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Synthesis of Compact Multidentate Ligands to Prepare Stable Hydrophilic Quantum Dot Fluorophores

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Abstract: We describe a simple and versatile scheme to prepare an array of heterofunctional multidentate ligands that permit strong and stable interactions with colloidal semiconductor nanocrystals (quantum dots, QDs) and render them soluble in aqueous environments. These ligands were synthesized by reacting various chain length poly(ethylene glycols) with thioctic acid, followed by ring opening of the dithiolane moiety, creating a bidentate thiol motif with enhanced affinity for CdSe−ZnS core−shell QDs. Functionalization with these ligands permits processability of the nanocrystals not only in aqueous but also in many other polar solvents. These ligands provide a straightforward means of preparing QDs that exhibit greater resistance to environmental changes, making them more amenable for use in live cell imaging and other biotechnological applications.

Introduction

Semiconductor nanocrystals (quantum dots, QDs) have generated a tremendous amount of interest in the past two decades.[1−5] This has been motivated by a desire to reach a fundamental understanding of several of their unique properties and by the wealth of potential applications involving the use of these materials, ranging from electronic devices to in vivo cellular imaging.[3−6] Colloidal luminescent semiconductor nanocrystals (luminescent QDs), in particular, those made of CdSe and CdTe, have been proposed as unique biomarkers.[7−10] They have the potential to overcome many of the limitations encountered by conventional organic fluorophores and genetically engineered fluorescent proteins in a variety of biological applications.[10−21] Luminescent QDs exhibit high chemical stability, high photobleaching thresholds, resistance to photodegradation, and readily tunable spectroscopic properties.[7−10,13,14] Colloidal QDs (core and core−shell) are often prepared from organometallic precursors using high-temperature solution chemistry routes.[1,22−25] As made, the inorganic materials are prepared in the presence of strongly coordinating organic ligands.[1,5,24,25] The most widely used surface-capping materials are ligand mixtures consisting of trioctylphosphine/trioctylphosphine oxide (TOP/TOPO) and long-chain alkyl−amines. Exchange of these ligands using postsynthetic processing techniques permits tailoring the solubility of the nanocrystals in various solvents with preservation of the optical properties.[1,5] Since the publication of the first reports on the design of hydrophilic QDs and the first proof of QD−protein conjugate formation,[7,8] there have been a number of studies aimed at designing a variety of surface functionalization schemes to

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render these inorganic fluorophores water-soluble and compatible with biological manipulations.[9,12,13,18,20,26] Many of the reported schemes, although providing aqueous solubility and coupling to biomolecules, often have several limitations which include short-term stability, sensitivity to pH, as well as weak and nonspecific ligand interactions with the QD surfaces.[7−13,18,20,26]

We have previously developed a strategy based on utilizing bidentate surface ligands composed of dihydrolipoic acid (DHLA) to render CdSe−ZnS core−shell nanocrystals water-soluble and biocompatible.[9,16] DHLA ligands provide stable interactions with QD surfaces due to the bidentate chelate effect afforded by the dithiol groups.[9,10] While homogeneous in basic buffer solutions, QD dispersions prepared by utilizing this method afford luminescent and functional materials for over a year.[9,10] However, macroscopic aggregation is observed when the local environment of these QDs is altered, such as when placed in acidic solutions, mixed with cationic lipids, or directly dispersed in the cytosol of cells.[9,10,27,28] These properties could be attributed to loss of water compatibility once the carboxylic acid end groups are no longer ionized. Furthermore, applying the conjugation approach based on the use of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC)[29] to DHLA, although simple in concept, is not reproducible with mercapto acetic acid or DHLA and resulted in macroscopic aggregates.[9,10] These limitations underscore the need for developing new approaches for surface functionalization of QDs to improve their stability and broaden their resistance to environmental changes. One of our long-term goals is to design ligands that permit preparation of compact hydrophilic QDs amenable to conjugation with a variety of biomolecules via simple covalent and noncovalent binding strategies. These issues are receiving considerable attention as they address many of the problems currently associated with the design and use of hydrophilic QDs and QD−bioconjugates.[18,26,30−37]

In this investigation, we report a general procedure for the design and preparation of aqueous soluble CdSe−ZnS core−shell nanoparticles featuring a variety of DHLA ligand derivatives appended with poly(ethylene glycols) (PEG) of various lengths to generate hydrophilic and biocompatible nanoparticles. This approach, as demonstrated by our previous work, utilizes the bidentate chelate interactions afforded by the dithiol domain of the DHLA with the QD surface. However, the means for achieving water solubility is governed not by the deprotonation of the carboxylic acid moieties but by the interactions of the hydrophilic poly(ethylene glycol) chain with the solvent. This presents an alternative mechanism for achieving water solubility of the QDs that is less sensitive to pH. As many conventional methods to prepare water-soluble QDs and final QD−bioconjugates necessitate the need for varying pH, these ligands represent a potential means to achieve aqueous solubility of QDs over a broad pH range based on the DHLA ligand motif. The present approach also has the advantage of providing rather compact hydrophilic QDs, which is particularly useful when employing QDs in Förster Resonance Energy Transfer (FRET) experiments since the efficiency of this process is strongly dependent on the separation distance between donor and acceptor fluorophores.[38,39] Furthermore, using mixed surface functionalities, these new ligands allow the design of nanocrystals that are water-compatible, multifunctional, and less pH-dependent. We also investigate the utility of QDs capped with these ligands for use in in vitro bioassays and show that they are better suited for intracellular imaging studies.

Experimental Section

1. Materials and Analysis. All syntheses were carried out under N_2 passed through an O_2 scrubbing tower unless otherwise stated. Air-sensitive materials were handled in an Mbraun M-150 glovebox, and standard Schlenk techniques were used in manipulation of air-sensitive solutions. All chemicals used in this work were obtained from Sigma-Aldrich and used without further purification. Solvents were obtained from Fisher Scientific and used as received. Chemical shifts for ^{1}H NMR spectra are relative to the residual protium ($CDCl_3$, $\delta = 7.26$ ppm; methanol-d_4, $\delta = 4.87$ ppm; or tetramethylsilane, $\delta = 0.0$ ppm). All J values are reported in hertz. The number of attached protons is found in parentheses, following the chemical shift value. Chromatographic purification (silica gel 60 Å, 230−400 mesh, Bodman Industries) of all newly synthesized compounds was accomplished on the benchtop. Materials were visualized with I_2 or ninhydrin spray on aluminum-backed TLC plates. Absorption spectra were recorded on an HP 8453 diode array spectrophotometer. Corrected photoluminescence spectra were recorded on a SPEX Fluorolog-3 spectrophotometer fitted with a red-sensitive R2658 Hamamatsu PMT.

