aggregates built up in the solution (Figure 3B and Supporting Information); at pH < 7 , aggregation occurred within a few hours of sample preparation. The PEG groups play a critical role in extending solubilization of QDs having carboxyl-terminal groups over a broad pH range. The stability of the QDs with PEG ligands even in acidic conditions has a significant impact for aqueous conjugation chemistry and

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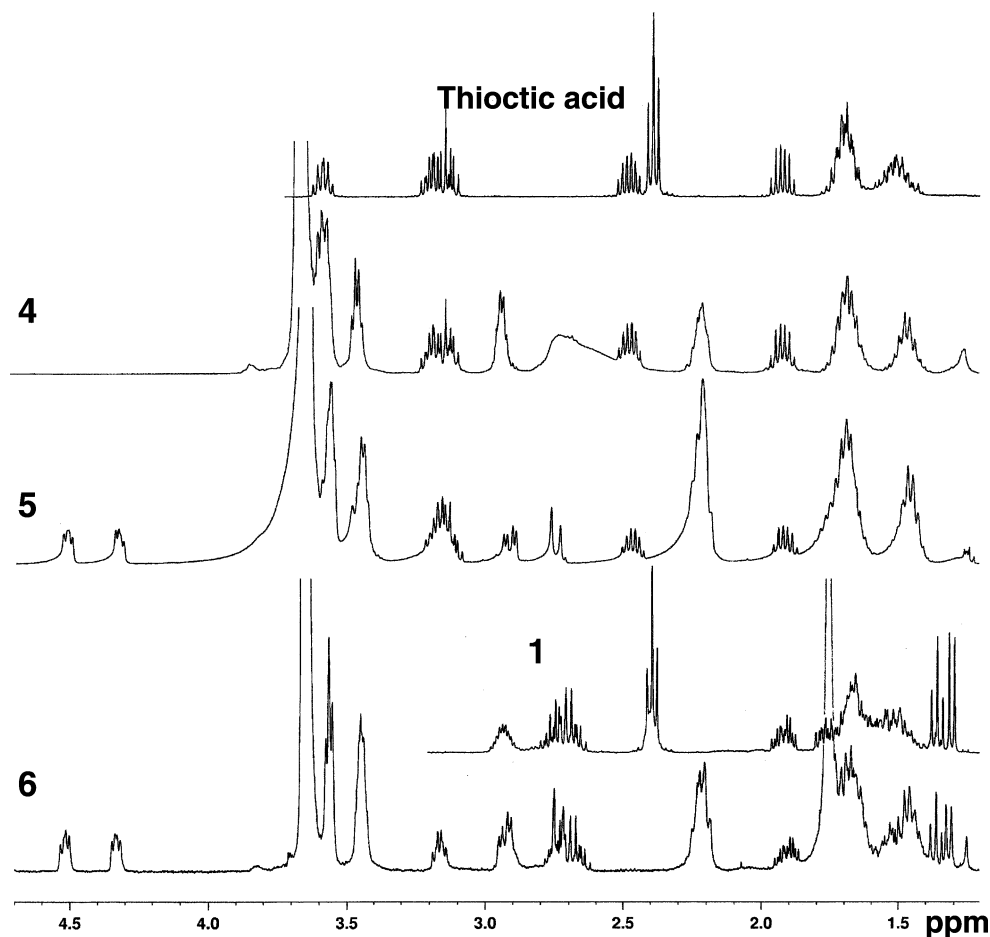


Figure 2. ^1H NMR spectra of compounds **4** (TA-PEG400-amine), **5** (TA-PEG400-biotin), and **6** (DHLA-PEG400-biotin) measured in CDCl_3 . The spectra of thioctic acid and DHLA (**1**) are shown for comparison. The large peak at ~ 1.75 ppm in the spectrum of **6** is attributed to water.

