Supporting Information.

Multifunctional Compact Zwitterionic Ligands for Preparing Robust Biocompatible Semiconductor Quantum Dots and Gold Nanoparticles

Kimihiro Susumu,[1] Eunkeu Oh,[1] James B. Delehanty,[2] Juan B. Blanco-Canosa,[3] Brandy J. Johnson,[2] Vaibhav Jain,[1] William Judson Hervey, IV,[2] W. Russ Algar,[2,4] Kelly Boeneman,[2] Philip E. Dawson[3] and Igor L. Medintz[2]

[1]Optical Sciences Division, Code 5611
[2]Center for Bio/Molecular Science and Engineering, Code 6900
U.S. Naval Research Laboratory
Washington, DC 20375

[3]Departments of Cell Biology and Chemistry
The Scripps Research Institute
La Jolla, CA 92037

[4]College of Science
George Mason University
Fairfax, VA 22030

Analytical Instrumentation. ^{1}H NMR spectra were recorded on a Bruker SpectroSpin 400 MHz spectrometer. Chemical shifts for ^{1}H NMR spectra are reported relative to tetramethylsilane (TMS) signal in the deuterated solvent (TMS, δ = 0.00 ppm). All *J* values are reported in hertz. Electronic absorption spectra were recorded using an HP 8453 diode array spectrophotometer (Agilent Technologies, Santa Clara, CA). Fluorescence spectra were collected using a Tecan Safire Dual Monochromator Multifunction Microtiter Plate Reader (Tecan, Research Triangle Park, NC) or a Spex Fluorolog-3 spectrophotometer (Jobin Yvon Inc, Edison, NJ) equipped with a red-sensitive R2658 Hamamatsu PMT detector. Fluorescence quantum yields were measured at room temperature with Rhodamine 6G in methanol (Φ_f = 0.93) as standard. The obtained fluorescence spectra were corrected using the spectral output of a calibrated light source supplied by the National Bureau of Standards. Dynamic light scattering measurements were performed on a CGS-3 goniometer system equipped with a HeNe laser illumination at 633 nm and a single photon counting avalanche photodiode for signal detection (Malvern Instruments). The autocorrelation function was performed by an ALV-5000/EPP photon correlator and analyzed using Dispersion Technology Software (DTS, Malvern Instruments). All QD solutions were filtered through 0.02 μm syringe filters (Whatman). The sample temperature was maintained at 20 °C. The autocorrelation function was the average of three runs of 10 sec each, and then repeated at different scattering angles ranging from 50° to 110°. The Laplace transform CONTIN analysis was applied to extract intensity versus hydrodynamic size profiles for the QD dispersions studied.[1] Laser Doppler velocimetry measurements were performed using a ZetaSizer NanoSeries equipped with a He-Ne laser source (λ = 633 nm) and an avalanche photodiode for detection, controlled with DTS software. Micromolar concentration solutions of QDs were loaded into disposable folded capillary cells, and data were collected at 20°C. Three runs of the measurements were performed for each sample. Mass spectrometry data of the ligand compounds were acquired on a quadrupole time-of-flight mass spectrometer (QSTAR Elite, AB Sciex, Foster City, CA). The instrument was operated in positive mode, with samples directly infused from the syringe pump through an IonSpray electrospray ionization source at a flow rate of 1.0 μL/min. Structural characterization of the QDs was carried out using a JEOL 2100-FS analytical high-resolution transmission electron microscope (HR-TEM) with a 200 kV accelerating voltage. Samples for TEM were prepared by spreading a drop of the QD dispersion

onto the holey carbon film on a fine mesh Cu grid (400 mesh) and letting it dry. The individual particle sizes were measured using Olympus measureIT and ImageJ; the average sizes and standard deviations were extracted from analysis of ~100 nanoparticles on average.

Characterization of Compact Ligand-Coated Quantum Dots and Gold Nanoparticles.

pH Stability Test. QD samples were dissolved in buffer solutions of different pH, and the QD concentrations were adjusted to ~0.5 μM. Buffer solutions used are as follows: 50 mM KCl + HCl for pH 2; 0.1 M AcOH + 0.1 M NaOAc for pH 3-6; 50 mM Tris + HCl for pH 7 and 9; 25 mM $NaHCO_3$ + NaOH for pH 11; 50 mM KCl + NaOH for pH 12 and 13. All samples were stored in a refrigerator and periodically taken out for collecting photographs.

Salt Stability Test. QD samples were dissolved in concentrated NaCl solutions, and the QD and NaCl concentrations were adjusted to ~0.5 μM and 3 M, respectively. All samples were stored in a refrigerator and periodically taken out for collecting photographs.

Contact Angles. Wettability of the ligands was determined by coating mica surfaces with **CL**-QDs and observing the resulting contact angles of water using sessile drop goniometry. In addition to providing an evaluation of QD wetting behavior, this measurement provides an indication of the relative hydrophilicity of the ligands used. Mica disks (AFM Quality V1, 10 mm diameter, Ted Pella) were cleaned in piranha solution (30/70 v/v mixture of 30% hydrogen peroxide and 70% sulfuric acid) for 45 min at room temperature, rinsed thoroughly in DI water (18.2 MΩ-cm from a Millipore Milli Q UV-Plus water purification system) and dried. The QDs were utilized as 5 μM solutions in water. Drops of 100 μL of each QD solution were applied to cleaned mica disks, distributed to provide complete coverage of the surface and allowed to dry at 40°C. Dry samples were visually homogenous and tinged with color from the QDs. Sessile drop goniometry was completed using a Ramé-Hart 100-00-115 Contact Angle Goniometer. DI water droplets of 3 μL were used, and reported values are the result of the average of four measurements collected over two samples which yielded deviations of <10% in all cases.

Preparation of 5xHis-MBP and Labeling with Texas Red Dye. The *E. coli* derived maltose binding protein (MBP) expressing a C-terminal pentahistidine sequence (5xHis-MBP) was

prepared as described.[2] The MBP used in the present studies was engineered to express a cysteine residue at position 95, replacing the native aspartic acid residue (5xHis-MBP95C, 95asp→cys). Texas Red C_2 maleimide dye (Life Technologies, Carlsbad, CA) was used to specifically label the 95C residue on 5xHis-MBP.[2,3] For labeling, MBP was reduced with dithiothreitol for 1 h, desalted over a PD-10 desalting column (GE Healthcare, Piscataway, NJ) and then mixed with the activated dye. MBP-Texas Red conjugates were purified from free dye over PD-10 columns. This procedure provided an average labeling ratio of ~1.0 Texas Red/MBP, as deduced from UV-vis absorption analysis (MBP ε~69,000 $M^{-1}cm^{-1}$ at 280 nm and Texas Red ε~80,000 $M^{-1}cm^{-1}$ at 595 nm, with an 18% Texas Red correction factor applied for absorbance at 280 nm).