2. Synthesis and Design. Synthesis of Poly(ethylene glycol)-Terminated Thioctic Acid Compounds. Poly(ethylene glycol) (PEG) of the desired average molecular weight is first appended onto thioctic acid (TA) using a dicyclohexylcarbodiimide (DCC)-mediated esterification reaction with a catalytic amount of 4-(dimethylamino)-pyridine (DMAP). The same reaction was used for attaching tetra(ethylene glycol), hexa(ethylene glycol), and various molecular weight PEGs onto the thioctic acid (Figure 1). For brevity, we limit our description to the preparation of three representative compounds, namely, **1**, **6**, and **7**. Additional information on the synthesis of compounds **2**−**5** and **8**−**11** is provided in the Supporting Information.

5-[1,2]-Dithiolan-3-yl-pentanoic Acid 2-{2-[2-(2-hydroxy-ethoxy)-ethoxy]-ethoxy}-ethyl Ester [TA-TEG]: Compound 1. Thioctic acid (6.19 g, 30 mmol), tetra(ethylene glycol) (58 g, 300 mmol), 4-(dimethylamino)-pyridine (1.1 g, 9 mmol), and dichloromethane (300 mL) were placed in a flask and degassed with a stream of N_2 for 20 min. The reaction mixture was cooled to 0 °C in an ice bath, and a solution of DCC (6.8 g, 33 mmol) in dichloromethane (20 mL) was added dropwise. The reaction mixture was stirred at 0 °C for 1 h before it was warmed to room temperature and stirred for 20 h. The precipitate that formed was filtered over a plug of Celite and washed with brine

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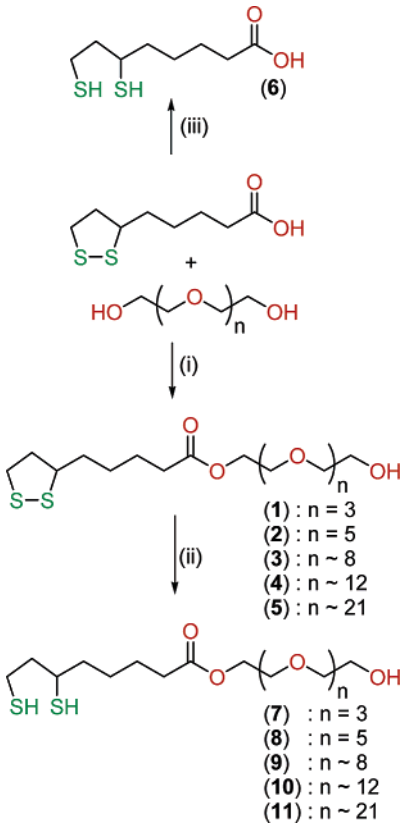

Figure 1. Synthetic route to compounds **1**−**11**. Reagents and conditions: (i) DCC, DMAP, CH_2Cl_2, 0 °C to room temperature; (ii) $NaBH_4$, EtOH/H_2O, rt; (iii) $NaBH_4$, $NaHCO_3$, H_2O, 0 °C.

(3 × 75 mL). The combined organic extracts were dried over $MgSO_4$, filtered, and evaporated to give a yellow oil. The crude product was purified by flash chromatography (ethyl acetate/methanol 95:5) and evaporated to give 7.1 g (62%) as a yellow oil. TLC (EtOAc/MeOH 9:1) R_f 0.4. ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 4.23 (t, J = 4.8 Hz, 2H), 3.70 (m, 12H), 3.65 (m, 2H), 3.15 (m, 2H), 2.45 (m, 1H), 2.36 (t, J = 7.4 Hz, 2H), 2.28 (s, 1H), 1.92 (m, 1H), 1.69 (m, 4H), 1.48 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 172.94, 72.17, 70.16, 70.05, 69.85, 68.68, 62.97, 61.14, 55.88, 39.78, 38.05, 34.13, 33.46, 28.24, 24.16.

Synthesis of Poly(ethylene glycol)-Terminated Dihydrolipoic Acid Compounds: Ring Opening. The ring opening of the dithiolane on the various thioctic acid-terminated PEG ligands (compounds **1**−**5**) and thioctic acid was carried out using sodium borohydride as described in Figure 1. While the conversion of thioctic acid (TA) to dihydrolipoic acid (DHLA) (**6**) takes place in strictly aqueous solvent,[9,40,41] the reduction of compounds **1**−**5** proceeded more cleanly when carried out in a mixture of ethanol and water.

6,8-Dimercapto-octanoic Acid [Dihydrolipoic Acid, DHLA]: Compound 6. Compound **6** was prepared as previously described,[9,40,41] with the following modifications. Thioctic acid (12.2 g, 59 mmol) was dissolved in 250 mL of 0.25 M $NaHCO_3$ and cooled to 0 °C in an ice bath. $NaBH_4$ (9.0 g, 238 mmol) was added slowly and the temperature kept below 4 °C while stirring for an additional 2 h. The reaction mixture was acidified with 6 M HCl to pH 1 and extracted with toluene (3 × 75 mL). The combined organic phases were dried over $MgSO_4$ and filtered. Evaporation of the solvent yielded 12.0 g (98%) of the desired product as a clear colorless oil.

6,8-Dimercapto-octanoic Acid 2-{2-[2-(2-Hydroxy-ethoxy)-ethoxy]-ethoxy}-ethyl Ester [DHLA-TEG]: Compound 7. Compound **1** (3.83 g, 10 mmol) was dissolved in 50 mL of 1:4 EtOH/water with stirring. $NaBH_4$ (416 mg, 11 mmol) was added and stirred for 60 min or until the solution became colorless. The reaction mixture was diluted with water (100 mL) and extracted with $CHCl_3$ (3 × 75 mL). The combined organic phases were dried over magnesium sulfate ($MgSO_4$), filtered, and evaporated. The residue was purified by flash chromatography (EtOAc/MeOH 95:5) and evaporated to give 3.4 g (88%) of the desired product as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 4.20 (t, J = 4.8 Hz, 2H), 3.65 (m, 12H), 3.57 (t, J = 4.5 Hz, 2H), 2.88 (m, 1H), 2.66 (m, 2H), 2.49 (s, 1H), 2.32 (t, J = 7.3 Hz, 2H), 1.87 (m, 1H), 1.7−1.35 (m, 7H), 1.32 (t, J = 8.0 Hz, 1H), 1.27 (d, J = 7.6 Hz, 1 H). ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 173.03, 72.18, 70.21, 70.10, 69.90, 68.73, 63.00, 61.18, 42.34, 38.89, 38.27, 33.56, 26.06, 24.10, 21.87.