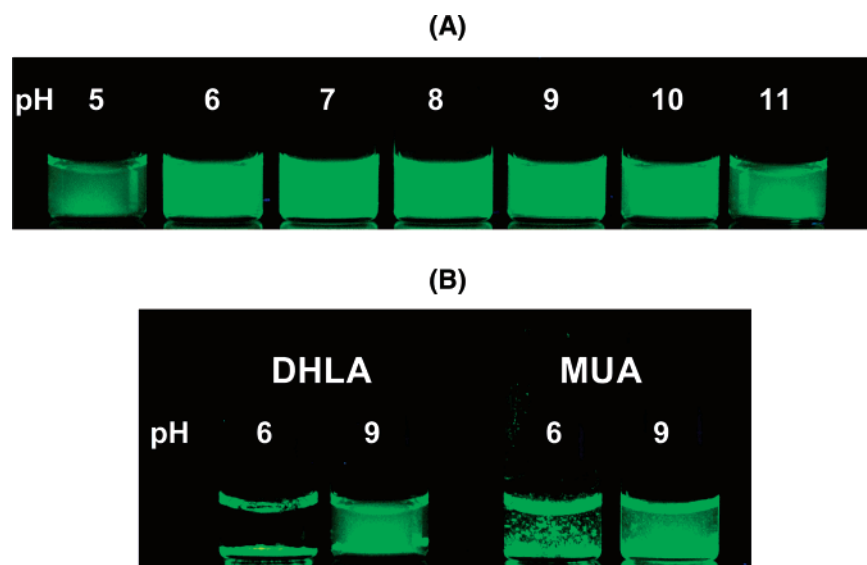


Figure 3. Luminescence image for a set of QD solutions in phosphate-buffered saline at various pH values. CdSe-ZnS samples emitting at 540 nm were used and were excited with a hand-held UV lamp at 365 nm; the image was collected at room temperature. (A) QD with DHLA-PEG600:DHHLA-PEG400-COOH (19:1 molar ratio) at pH 5–11. (B) QD with DHLA at pH 6 and 9, and QD with MUA at pH 6 and 9. Images were collected 2 days after sample preparation.

cell imaging studies. The PEG modules (PEG400 and 600) we used to ensure water solubility of the QDs are relatively short. Such compact size of surface ligands on QD is quite useful not only for ensuring detectable interaction between QD and target

entities but also for efficient cell delivery and the following imaging studies.^{3,21}

FT-IR spectra were measured for QDs after cap exchange reactions (using ligand mixtures) to verify that both ligands were

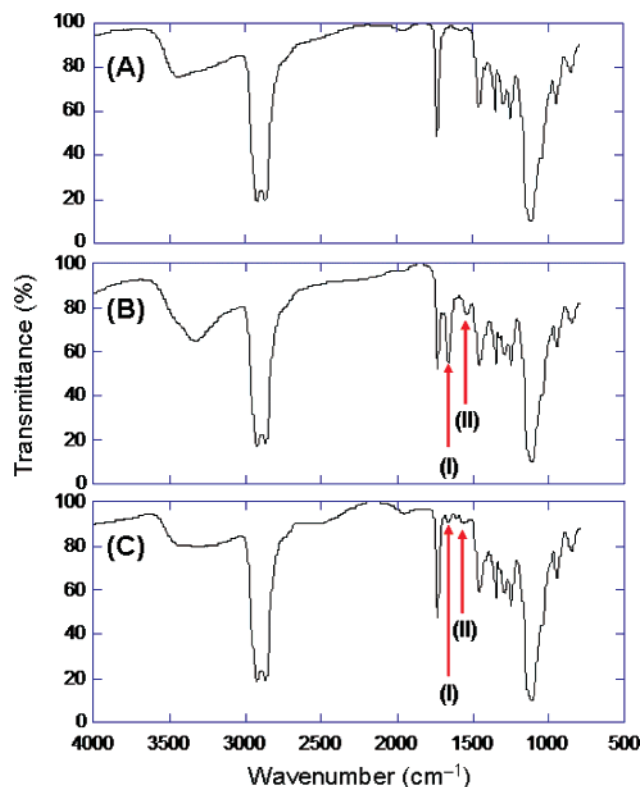


Figure 4. FT-IR spectra of QDs with different surface ligands: (A) DHLA-PEG600; (B) DHLA-PEG600:DHLA-PEG400-biotin (4:1 molar ratio); (C) DHLA-PEG600:DHLA-PEG400-COOH (19:1 molar ratio). (I) and (II) can be assigned as amide I and amide II bands, respectively.