Self-Assembly of Quantum Dot-Protein Conjugates and Characterization Using Agarose Gel Electrophoresis. 550 nm emitting CdSe-ZnS QDs cap exchanged with **CL1, CL2** and **CL4** were self-assembled with 5xHis-tagged MBP labeled with Texas Red. Samples were prepared by incubating increasing molar ratios of the MBP with the same concentration of the QDs; 5 pmol of QDs was utilized per sample. Assembled samples were mixed with 30% glycerol loading buffer and then run on 1.5 % agarose gel using 1x TBE buffer (90 mM Tris borate, 2mM EDTA, pH 8.3) for ~10 min with a field strength of ~10 V/cm gel length. Fluorescent images were collected on a Kodak 440 Digital Image Station (Rochester, NY) using 365 nm excitation and 525 nm band-pass or 595 nm long-pass cut-off filters.

EDC Coupling. Typical reaction conditions used are as follows. 550 nm emitting **CL1**-QDs (33.7 µM, 29.7 µl, 1.0 x 10^{-9} mol), Lissamine rhodamine B ethylenediamine (211 µM in DMSO:H_2O (~3:7 (v/v)), 71 µl, 1.5 x 10^{-8} mol), sulfo-NHS (200 mM in 10 mM phosphate buffer (pH 7.0), 50 µl, 1.0 x 10^{-5} mol) and additional 10 mM phosphate buffer (pH 7.0, 99.3 µl) were mixed in a 1.5 mL Eppendorf tube. EDC (1.0 M in 10 mM phosphate buffer pH 7.0, 50 µl, 5.0 x 10^{-5} mol) was added to the mixture to initiate the reaction. After stirring for 2 h at room temperature, the mixture was loaded onto a PD-10 desalting column and elution of the QD product (band) was traced by hand-held UV lamp. The first emitting single band was collected and used for spectroscopic analysis without further purification. For control reactions without EDC, extra buffer was used to adjust the final concentration of the reaction mixture. For EDC

coupling to AuNPs, a Cy3-labeled peptide (CSTRIDEANQRATKLP_7SH_6) was utilized. The N-terminal cysteine on this peptide was labeled with Cy3-maleimide (GE Healthcare) and the peptide purified as described.[4] The EDC reaction targets the unique lysine-amine near the C-terminus in this sequence.

FRET Analysis. FRET efficiency *E* was determined using:

$$E_n = \frac{(F_D - F_{DA})}{F_D} \qquad \text{(Eq. 1)}$$

where *n* is the ratio or valence of dye-acceptors per QD and F_D, F_{DA} designate the fluorescence intensities of the QD donor alone and donor in the presence of acceptor(s), respectively.[5] Data from FRET efficiency were analyzed using Förster theory to determine values for the center-to-center (QD-to-dye) separation distance *r* by using Equation (2) which presumes a centro-symmetric distribution of dye-acceptors around the central QD:[3]

$$r = \left(\frac{n(1 - E_n)}{E_n} \right)^{1/6} R_0 \qquad \text{(Eq. 2)}$$

where R_0 is the Förster distance corresponding to a FRET efficiency *E* of 50% for a configuration consisting of a single QD donor coupled to a single dye-acceptor and is defined by:[5]

$$R_0 = 9.78\text{x}10^3[\kappa^2 \breve{n}^{-4} Q_D I]^{1/6} \qquad \text{(Eq. 3)}$$

where $\breve{n}$ is the refractive index of the buffer medium, Q_D is the fluorescence quantum yield (QY) of the donor, *I* is the spectral overlap integral, and κ^2 is the dipole orientation factor. We use a κ^2 of 2/3 which is appropriate for the random dipole orientations found within these self-assembled configurations as described.[3]

Cellular Delivery of Compact Ligand Coated Quantum Dots

***Cell Culture*.** COS-1 cells (African green monkey kidney cell line, ATCC, Manassas, VA) were cultured in complete growth medium (Dulbecco's Modified Eagle's Medium, DMEM) supplemented with 1% (v/v) antibiotic/antimycotic and 10% (v/v) heat inactivated fetal bovine

serum (ATCC). Cells were cultured in T-25 flasks and incubated at 37ºC under 5% CO_2 atmosphere and subcultured every 3-4 days as described previously.[6] For microinjection, the cells were seeded onto 35 mm coverslip bottom plastic dishes (BD Biosciences, Bedford, MA) coated with poly-L-lysine and 50 μg/mL fibronectin (Sigma Aldrich, St Louis, MO) in sodium bicarbonate buffer pH 8.5. Approximately 1.5 x 10^4 cells were seeded per well and cultured overnight.

Microinjection and Imaging. Stock solutions of 5-10 μM QDs surface-functionalized with the indicated ligands were prepared in 1x phosphate buffered saline (PBS, 137 mM NaCl, 10 mM phosphate, 3 mM KCl, pH 7.4), diluted to 2 μM in PBS and filtered through an Anotop 10 0.2-micron inorganic membrane syringe filters (Whatman, Maidstone, England) prior to microinjection. Femtoliter aliquots of QDs were directly injected into adherent COS-1 cells using an InjectMan® NI2 micromanipulator equipped with a FemtoJet programmable microinjector (Eppendorf, Westbury, NY).[7,8] During microinjection, the cells were buffered in DMEM supplemented with 25 mM HEPES pH 7.4 (Life Technologies, Carlsbad, CA). Epifluorescence image collection was carried out using an Olympus IX-71 total internal reflection fluorescence microscope equipped with a 60x or 100x oil immersion lens where samples were illuminated with a Xe lamp for UV excitation or a visible/bright light source. Differential interference contrast images (DIC) were collected using the bright light source. Filters utilized to collect spectrally resolved images include those for green 550 nm emitting QD photoluminescence (PL) (excitation D420/40, dichroic T495LP, emission D525/35) and red 625 nm emitting QD PL (excitation D420/40, dichroic D595LP, emission D630/60). Cellular micrographs were captured with a DP71 color digital camera (Olympus, Center Valley, PA). Images were analyzed and prepared using DP Manager Software (Olympus) and Adobe Photoshop.