Characterization of the ligand compounds **1**−**11** was accomplished by ^{1}H and ^{13}C NMR spectroscopic techniques. The NMR data indicate that when appending thioctic acid (TA) to the various poly(ethylene glycols), the resulting spectrum is essentially a composite of the two starting materials. Figure 2 shows the spectra of TA and compound **2** (TA-TEG); similar spectra were obtained for compounds **3**−**5**. The main contribution from the PEG moiety appears as a large broad multiplet at 3.5−3.6 ppm with an additional two-proton triplet at 4.1 ppm. The absence of the carboxylic acid resonance (broad peak at 11 ppm) of compounds **2**−**5**, but present in the thioctic acid spectrum, is further evidence of successful coupling (see Supporting Information). Figure 3 shows that the oxidized and reduced forms of related structures (compounds **2** and **8**) have very disparate spectra and are easily distinguishable. The thiol protons of compound **8** are clearly discernible as seen by the well-resolved triplet and doublet at 1.30 and 1.25 ppm, respectively, with integrated intensities of one proton each. Furthermore, new resonances are observed for compound **8** at 2.8 and 2.6 ppm but are conspicuously absent from precursor compound **2**, while resonances at 3.0 and 2.3−2.35 ppm for **2** are absent in **8** (Figure 3). Similar differences between the open and closed forms are observed for the other compounds, namely, **3** versus **9**, **4** versus **10**, **5** versus **11**, and **TA** versus **DHLA**. It is these characteristic resonances that lead us to unambiguously identify the compounds prepared in this investigation. Starting from commercially available precursors, it is possible to quickly and efficiently prepare large amounts of the poly(ethylene glycol)-modified dihydrolipoic acid ligands.

We have also investigated the DCC-based esterification of dihydrolipoic acid with oligo- and poly(ethylene glycols). This route, however, proved to be less efficient in that the products were isolated as mixtures consisting of the reoxidized form of the ligand and desired product. Thus, the higher yields and ease of separation using the procedure outlined above provided the cleanest materials.

3. Amylose Resin Assay of MBP-5HIS to QDs. Affinity columns (25 mm × 76 mm) of amylose resin (New England Biolabs, Beverly, MA) were setup and equilibrated with 10 mM Na−tetraborate buffer (pH 9.55).[9] Then, 100 pmol of CdSe−ZnS QDs capped with **6**, **10**, and a 1:1 mixture of **6** and **10** was mixed with the equivalent of 20 maltose binding proteins appended with an oligohistidine tail (MBP-HIS) per QD in 200 μL aliquots of 10 mM borate buffer at pH 9.55 and allowed to self-assemble for 1 h.[9,16] Duplicates of each sample tested were loaded onto the amylose resin pre-equilibrated in buffer. After binding to the column, samples were washed with 1 mL of buffer, eluted with 20 mM maltose in buffer, and the eluant captured. The eluant was brought up to 500 μL with 20 mM maltose in buffer. A quantity of 100 μL of all samples was then loaded onto a microtiter well plate in triplicate, and the PL was measured on a Tecan Safire plate reader (Tecan US, Research Triangle Park NC). By normalizing the PL of the DHLA-capped QD self-assembly mixture to 100%, we could estimate the percent of each QD sample binding to the column

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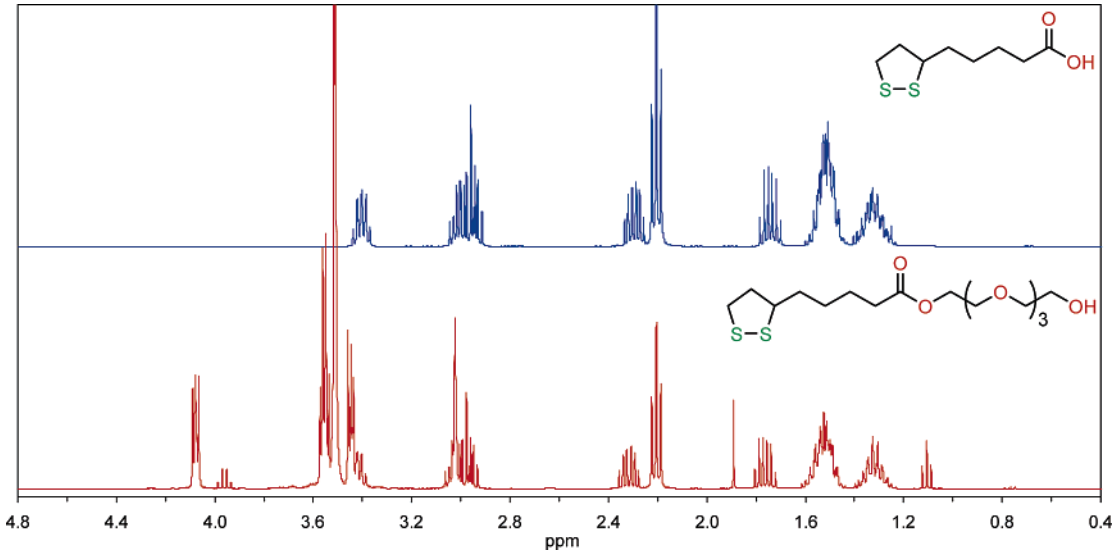

Figure 2. ^{1}H NMR spectra highlighting the coupling of tetraethylene glycol to thioctic acid: TA and compound **1**. (Additional ^{1}H NMR spectra for the other PEG-terminated TA molecules are provided in the Supporting Information.)