indeed attached to the QD surface after cap exchange (Figure 4). Furthermore, data were collected using QDs having rather low fractions of COOH- or biotin-terminated ligands, because these are the samples that will eventually be employed in designing practical assays; spectra for the pure ligands are provided in the Supporting Information. The entire features of DHLA-PEG600-capped QDs were essentially the same as the spectrum of free DHLA-PEG600 ligand. The sharp peak at 1734 cm^{-1} was ascribed as the C=O stretch mode of the ester group. The FT-IR spectra of QDs capped with DHLA-PEG600:DHLA-PEG400-biotin (4:1) and QDs capped with DHLA-PEG600:DHLA-PEG400-COOH (19:1) also have spectral features similar to those of DHLA-PEG600-capped QDs. This is not surprising since those QDs with mixed ligands still have DHLA-PEG600 as the major surface ligand. However, Figure 4 also shows two additional distinct bands between 1500 and 1700 cm^{-1} (1666 and 1541 cm^{-1} for QD with DHLA-PEG600:DHLA-PEG400-biotin (4:1), and 1666 and 1564 cm^{-1} for QD with DHLA-PEG600:DHLA-PEG400-COOH (19:1)). Those two bands can be assigned to amide I (C=O stretch) and amide II (N-H bending) bands in ligands **6** and **8**, respectively, and are exclusively from either DHLA-PEG400-biotin or DHLA-PEG400-COOH; DHLA-PEG600 does not have an amide bond. The present FT-IR data qualitatively indicate that both ligands are present on the QD surface after cap exchange with a mixture of two ligands.

Optical Properties. Absorption and fluorescence spectra were measured for QDs capped with the original TOP/TOPO ligands and dispersed in toluene and for the hydrophilic QDs capped with the new DHLA-PEG derivatives in H_2O (Figure 5). Absorption spectra measured before and after the cap exchange

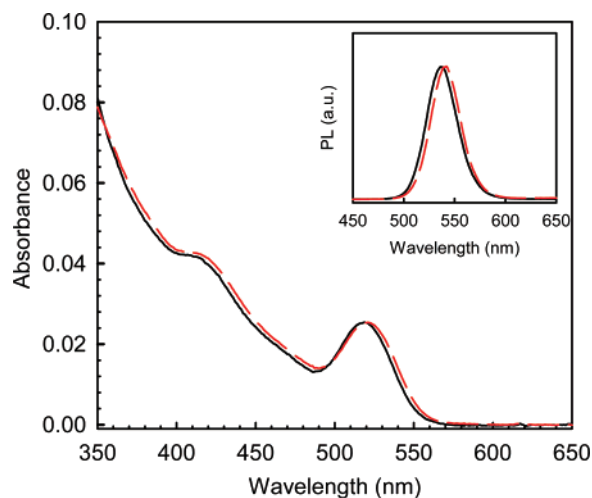


Figure 5. Absorption and fluorescence (inset) spectra of QD with TOP/TOPO ligands in toluene (black line) and QD with a mixture of DHLA-PEG600 (2):DHLA-PEG400-biotin (**6**) (4:1 molar ratio) in H_2O (broken red line). $\lambda_{\text{exc}} = 400\text{ nm}$.

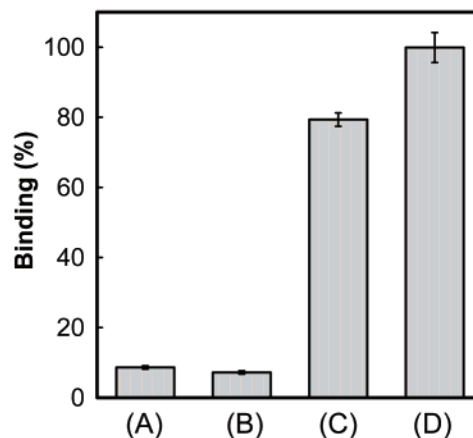
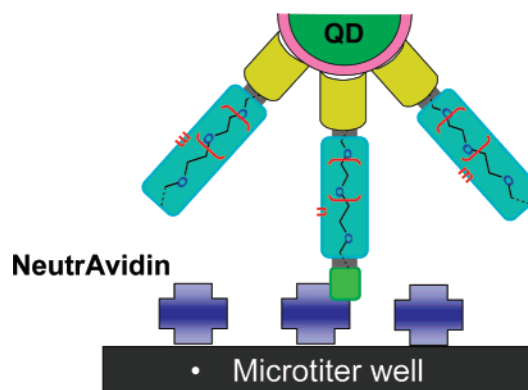