Quantum Dot Uptake with Cell Penetrating Peptide. The sequence of the cell-penetrating peptide (CPP) used in this study is R_9GGLA(Aib)SGWKH_6, where Aib is the artificial residue alpha-aminoisobutyric acid.[6] All QD delivery experiments were performed on adherent cells seeded into the wells of Lab-Tek 8-well chambered #1 borosilicate cover glass (Nalge Nunc, Rochester, NY) previously coated with 2 μg/mL fibronectin. For preparation of CL-QD-CPP

complexes, the CPPs were first diluted into DMEM containing 25 mM HEPES (DMEM-HEPES, Life Technologies, Carlsbad, CA). The appropriate amount of CL-QDs was then added and CPP-QD complex formation was allowed to proceed for approximately 10-15 minutes. For cellular deliveries, 100 nM of unconjugated QDs or QD-CPP complexes (assembled with 25-50 CPP per QD) were diluted into DMEM containing 25 mM HEPES (Life Technologies, Carlsbad, CA) and incubated on the cells for 1-2 hours. Endosomes were counterstained by inclusion of 30μg/mL AlexaFluor 647-transferrin (Life Technologies) during QD delivery. For subsequent imaging, cells were washed with 1x PBS, fixed with 3.7% paraformaldehyde in PBS and cell nuclei were stained with DAPI (Sigma Aldrich) unless otherwise indicated. The intracellular distribution of delivered QDs was observed with differential interference contrast (DIC) and epifluorescence microscopy using the Olympus IX-71 microscope equipped with a 60x oil immersion lens. Samples were excited using a Xe lamp and images were collected using standard filter sets for DAPI, FITC (for QDs) and Cy5 (for AlexaFluor 647-transferrin).

Cellular Proliferation Assays. Cellular toxicity was assessed by measuring cellular proliferation using the CellTiter 96 Cell Proliferation Assay (Promega, Madison, WI).[6] The assay is based upon the conversion of a tetrazolium substrate to a formazan product by viable cells at the assay end point. Cells (1×10^4 cells/well) were cultured in 96-well microtiter plates and then incubated with increasing concentrations of QDs, free CPP or QDs complexed with CPP in DMEM containing 25 mM HEPES for 2 hours. Materials were subsequently replaced with complete growth medium and cells cultured for 72 h prior to assaying viability.

References

(1) Pons, T.; Uyeda, H. T.; Medintz, I. L.; Mattoussi, H. *J. Phys. Chem. B* **2006**, *110*, 20308-20316.
(2) Medintz, I. L.; Goldman, E. R.; Lassman, M. E.; Mauro, J. M. *Bioconjugate Chem.* **2003**, *14*, 909-918.
(3) Clapp, A. R.; Medintz, I. L.; Mauro, J. M.; Fisher, B. R.; Bawendi, M. G.; Mattoussi, H. *J. Am. Chem. Soc.* **2004**, *126*, 301-310.
(4) Sapsford, K. E.; Farrell, D.; Sun, S.; Rasooly, A.; Mattoussi, H.; Medintz, I. L. *Sensors and Actuators B-Chemical* **2009**, *139*, 13-21.
(5) Lakowicz, J. R. *Principles of Fluorescence Spectroscopy*; 3rd ed.; Springer: New York, 2006.
(6) Delehanty, J. B.; Bradburne, C. E.; Boeneman, K.; Susumu, K.; Farrell, D.; Mei, B. C.; Blanco-Canosa, J. B.; Dawson, G.; Dawson, P. E.; Mattoussi, H.; Medintz, I. L. *Integr. Biol.* **2010**, *2*, 265-277.
(7) Mei, B. C.; Susumu, K.; Medintz, I. L.; Delehanty, J. B.; Mountziaris, T. J.; Mattoussi, H. *J. Mater. Chem.* **2008**, *18*, 4949-4958.

(8) Boeneman, K.; Delehanty, J. B.; Susumu, K.; Stewart, M. H.; Medintz, I. L. *J. Am. Chem. Soc.* **2010**, *132*, 5975-5977.
(9) Susumu, K.; Medintz, I. L.; Delehanty, J. B.; Boeneman, K.; Mattoussi, H. *J. Phys. Chem. C* **2010**, *114*, 13526-13531.
(10) Susumu, K.; Uyeda, H. T.; Medintz, I. L.; Pons, T.; Delehanty, J. B.; Mattoussi, H. *J. Am. Chem. Soc.* **2007**, *129*, 13987-13996.
(11) Susumu, K.; Mei, B. C.; Mattoussi, H. *Nat. Protoc.* **2009**, *4*, 424-436.

Supporting Figures:

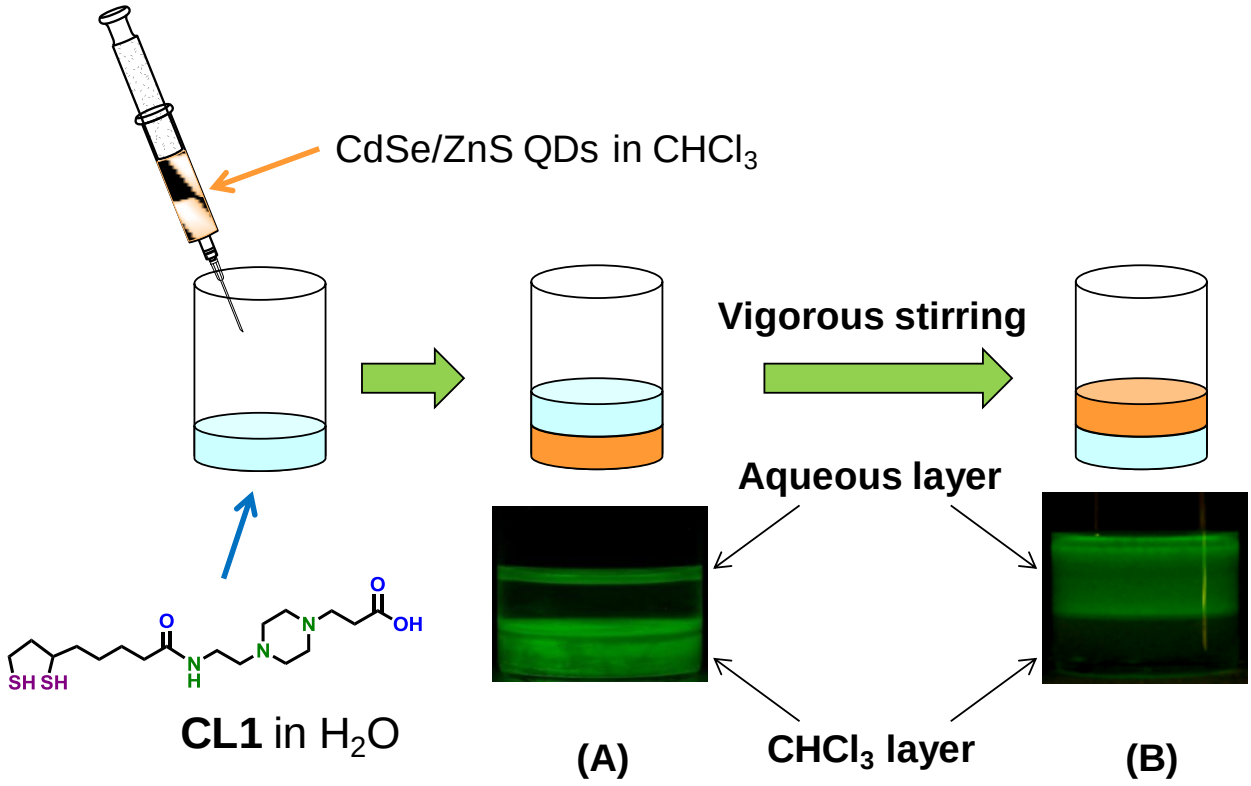


Figure S1. Schematic representation of cap exchange process using the compact ligand **CL1** as an example. Luminescence images of 550 nm emitting CdSe-ZnS QDs before (A) and after (B) cap exchange in a biphasic mixture are also shown.