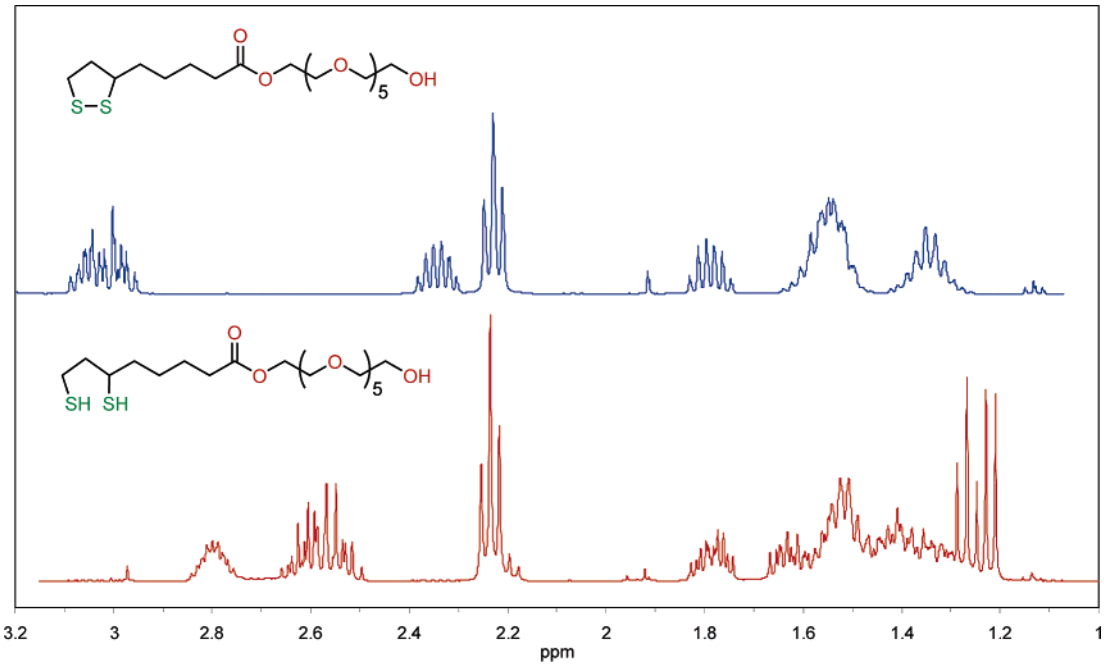

Figure 3. Comparative ^{1}H NMR spectra of the upfield region displaying the characteristic shifts resulting from the reduction of the dithiolane moiety; compounds **2** and **8** are shown.

and being released with maltose relative to the solution containing DHLA-capped QDs (Figure 4).

4. Live Cell Imaging. HeLa and NRK cells were cultured in DMEM supplemented with 10% fetal bovine serum (Invitrogen Life Technologies). Cells were grown on coverslips (Fisher Scientific) and imaged in 10 mM Hanks buffered salt solution, Hepes, and 5% FBS, pH 7.4. QDs capped with DHLA-PEG600 (compound **10**) were diluted in injection buffer (140 mM KCl/10 mM Hepes, pH 7.4) and microinjected into cells using a Narashige microinjector (Japan). Cells were imaged using an Olympus IX70 microscope equipped with a 75 W xenon lamp source, a 480/40 nm excitation filter, and appropriate emission filters (Chroma Technologies Corp., Brattleboro, VT). Images were acquired with an ORCA-ER camera (Hamamatsu Photonics). Image acquisition, processing, and analysis were done using MetaMorph (Universal Imaging, Downingtown, PA).

Results and Discussion

1. Synthesis and Design. Utilizing an excess of the PEG in the esterification reaction substantially minimizes formation of the bisubstituted species and simplifies the chromatographic purification of compounds **1**−**5**. Prior to chromatography, much of the unreacted glycol is removed by extraction with water, thus greatly simplifying the purification process. Since the bisubstituted species exhibits an R_f value larger than that of the desired monosubstituted product, it is easily separable by flash column chromatography. The subsequent ring opening process is facile and can be monitored visually by following the gradual disappearance of the yellow color of the precursor to a final colorless solution (final product). Completion of the reaction is

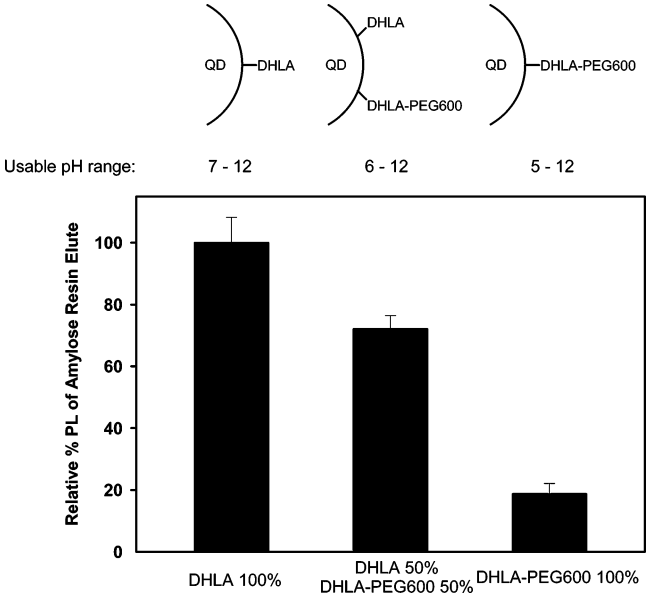

Figure 4. Amylose resin assay testing the ability of MBP-5HIS to attach to QDs capped with DHLA (compound **6**), DHLA-PEG600 (compound **10**), and a 50:50 mixture of compounds **6** and **10**. Even though they are not as efficient as the pure DHLA capping, the mixed cap QDs conjugated to MBP-5HIS showed efficient binding to amylose resin and release by soluble maltose.

clearly reflected by the disparate NMR spectra of the oxidized and reduced forms of the ligands (Figure 3).

The synthetic route described here, which begins by appending the poly(ethylene glycol) chain of desired length onto thioctic acid followed by reduction with $NaBH_4$, provides a simple, reproducible, and efficient scheme to prepare our desired ligands. It is substantially better than the reverse route where ring opening of thioctic acid is followed by coupling of the poly(ethylene glycol). This route exhibits an overall lower yield and a more difficult separation of the PEG-modified DHLA, due to the formation of the reoxidized species and other side products. This synthetic route provides a general and relatively simple means to prepare large quantities of heterobifunctional linkers based on poly(ethylene glycol) chains in which one end is modified with thioctic acid/dihydrolipoic acid, thus maintaining the bidentate surface functionality of the final compound. It is then possible to modify the other end of the polymer chain to provide other functional moieties for further covalent coupling strategies.