Figure 6. Surface binding assays of biotin-modified QDs to NeutrAvidin. (Top) Schematic representation of the binding assay used; m and n designate the number of ethylene oxide repeating units. (Bottom) Binding (%) of QDs with different surface ligands: (A) DHLA (**1**), (B) DHLA-PEG600 (**2**), (C) 1:DHLA-PEG400-biotin (**6**) (4:1 molar ratio), and (D) 2:6 (4:1 molar ratio).

were essentially unchanged, though occasionally a few nanometers red-shift of the lowest absorption maximum of the hydrophilic QDs was measured compared with that of QDs capped with TOP/TOPO ligands. The fluorescence spectra of those QD solutions also showed similar trends (see Figure 5, inset). These optical features indicate that cap exchange with

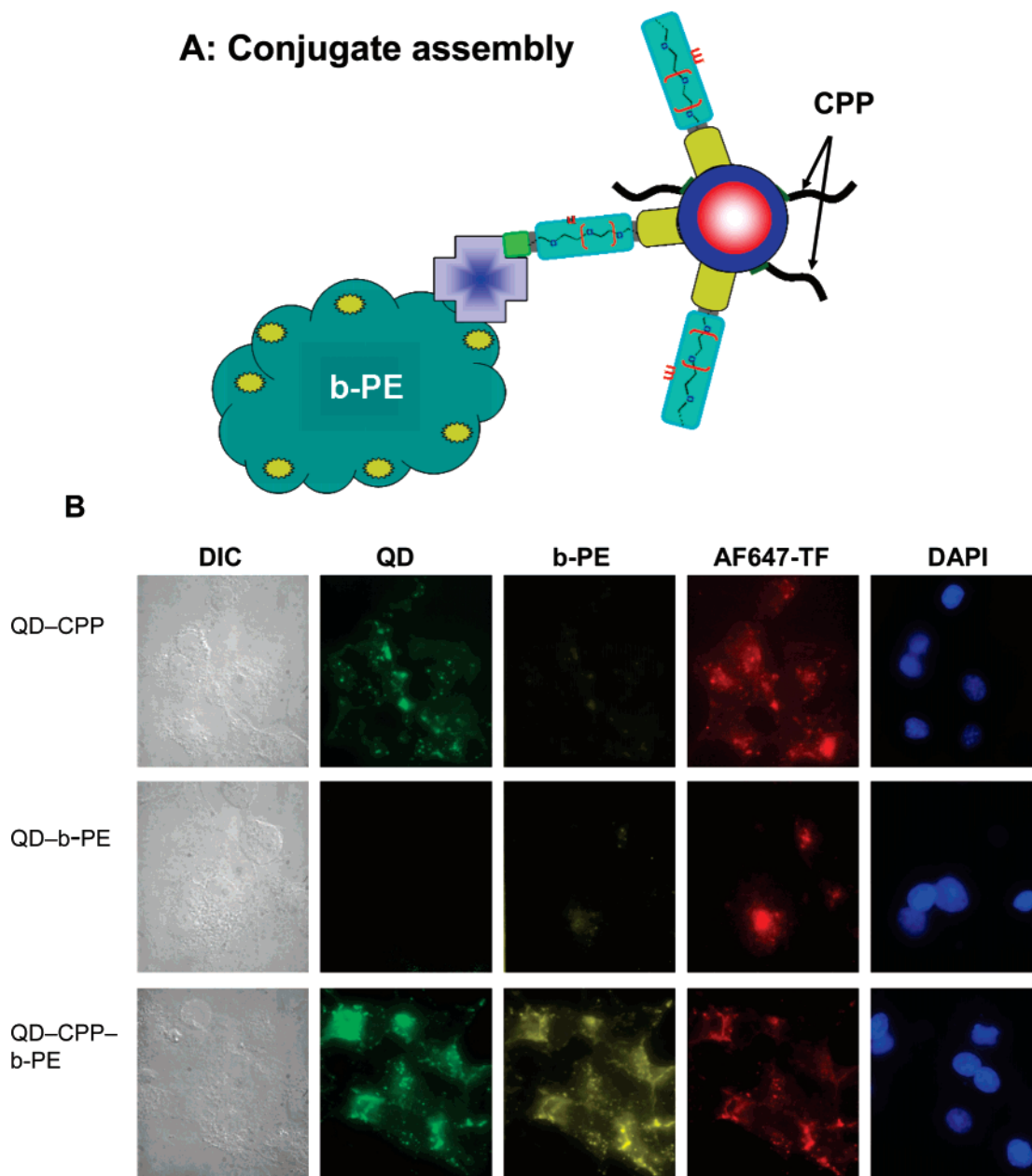


Figure 7. Cellular uptake of biotinylated 540-nm QD–b-phycoerythrin bioconjugates. (A) Schematic representation of the QD bioconjugate. Each QD is conjugated to ~ 1 b-PE-streptavidin and ~ 25 CPPs on average. (B) Representative images of COS-1 cells incubated with ~ 75 nM QD–CPPs (top), 75 nM QD–b-PE (middle), and ~ 75 nM QD–CPP–b-PE (bottom); QDs were capped with DHLA–PEG600:DHLA–PEG400–biotin (9:1). For each series, the corresponding DIC, 540-nm QD, b-PE, AF647-TF, and DAPI fluorescence images are shown. Excitation and emission selections are described in the Experimental Section.