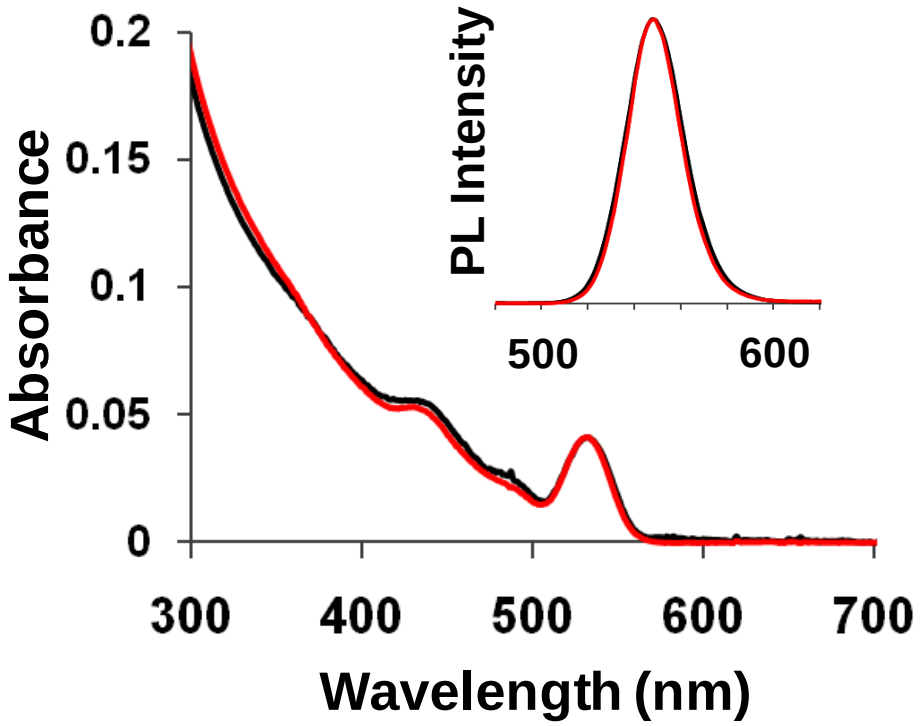


Figure S2. Absorption and fluorescence (inset) spectra of CdSe-ZnS QDs with original hydrophobic ligands in hexane (black line) and with **CL1** in H_2O (red line).