2. Preparation of DHLA-PEG-Capped Hydrophilic CdSe−ZnS Core−Shell QDs. Exchanging the native capping shell (TOP/TOPO/hexadecylamine) with our newly designed DHLA-PEG ligand series to render the nanocrystals water-soluble was carried out using a procedure similar to that for DHLA functionalization described earlier with several modifications.[9,40,41] The nanocrystal fluorophores were synthesized using known literature procedures.[1,2,22−24,42] Briefly, samples were prepared utilizing a stepwise approach consisting of core nanocrystal growth, overcoating with a thin layer of ZnS, size selective precipitation, surface ligand exchange, and purification. The use of DHLA in the ligand exchange process with the native TOP/TOPO to generate stable, aggregate-free aqueous dispersions of nanoparticles was carried out as described previously.[9,15] Precipitated QDs (∼100−300 mg) obtained from centrifugation in ethanol are dispersed in pure DHLA (∼1 mL) and heated to ∼70 °C for ∼2−3 h until the solution becomes homogeneous. Dimethyl formamide (5 mL) is added to the solution, and the nanocrystals are precipitated with potassium *tert*-butoxide (∼400−600 mg) and centrifuged to produce a pellet of QDs and excess potassium *tert*-butoxide. The supernatant, free of QDs, is discarded, and the precipitate is dispersed in water and purified using concentration/dilution with a membrane separation filter (Millipore, M_w cutoff ∼50 000 Da) with water through four cycles. However, achieving water compatibility via surface ligand exchange of QDs with the various PEG-terminated DHLA ligands (compounds **9**−**11**) and mixtures of DHLA (compound **6**) with DHLA-PEG600 (compound **10**) required the use of a modified procedure outlined below. Mixtures of DHLA (compound **6**) with **9** or **11** proceeded with similar results.

Surface ligand exchange of the native TOP/TOPO ligands with compounds **7** and **8** resulted in dispersions of QDs that were not soluble in water, however, readily formed homogeneous solutions in polar organic solvents, such as methanol, ethanol, or DMF. It should be noted that QDs coated with TOP/TOPO cannot be dispersed in these polar organics, thus highlighting the ability of these DHLA-modified ligands to tailor the solubility based on the poly(ethylene glycol) chain lengths. While not dispersible in aqueous environments, QDs coated with these ligands which are soluble in polar organics can find potential utility in a variety of other applications, such as sol−gel processing employed in the fabrication of thin solid films, with high density of QDs for use in amplified stimulated emission (ASE) experiments.[43]

Preparation of nanocrystals capped with compounds **9**, **10**, and **11** was carried out as follows. TOP/TOPO-capped nanoparticles (100−300 mg) after size selective precipitation with ethanol were suspended in 1−2 mL of surface-capping ligand **9**, **10**, or **11**, mixed with 1 mL of methanol, and heated to ∼60 °C overnight under N_2 with stirring. The resulting homogeneous sample was cooled to room temperature and diluted with hexanes, ethanol, and chloroform to produce a monophasic turbid solution (solvent-induced precipitation of the newly capped QDs). The solution was centrifuged to produce a pellet of nanoparticles, while the supernatant, free of color, was discarded. The precipitate was dispersed in water, providing a clear homogeneous dispersion of nanocrystals, and was purified using four cycles of concentration/dilution with an Ultra-free centrifugal filtration device (Millipore, M_w cutoff ∼50 000 Da). These procedures permit elimination of soluble organics and other materials from the solution and provide homogeneous aggregate-free QD dispersions with concentrations ranging from 5 to 150 μM that were stored for further use. Homogeneous aqueous dispersions of these hydrophilic QDs exhibited absorption and emission characteristics virtually identical to those of the native materials with photoluminescence efficiencies of ∼25−30%, lower than what is measured in organic solutions (usually up to ∼60−80%).

3. QDs Capped with Mixtures of DHLA and DHLA-PEG ligands. Exchanging the native capping shell on the QDs with mixtures of DHLA (compound **6**) and one of the longer PEG-

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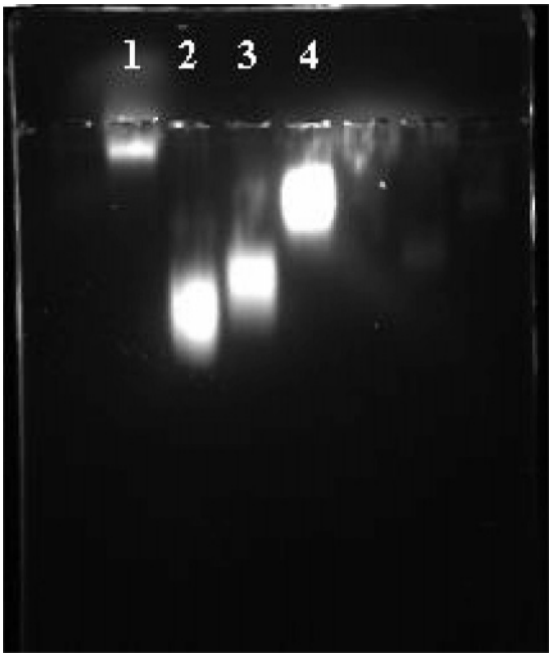

Figure 5. Gel shift of CdSe−ZnS QDs coated with various ratios of **10** to **6** in 1% agarose gel buffered with Tris borate EDTA buffer. Lane 1, 1:0 **10**:**6**; lane 2, 1:1 **10**:**6**; lane 3, 3:1 **10**:**6**; lane 4, 10:1 **10**:**6**.

terminated ligands, namely, **9**, **10**, or **11**, required addition of ∼10 mg of potassium *tert*-butoxide to achieve complete aqueous dispersion of the sample, an amount much smaller than what was required (∼400−600 mg) when using only DHLA. Presumably, the base is needed for the ionization of the carboxyl moieties in order to facilitate dispersion in water. Confirmation of the cap exchange with a mixture of DHLA and compound **9**, **10**, or **11** can also be obtained by investigating the effects of surface charges using gel electrophoretic mobility shift. Figure 5 shows the gel mobility shift of a series of QD dispersions capped with a mixture of DHLA (compound **6**) and PEG-modified DHLA compound **10**. The extent of the mobility shift is seen as a function of the molar ratio of the two compounds. The molar ratios of compound **6** to compound **10** ranged from 1:1, 1:3, and 1:10 during the surface ligand exchange process. The higher the molar ratio of **6**:**10**, the further the QDs migrated to the positive electrode. As seen in lane 1 of Figure 5, QDs coated with 100% of compound **10** show little or no shift from the loading well under the applied voltage, due to the nonionized nature of these QD systems. When 50% of compound **10** is replaced with **6** (lane 2 in Figure 5), a substantial gel shift was measured, which we attribute to the finite electrophoretic mobility brought about by the presence of charged DHLA ligands on the QD surface. Similarly, QDs coated with a mixture of **6** and **10** but at lower concentrations of **6** (30% in lane 3 and 10% in lane 4) resulted in smaller shifts relative to the case shown in lane 2. As the amount of charge is increased (with increasing DHLA fraction), an increase of the mobility is also observed, which tracks well in a linear fashion. This reflects a lower electrophoretic mobility of the QDs, due to a smaller density of surface charges when the fraction of **6** on the QD surface is reduced. QDs capped with DHLA (compound **6**) showed an even more pronounced gel mobility shift.[44]