hydrophilic ligands essentially has no effect on the spectroscopic properties of the inorganic QD cores. The relative fluorescence quantum yields measured for the water-soluble QDs were $\sim 50\%$ as compared to the native TOP/TOPO-capped QDs. Decrease of the fluorescence quantum yields of QDs following transfer into aqueous solutions has been commonly observed.^{11,22,33} The quantum yield values for our materials ranged from 10 to 30% for QDs capped with either pure or mixed surface ligands.²²

Surface Binding Assay. Once cap exchange and dispersion into aqueous solutions was achieved, we further carried out targeted biological assays to demonstrate biocompatibility of

the QDs with the newly functionalized QDs. For example, the binding properties of QDs capped with a mixture of DHLA–PEG600 (2) and biotin-terminated DHLA–PEG400 (6) were investigated for their ability to bind with NeutrAvidin-functionalized substrates. Avidin (or streptavidin)–biotin binding has one of the highest noncovalent affinities known ($K_D \approx 10^{15} \text{ M}^{-1}$) and has been widely used in a variety of biological targeting studies.²⁷ NeutrAvidin, which is a deglycosylated form of avidin, has low nonspecific binding properties compared with avidin or streptavidin, while it maintains the strong binding affinity with biotin. Following incubation of the NeutrAvidin-functionalized plate wells with the QD solutions, the wells were rinsed three times with PBS buffer, and the fluorescence signals were collected. Figure 6 shows the fluorescence intensities

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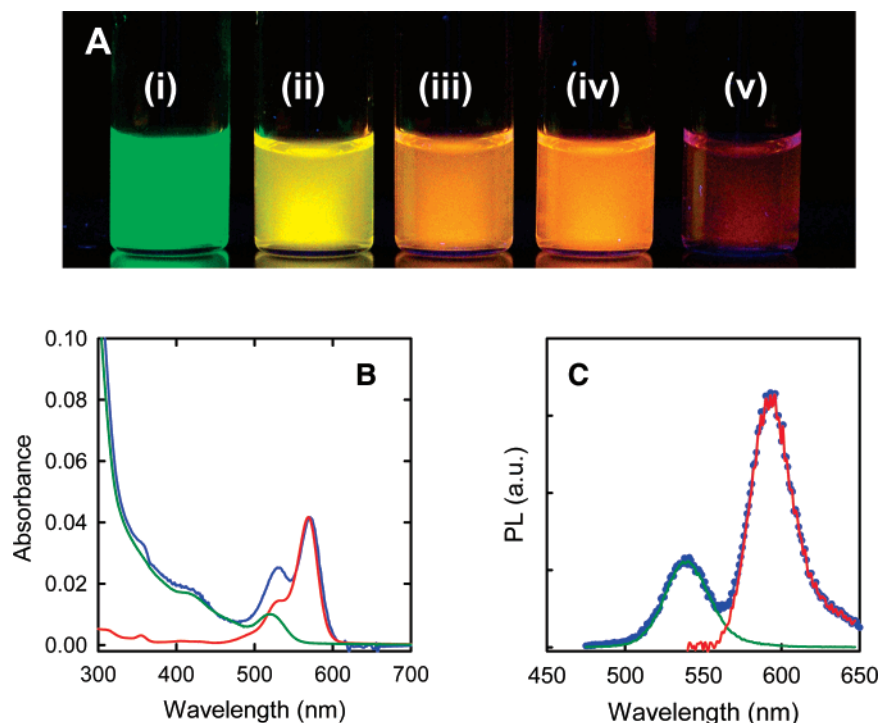