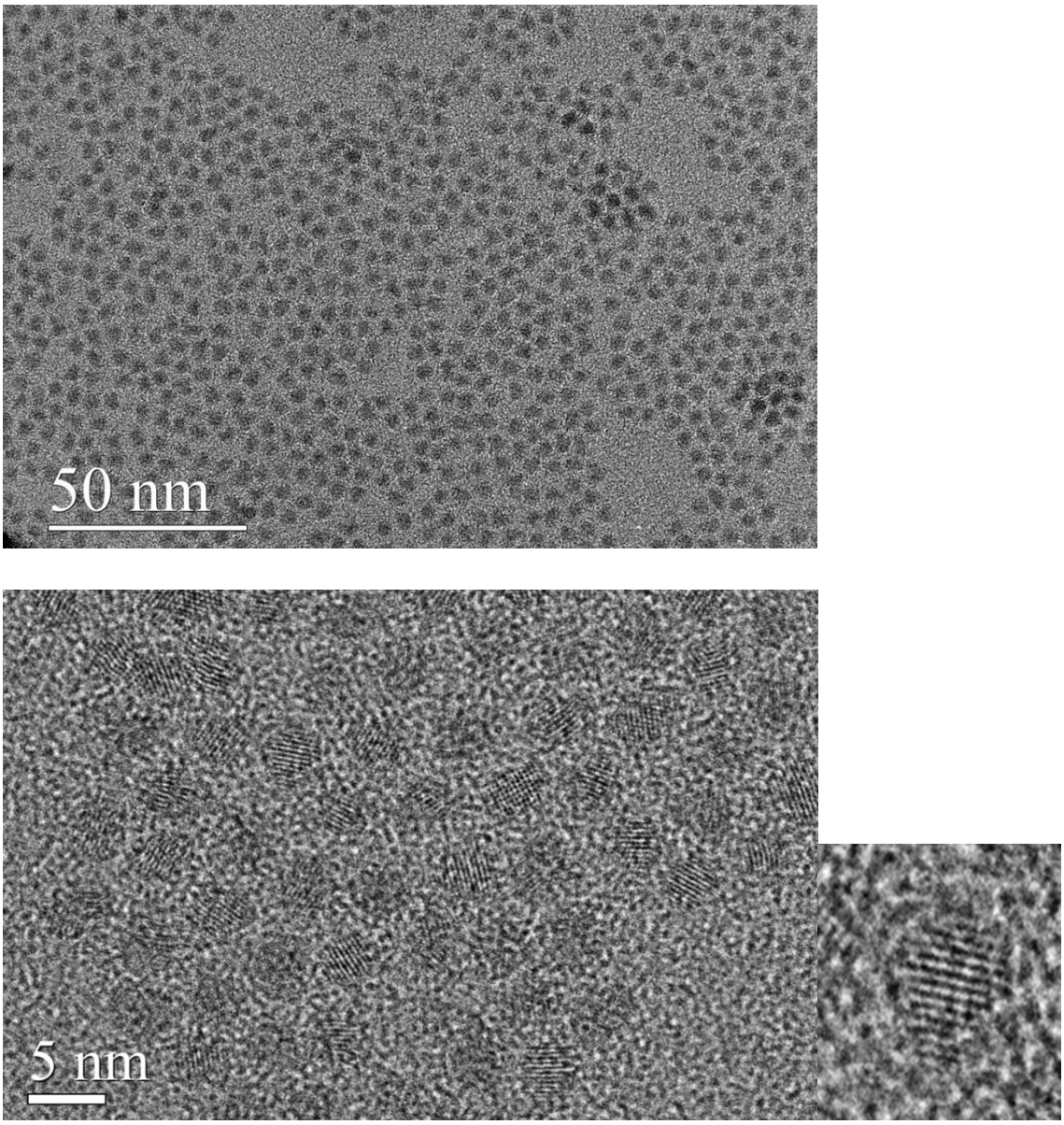
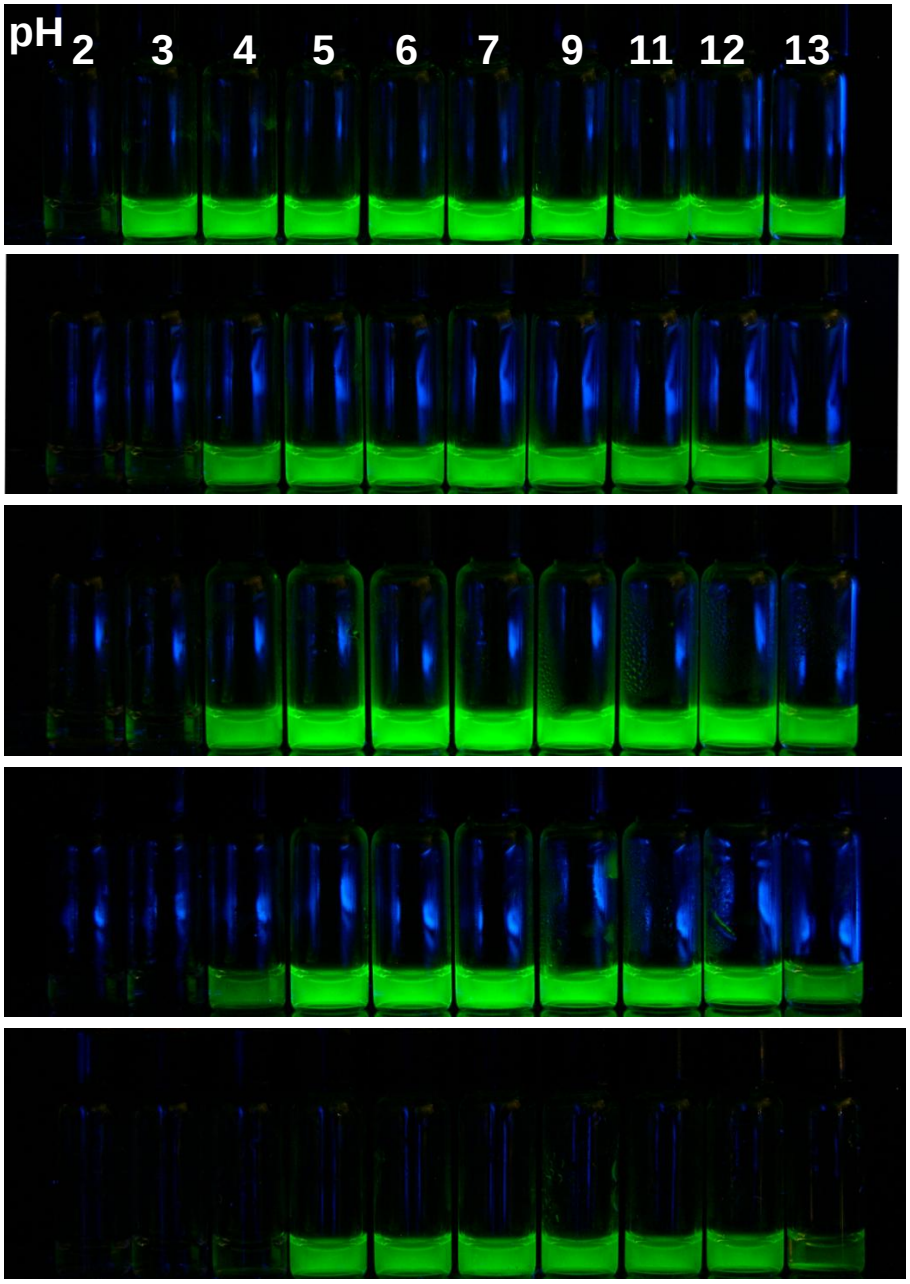