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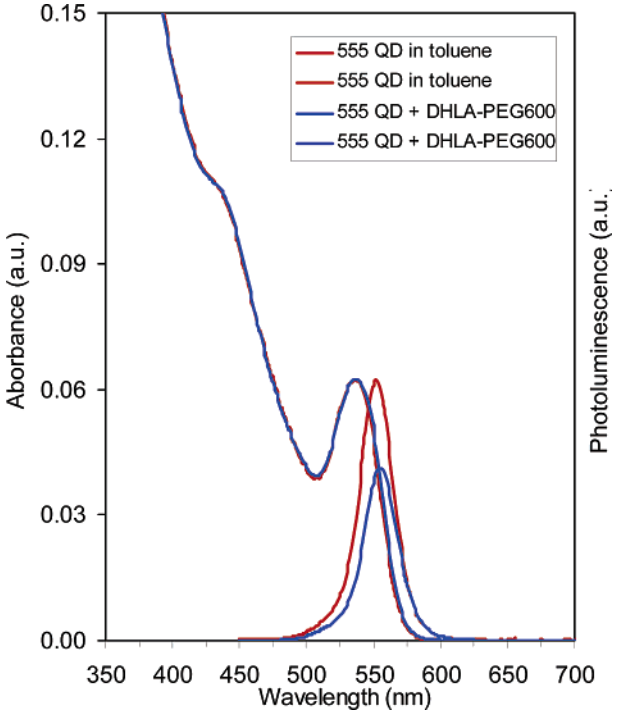

Figure 6. Spectra of TOP/TOPO-capped nanocrystals in toluene and after surface ligand exchange with DHLA-PEG600 (compound **10**) in water.

Surface ligand exchange of the QDs with compound **6**, **9**−**11**, or any mixture of **6** and **10** to produce water-soluble QD dispersions exhibited absorption profiles that were virtually unchanged from the organic growth solutions. Presumably, the length of the chain in the PEG is responsible for achieving complete aqueous dissolution of the QDs as surface ligand exchange using compounds **7** and **8** resulted in QDs that were *partially* soluble in water. These results suggest that a minimum poly(ethylene glycol) length is needed to achieve homogeneous aqueous dispersions, which correspond to a chain length of approximately 8 ethylene oxide units or a PEG with average molecular weight of 400 when appended to dihydrolipoic acid.

It is important to note that the surface ligand exchange process with either pure PEG-modified DHLA, DHLA, or a mixture of the ligands is similar for all cases, and that the methodology is quite general for this class of molecules.

4. Optical Characterization. Estimates of the photoemission quantum yields were obtained by comparing the integrated emission from rhodamine 6G in ethanol (QY = 0.90) or other standards to that of the CdSe−ZnS nanoparticles whose emission spectral profile closely matched that of the standard. Concentrations were adjusted such that both reference and QD samples gave optical densities of ∼0.05 at the excitation wavelength, and solvent refractive indexes were taken into account.[45] Fluorescence spectra were collected between 450 and 700 nm at room temperature and integrated on a wavenumber scale. The differences in the absorption and photoemission spectral profiles of the QDs measured in toluene and QDs that have been transferred into water using ligand **10** are negligible with peak shifts of less than 3 nm in all cases (Figure 6). The same trend is observed when compounds **9**−**11** or mixtures of **6** and any of these compounds are used, indicating that the surface ligands have no effect on the electronic properties of

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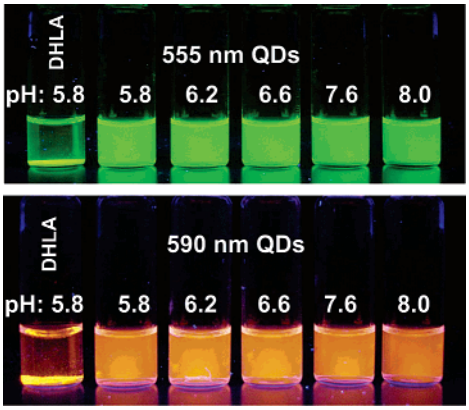

Figure 7. (A) Luminescence image set of 555 nm emitting CdSe−ZnS QD solutions in phosphate buffer saline at various pH values after 2 days at room temperature: (vial 1) QDs coated with compound **5** at pH 5.8; (vials 2−6) QDs coated with compound **10** at pH 5.8, 6.2, 6.6, 7.6, and 8.0, respectively. (B) Same set as above with 590 nm emitting QDs. Samples were excited with a hand-held UV lamp at 365 nm.

the inorganic QD core. However, although the absorption and emission spectral properties of the organic soluble and aqueous soluble QDs are nearly identical, there is a noticeable decrease of the photoluminescence quantum yield for the QDs in aqueous dispersions (Figure 6). The quantum yields are approximately 70−80% for the QDs measured in toluene or hexane solutions and drop to 25−30% for the QDs coated with compound **9**, **10**, or **11** and dispersed in water. This is not uncommon, as reported quantum yields for QDs transferred into buffer solutions are also smaller than those measured in organic solutions (∼10−30%).[7−10,36] Concentrations were estimated from measurements of the absorption spectra[9,23] or using the size-dependent extinction coefficient at 350 nm, as done by Leatherdale et al.[46] Concentrations obtained from both methods were within experimental error.

5. Stability Measurements and Noncovalent Self-Assembly with HIS-Terminated Proteins. The pH stability of aqueous dispersions of QDs coated with DHLA-PEG compound **9**, **10**, or **11** was investigated. Figure 7 shows a solution of QDs capped with compound **6** at pH 5.8 side by side with solutions of QDs capped with compound **10** in various PBS solutions whose pH ranged from 5.8 to 8. Aqueous dispersions of QDs capped with DHLA (compound **6**) showed that at pHs lower than 7, aggregation of the nanoparticles occurs, signifying the principal role that the carboxylate functional group plays in determining the solubility of the complex in aqueous solutions. This is clearly seen in vial 1 of Figure 7A,B, where QDs capped with pure DHLA were used. For QDs functionalized with ligand **10**, optically clear homogeneous solutions were obtained at pH values of 5.8 and above (vials 2−6 of Figure 7A,B). QD samples capped with ligand **9** or **11** exhibited similar pH stability trends. Aqueous dispersions of QDs coated with mixtures of compounds **6** and **10** remained homogeneous at pH 5−7 after preparation; however, aggregation build-up was observed after 1 day while maintaining their luminescence when the fraction of DHLA in the ligand mixture exceeds 50%, which underscores the importance of maintaining relatively high pH conditions for QDs that contain DHLA to remain in solution.