Figure 8. (A) Luminescence image for solution fractions of 540-nm emitting QDs reacted with the dye after gel filtration: (i) no EDC, (ii) 0.01 M EDC, (iii) 0.04 M EDC, (iv) 0.1 M EDC, and (v) Lissamine rhodamine B ethylenediamine only. Samples do not necessarily have the same QD and dye concentrations. The low signal for sample (v) is due to poor excitation of the dye at 365 nm (provided by a UV lamp). (B) 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 H₂O (red line). (C) 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). Fluorescence spectra were generated using 300 nm excitation.

measured for four different QD solutions: one capped with DHLA (**1**), one capped with DHLA-PEG600 (**2**), one capped with a mixture of **1** and DHLA-PEG400-biotin (**6**) (4:1), and one capped with a mixture of **2** and **6** (4:1).

The data clearly show that only wells incubated with QDs capped with a mixture of biotin-terminated DHLA-PEG produced large signal-to-background fluorescence ratios. This result indicates that efficient and specific interactions between the biotin groups on the QD surface and NeutrAvidin on the substrate drive the surface capture of the QDs; control samples using DHLA or DHLA-PEG600-capped QDs showed negligible interactions with NeutrAvidin (see Figure 6). Also, comparison of biotin-functionalized QDs with different hydrophilic ligands (**1:6** (4:1) and **2:6** (4:1)) indicates that long PEG chains surrounding biotin ligand did not disrupt efficient binding between biotin and NeutrAvidin. This promising preliminary result was further complemented by cell-labeling assays.

Cellular Uptake of Quantum Dot-b-Phycocerythrin Conjugates. The ability of QDs functionalized with a mixture of DHLA-PEG600:DHLA-PEG400-biotin and CPP to mediate cellular uptake and delivery of specific protein-attached cargoes was also investigated. QDs emitting at 540 nm, capped with a DHLA-PEG600:DHLA-PEG400-biotin (9:1) mixture, were exposed to monofunctionalized streptavidin-b-phycoerythrin (b-PE) at a 1:1 molar ratio. This multisubunit phycobilin light-harvesting protein has a molecular weight of ~240 000 and ~34 separate chromophores (open-chain tetrapyrroles) within its structure, and its intense brightness has led to its use as a fluorescent marker.^{34,35} Similar to what has been previously

described,²¹ the QD-b-PE conjugates were also self-assembled with cell-penetrating peptides (~25 CPPs per QD) to mediate cellular uptake (see Figure 7A). COS-1 cell cultures were separately incubated with 540-nm QD-CPP, QD-b-PE, and QD-CPP-b-PE assemblies. Each row of panels in Figure 7B shows four representative images, consisting each of 540-nm QDs, b-PE, AF647-transferrin, and DAPI emissions for the three nanoassemblies used. In addition, DIC images of the cell cultures are provided. The brightness of the b-PE is apparent in the micrographs, even though a low ratio of 1 b-PE per QD was used. We should note that, due to a slight leakage of the b-PE emission into the QD channel, the nanocrystal contribution was “corrected” using intensity ratios between the two channels measured for b-PE alone (side-by-side fluorescence spectra of QDs and b-PE are provided in the Supporting Information). However, such correction is only partial, since the QD signal is likely reduced while that of the protein is enhanced, due to FRET interactions between QD and b-PE in the conjugate. The patterns of staining from the QD, b-PE, and AF647-TF channels can all be superimposed, confirming that the b-PE remains attached to the QDs and essentially localized within endosomes. It is also worth noting that attachment of the b-PE on the QD surface did not preclude self-assembly of the smaller CPP nor its availability to mediate cellular delivery.