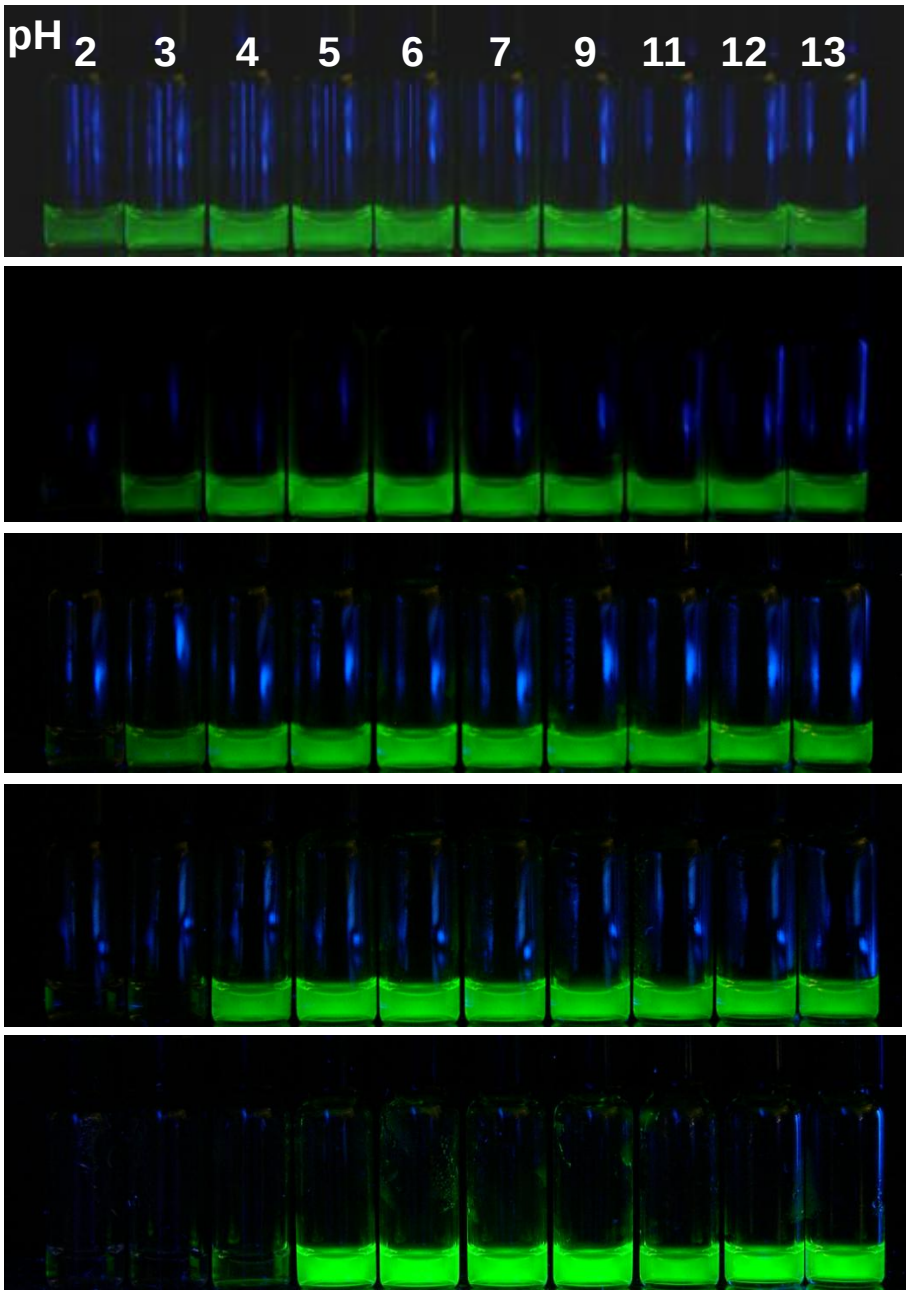