It is important to note that compounds **1**−**5** and **7**−**11** could also be used to functionalize other nanoparticles and surfaces, such as those made of gold and silver.[47,48] In particular, due to the strong affinity of thiols to gold surfaces, our designed ligands could provide unique flexibility in preparing functionalized gold surfaces and nanoparticles for use in targeted experiments.[47−49]

Whereas QDs coated with compound **6** resulted in aqueous dispersions that were aggregate-free in the pH regime of 7−12, the use of compound **10** in the surface ligand exchange process resulted in aggregate-free solutions from pH ∼5 to 12 over at least 1 year after preparation. Furthermore, NMR spectra of DHLA-PEG ligands taken several months after preparation showed no signs of degradation, indicating that the ligands themselves are also stable. The gain in pH stability is substantial as it progresses into the acidic regime.[50] By using mixtures of ligands **6** and **10**, for example, we have obtained nanoparticles that were luminescent and homogeneous in the pH range of ∼6−12 and, thus, provided a rationale for exploring additional properties conferred with these ligand mixtures. We have previously demonstrated that QDs capped with DHLA (compound **6**) undergo electrostatic and metal-affinity-driven self-assembly with maltose binding protein appended at its C-terminal with a leucine zipper or an oligohistidine attachment domain, MBP-zb or MBP-5HIS, respectively; the formed QD−MBP conjugates were found to tightly bind to amylose resin and elute with excess maltose.[9,16] Figure 4 shows the results of an amylose resin assay applied to three QD solutions incubated with MBP-5HIS, one capped with DHLA (ligand **6**), one capped with DHLA-PEG600 (ligand **10**), and one with a 1:1 mixture of ligands **6** and **10**, to verify the ability of MBP-5HIS to self-assemble on these three variations of QD−ligand complexes. The metal-affinity self-assembly process for QDs capped with compound **6** is highly efficient, whereas for QDs coated with **10**, nonspecific interactions may be responsible for the small QD−protein complex recovered (Figure 4).[9,16] The binding efficiency of MBP-HIS to QDs capped with a mixture of DHLA and DHLA-PEG600 (compound **10**) is slightly lower than that of QDs capped with DHLA; however, it is much higher than what is measured for QDs capped with PEG-terminated DHLA. We believe that the longer PEG molecules may prevent the MBP-5HIS tail from interacting with the QD zinc and sulfur atoms.[39] Nonetheless, this assay proves that the new PEG-terminated ligands do not alter the ability of QDs partially capped with DHLA to self-assemble with HIS-terminated proteins. The self-assembly process for QDs coated with a 1:1 mixture of **6** and **10** is effective and represents a balance between the ability to self-assemble protein onto the QD and maintain a relatively broad usable pH range.

In addition to the pH stability, we investigated the behavior of QDs capped with DHLA-PEG ligands in aqueous solutions at various ionic strengths. In particular, the photoluminescence properties of two sets of QDs dispersed in buffer solutions at neutral pH were monitored as the concentration of NaCl was increased from 0 to 1 M; one set of 510 nm emitting QDs capped

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(50) QDs capped with compounds **6**, **9**, **10**, and **11** or any mixtures of these are still stable at pH values larger than 12. We did not explore that pH range any further because they are not practically useful for biomotivated applications.

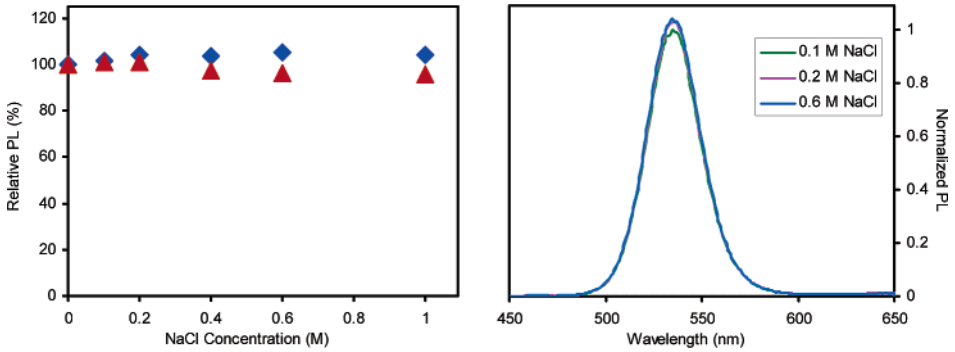

Figure 8. (A) Effect of increasing ionic strength on the photoluminescence of 510 nm emitting CdSe−ZnS QDs coated with compound **11** (◆) and 535 nm emitting CdSe−ZnS QDs coated with compound **10** (▲). Dispersions of QDs were placed in fluorescence cuvettes (1 cm optical path) containing an equal concentration of QDs but various concentrations of sodium chloride. The resulting photoluminescence spectra were background-corrected, integrated, and normalized to the emission form slat-free solution. The PL emission from QDs with no NaCl added was set to 100%. (B) Photoluminescence spectra of 535 nm emitting CdSe−ZnS QDs coated with compound **10** at three ionic strength concentrations. The 0.2 and 0.6 M solutions were normalized against the 0.1 M NaCl solution.