EDC Coupling. In order to demonstrate easy access to biochemical conjugation, the QDs with DHLA-PEG-COOH ligands were tested for coupling to amine-terminated dyes using EDC, which is commonly used for coupling of water-soluble dyes to an array of biological molecules including peptides, proteins, and antibodies by targeting amine and/or carboxyl groups. In this study, QDs capped with DHLA-PEG600:

(34) Glazer, A. N. *Methods Enzymol.* **1988**, *167*, 291–303.

(35) Glazer, A. N. *J. Appl. Phycol.* **1994**, *6*, 105–112.

DHLA-PEG400-COOH (19:1) were reacted with Lissamine rhodamine B ethylenediamine in the presence of EDC and sulfon-NHS in PBS buffer solution. After the reaction was complete, excess unreacted dyes and reagents were removed by size exclusion gel filtration.

No precipitation or aggregation buildup was observed either during or after the reaction was complete. Absorption spectra measured from the eluted solutions indicate that all the first major fractions collected off the gel filtration column included QDs. There is also a distinct change in the solution fluorescence for reactions carried out in the presence of EDC (Figure 8A). Both absorption and emission spectra collected from the fractions containing QDs showed significant contributions (new bands) at 571 nm for the absorption and 592 nm for the fluorescence, identical to the absorption and fluorescence maxima of the rhodamine dye alone (Figure 8B,C). In comparison, for the reaction without EDC (control experiment), the first major fractions collected from the gel column contained only unreacted QDs. Since the rhodamine dye has a very small absorption at the excitation wavelength we used (at 300 nm), we attribute the fluorescence contribution from the rhodamine dye primarily to energy transfer from the bound and well-excited QDs.^{18,19} Though the reaction conditions were not fully optimized, the present result is promising and proves that we can use our ligand design to covalently attach a variety of biological entities and organic molecules to the QD surface in aqueous media. Further optimization of the EDC couplings with a variety of target molecules is one of our future goals. This result contrasts with the macroscopic aggregation commonly encountered when using QDs capped with DHLA or other short-chain thiol-alkyl-COOH ligands.¹⁶ For these materials, the EDC coupling involves reduction of the pH to ~6 and activation of the carboxyl groups, which in combination reduces the QDs stability in water and often results in aggregate formation, though fluorescence of the nanocrystals is usually preserved.

Conclusions

In this study, we have successfully demonstrated a simple and efficient synthetic procedure to prepare a series of new capping ligands appended with terminal functional groups that

can promote the compatibility of luminescent QDs with buffer solutions and coupling to biomolecules. These ligands are modular and consist of a short poly(ethylene glycol) (PEG) segment, to which we attached an anchoring DHLA group on one end to drive binding to the QD and a variety of biologically reactive/relevant groups at the other end for attaching bioreceptors. Cap exchange with these hydrophilic ligands provided QDs that are water-soluble over extended periods of time and over a relatively broad pH range. Inserting a PEG segment within the ligand allows us to attach a variety of functional groups on the QDs without compromising water-solubility. Furthermore, we find that the use of a mixed-ligand strategy, achieved by simply varying the fraction of DHLA-PEG ligand used in the mixture during cap exchange, is very effective in fine-tuning the functionality of the QD surface. For example, mixed-cap biotin-functionalized QDs showed strong and specific interactions with NeutrAvidin in surface binding assays as well as inside live cells. We also implemented EDC coupling to attach COOH-functionalized QDs to amine-functionalized dyes. This ubiquitous covalent coupling technique opens up easy access to a variety of biological entities and offers additional options for biological assay design. Further studies of these surface-functionalized QDs and coupling with a variety of biomolecules and their use in developing biological assays are in progress.

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Supporting Information Available: Luminescence images of solutions of DHLA- and MUA-capped green-emitting QDs, FT-IR spectra of free ligands, and photoemission spectra of the QDs and b-PE used in cell imaging. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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