Figure S3. High-resolution TEM images of 550 nm emitting CdSe-ZnS QDs with original hydrophobic ligands. Average size of the QDs is 4.0 ± 0.29 nm.

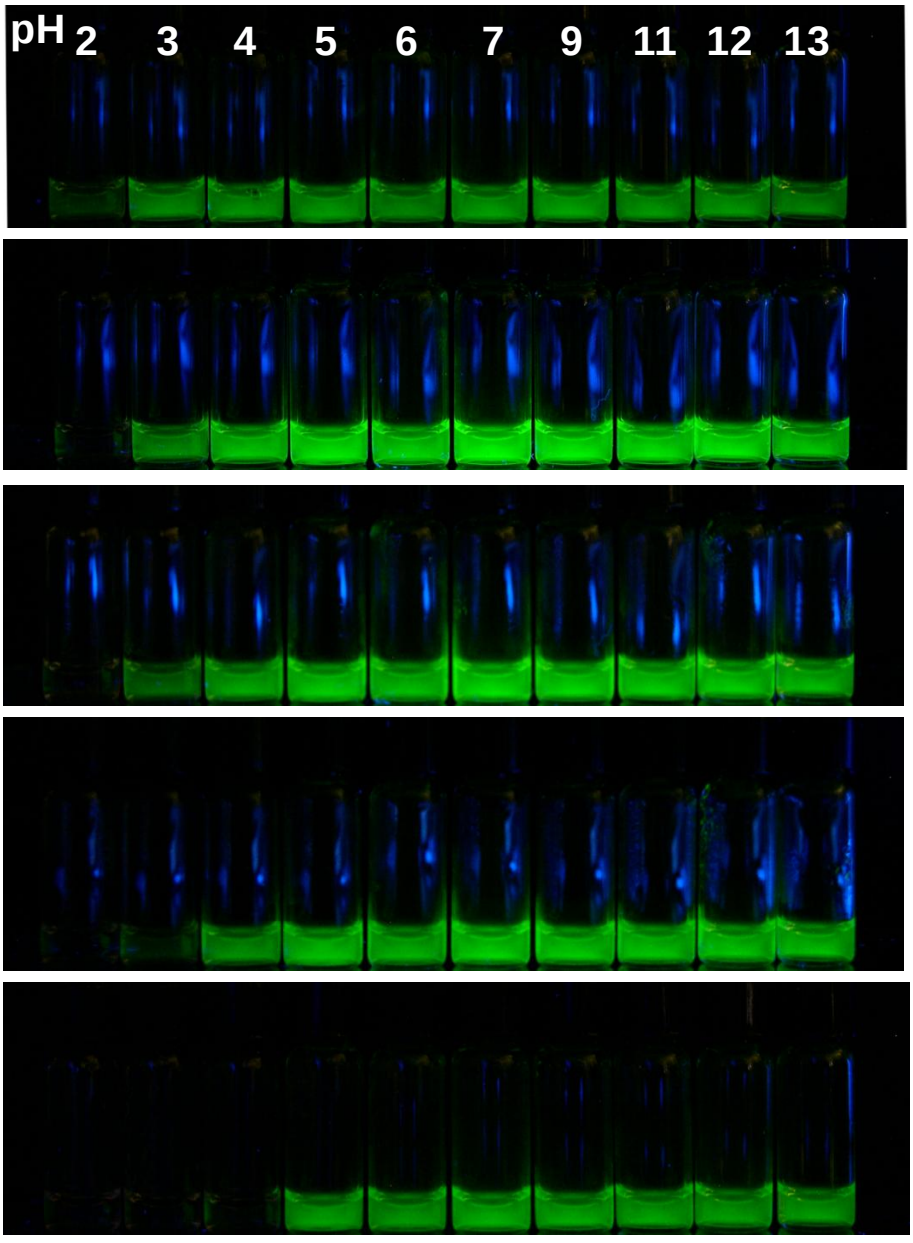
(A) **CL1**-QD



(B) **CL2**-QD



(C) **CL3**-QD



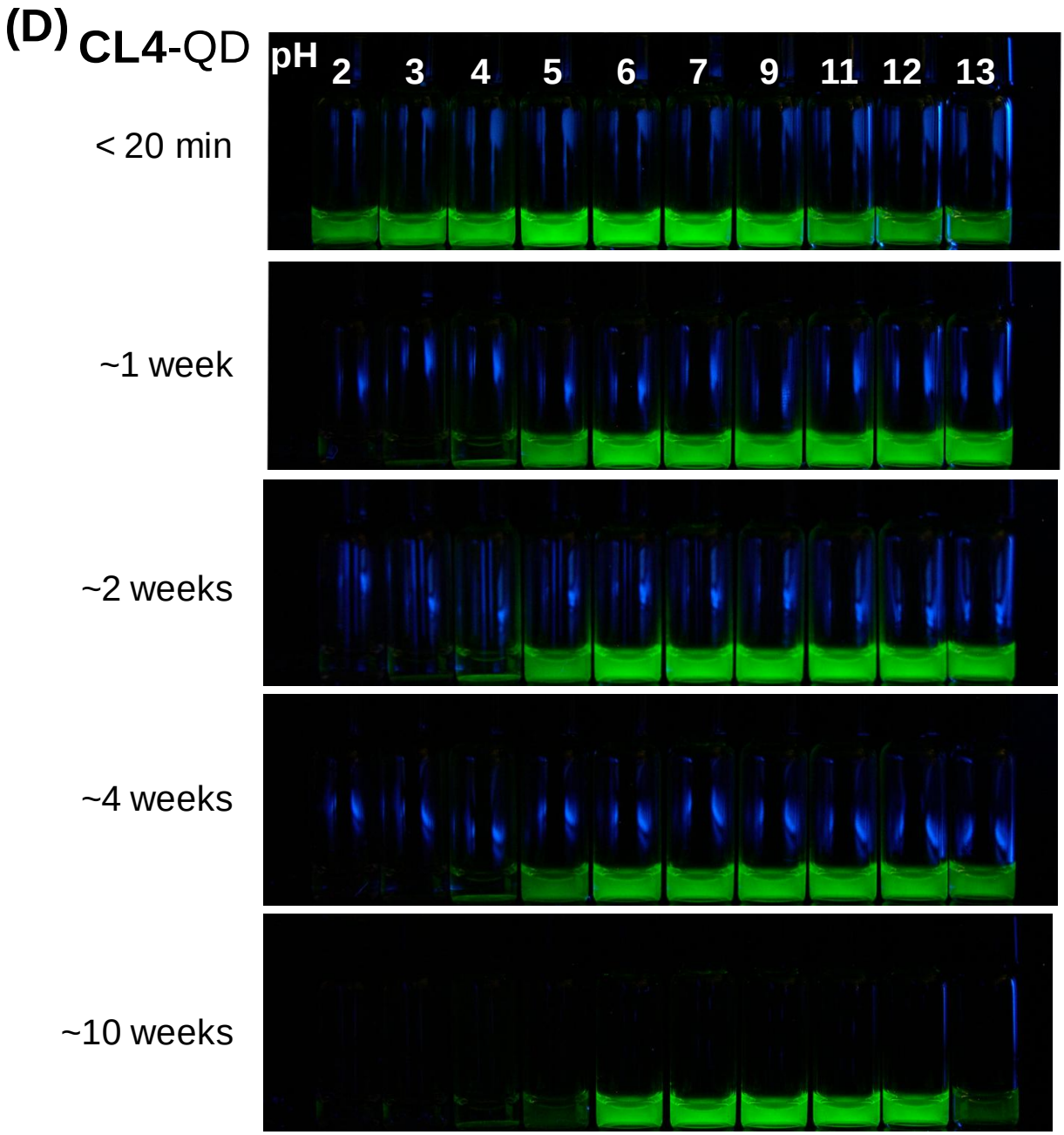


Figure S4. Luminescence images for a set of 0.5 μM QD solutions in different buffers at pH 2 ~ 13: (A) **CL1**-QD; (B) **CL2**-QD; (C) **CL3**-QD; (D) **CL4**-QD. CdSe-ZnS QDs emitting at 550 nm were used and were excited with a hand-held UV lamp at 365 nm.

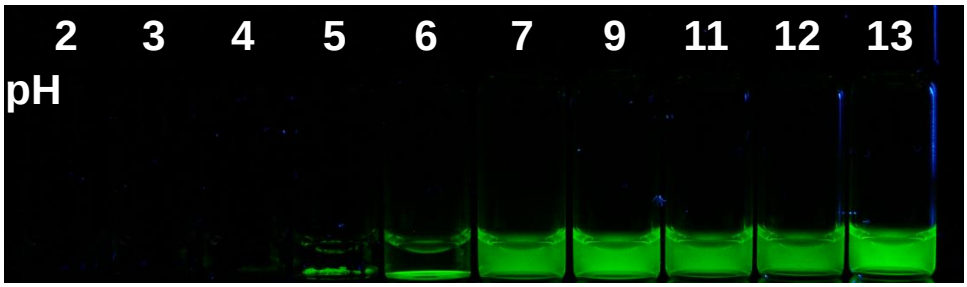


Figure S5. Luminescence images of DHLA-coated CdSe-ZnS QDs in different buffers at pH 2 ~ 13 after 4 days. CdSe-ZnS QDs emitting at 550 nm were used and were excited with a hand-held UV lamp at 365 nm.

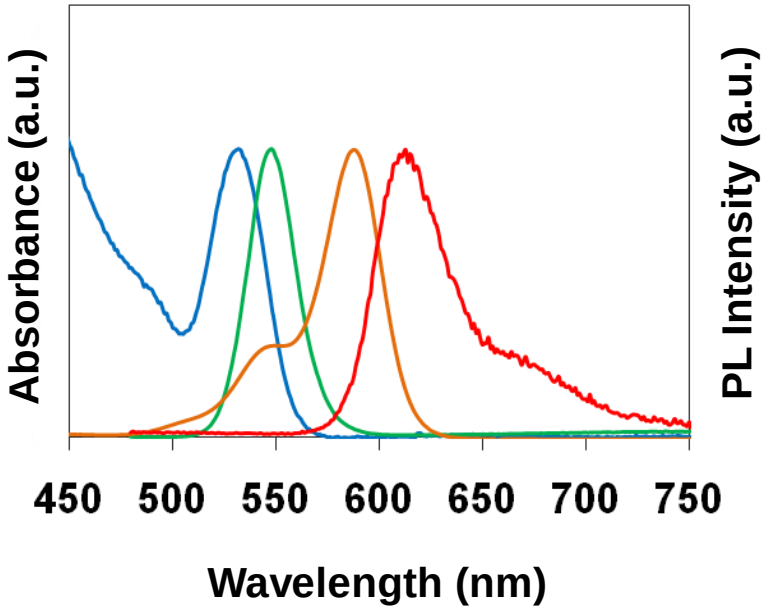


Figure S6. Normalized absorption spectra of 550 nm emitting QD (blue line) and Texas Red (orange line), together with the fluorescence spectra of the QD (green line) and Texas Red (red line). The spectral overlap integral between the QD fluorescence and Texas Red absorption was calculated to be 2.98 x 10^{-13} $M^{-1}cm^3$.[3,9] For the 550 nm emitting QD of which fluorescence quantum yield is 15 %, Förster distance, corresponding to 50 % energy transfer efficiency, was estimated to be 4.5 nm.