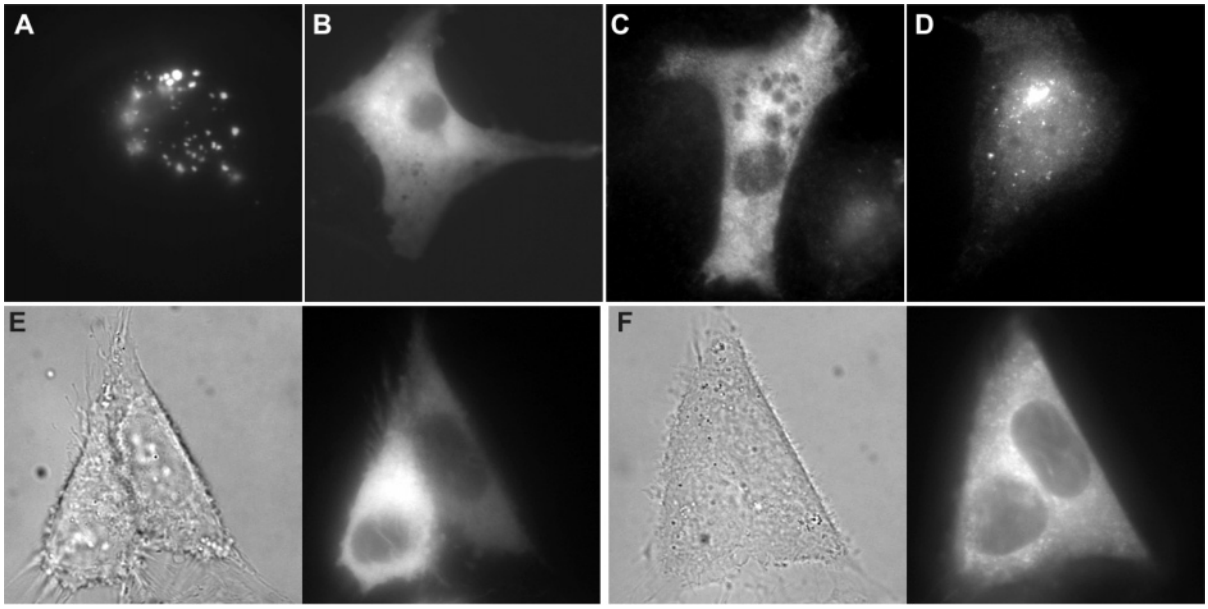

Figure 9. Distribution of DHLA-PEG600-capped QDs in live cells. Mammalian cells (Hela-A,E,F and NRK-B,C,D) were grown in culture media and injected with a 10 μM QD solution. The images show epifluorescence images of cells 5 min after being injected with (A) DHLA-capped QDs, (B) QDs capped with DHLA-PEG600 (compound **10**), and (C) QDs capped with compound **11**. (D) Epifluorescence image of cells 14 h after being microinjected with QDs capped with DHLA-PEG600. (E and F) Bright field (left) and epifluorescence (right) images of cells injected with QDs coated with DHLA-PEG600 (E) 5 min and (F) 35 min after being microinjected. Note that cells shown in (E) have different amounts of QDs, as judged by their fluorescence, but within 30 min, they fuse together causing redistribution of their cytoplasm, thus the QD fluorescence.

with compound **10** (DHLA-PEG600) and one set of 535 nm emitting QDs capped with compound **11** (DHLA-PEG1000), each set having a fixed QD concentration, were studied. Over the concentration range of NaCl used (0−1.0 M), we observed little or no change in both the PL intensity and the characteristics of the emission spectra for both cases (see Figure 8). These experiments further highlight the stability of QDs capped with these new ligands in high ionic strength conditions. Moreover, the derived results are directly applicable to intracellular and in vivo studies, where the ionic concentration is known to be high. The data shown in Figure 8 are consistent with our previous observations using DHLA-capped QDs electrostatically self-assembled with engineered protein to form QD bioconjugates, where insignificant effects of salt concentration on the conjugates and their spectroscopic properties were observed.[9] The observations using QDs capped with PEG-terminated ligands confirm and extend those results.

6. Cellular Imaging Studies. One of the main limitations of currently available QDs for intracellular imaging is their tendency to aggregate in the cytosol and their sensitivity to pH changes. The pH in the cytosol is close to 7.3, but it drops below 5 in some organelles. For example, dispersions of DHLA-capped QDs are unstable at pH values below 7, and they aggregate soon after they are introduced into the cytoplasm by microinjection (Figure 9A). Precipitation and aggregation of DHLA-capped QDs in the cell cytoplasm is also observed using other approaches, such as cationic lipid-based loading and scrape loading.[27,28] In light of the enhanced pH stability of QDs capped with the new DHLA-PEG ligands, we tested their use for imaging the cytoplasm of live cells. The results presented here were obtained using microinjection of QDs directly into the cytoplasm, but other approaches for introducing QDs into live cells yielded similar results. QDs capped with either compound **10** (Figure 9B) or **11** (Figure 9C) dispersed better than the

DHLA-capped QDs when injected into the cytosol. Over the subsequent 12−24 h, a fraction of QDs slowly aggregated, as assayed by the appearance of punctate QD fluorescence (Figure 9D). Microinjection of QDs capped with PEG-terminated monothiol ligands showed homogeneous distribution in the cytoplasm of HeLa cells up to 2 h following reagent delivery.[51] Since poly(ethylene glycols) are capable of binding and fusing lipid membranes,[52] it is thus possible that the aggregates of nanocrystals that appear upon longer incubation are due to binding of the PEG-capped QDs to the membrane of the intracellular organelles. However, from the present set of data, we cannot unequivocally distinguish if the punctuates of fluorescence are due to aggregation or to QDs binding to membranes of the organelles present in the cytoplasm. In agreement with the potential fusogenic role of PEG, we occasionally observed that cells injected using a relatively high concentration (>10 μM) of DHLA-PEG-capped QD solutions fused together several minutes after loading (Figure 9E,F). When monitored over a period of at least 2 days, the cells labeled with DHLA-PEG-capped QDs were alive and showed no obvious growth defects, compared to unlabeled cells. This suggests that QDs prepared in this method could be useful for long-term cellular imaging.

Conclusions

We have designed and synthesized a new series of organic ligands that are capable of converting hydrophobic nanocrystals to water-soluble QDs. This synthetic approach is based on a simple modification of thioctic acid with various lengths of poly(ethylene glycols). Key features of the nanoparticles prepared using these PEG-modified ligands include the ability to prepare aqueous solutions of QDs that are aggregate-free and chemically stable over long periods of time (several months). By using mixed compositions of surface ligands with DHLA, we are able to assess the degree of charge on the nanoparticle surface by gel electrophoresis and to employ a metal-affinity-driven self-assembly method to form QD−protein conjugates.

One limitation of using organic fluorophores in many cellular applications is their sensitivity to the environment. For example, across the compartments of a live cell, the concentrations of protons can vary by almost three log units and the concentration of calcium varies by five log units. Improving the stability of these QDs and the ability to conjugate them to molecules will make QDs more useful for numerous biological assays both in vitro and in living cells. The use of QDs in living cells will be further facilitated by the improved dispersion in the cytoplasm of live cells versus QDs solubilized by a charge-driven mechanism, such as DHLA, mercapto acetic acid, or other mercapto carbonic acids. These PEG-capped QDs have no obvious cytotoxic effects on tissue culture cells observed over a two day period. This should encourage their use for intracellular labeling and long-term imaging. This method of QD solubilization is an important step in creating all-purpose biocompatible QDs.

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Supporting Information Available: Detailed synthesis of all the TA-PEG and DHLA-PEG ligands prepared along with the corresponding NMR spectra. Chart of solubility for QDs capped with DHLA-PEG ligands. This material is available free of charge via the Internet at http://pubs.acs.org.

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