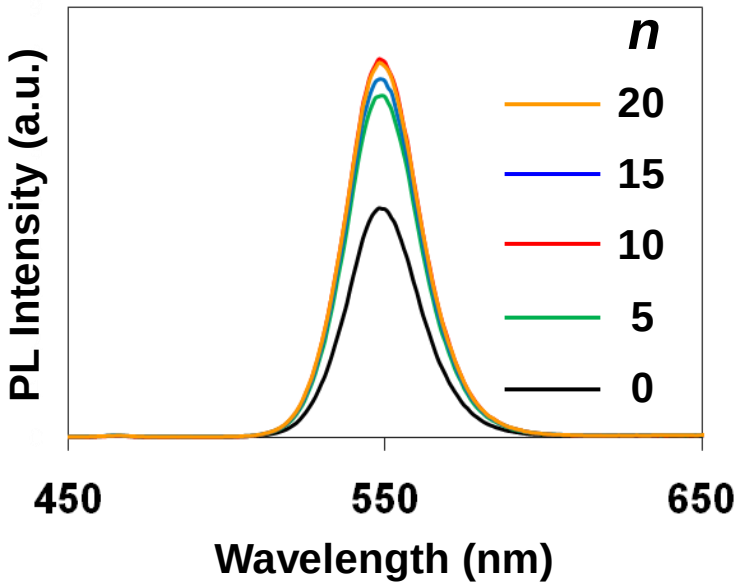


Figure S7. Fluorescence intensity changes of 550 nm emitting CdSe-ZnS **CL1**-QDs titrated with "*n*" equivalents of His-tagged MBP.

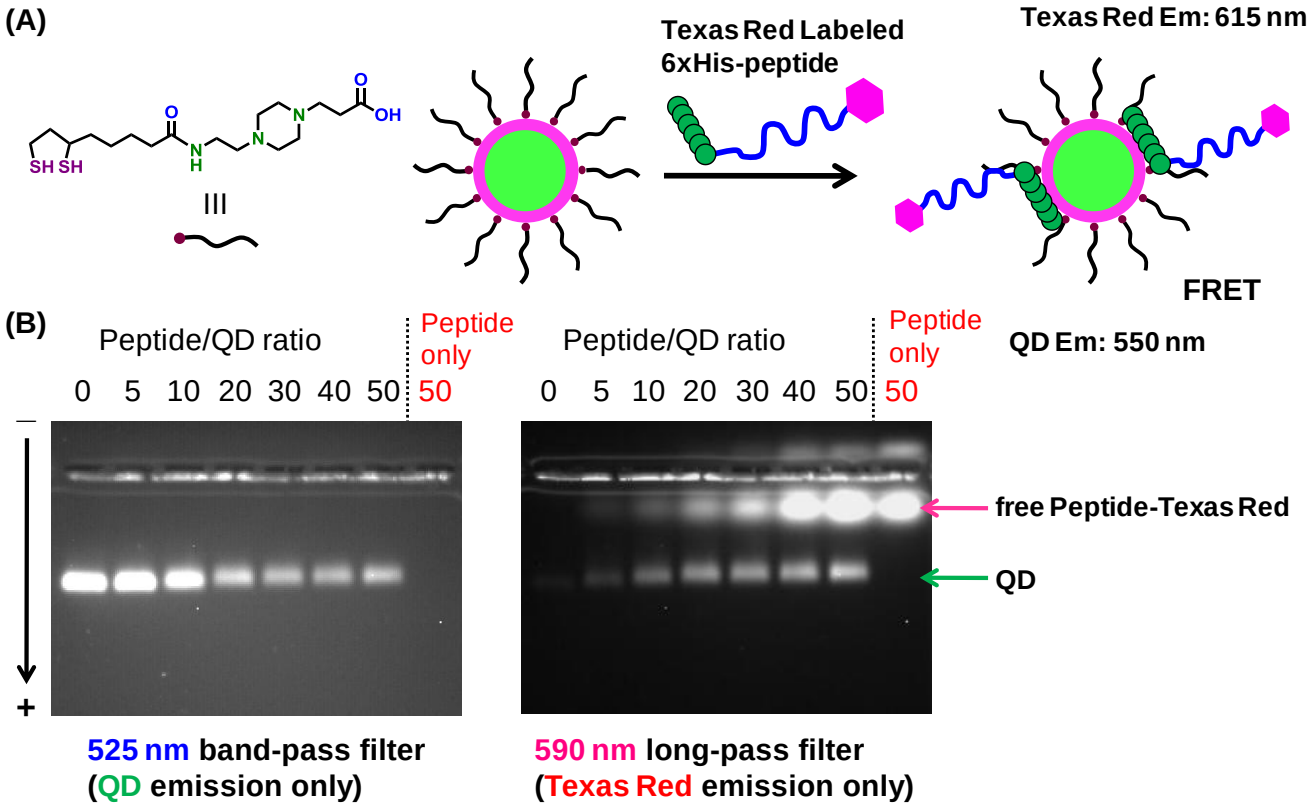


Figure S8. (A) Schematic representation of the FRET assay used to investigate direct assembly of His-tagged peptide labeled with Texas Red onto QD surface. (B) Gel electrophoresis images of 550 nm emitting **CL1**-QD self-assembled with "*n*" equivalents of 6xHis-peptide-Texas Red. Lane labeled "0" contains only QD while that labeled "Peptide only" contains only His-peptide-Texas Red. Gels were run on 1.5 % agarose gel in 1x TBE buffer (pH 8.3) for ~10 min, and the images were captured with 525 nm band-pass and 590 nm long-pass filters to highlight the QD and Texas Red fluorescence, respectively.

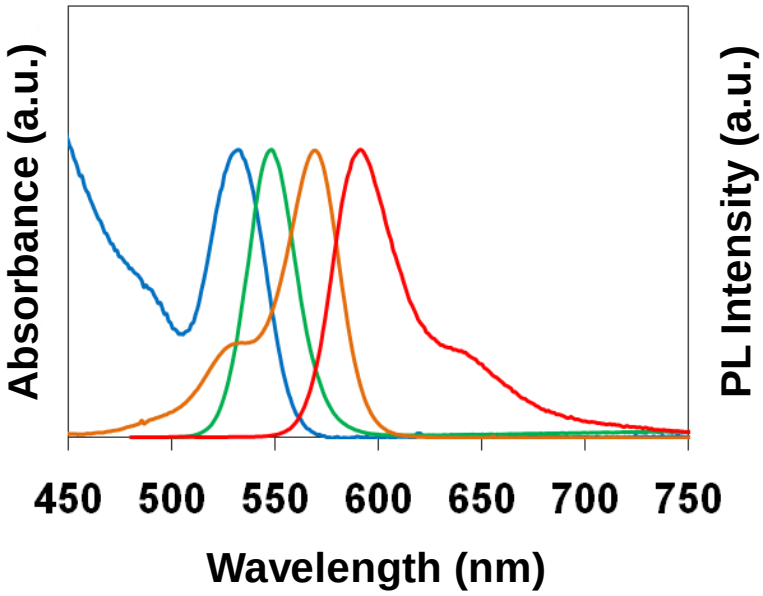
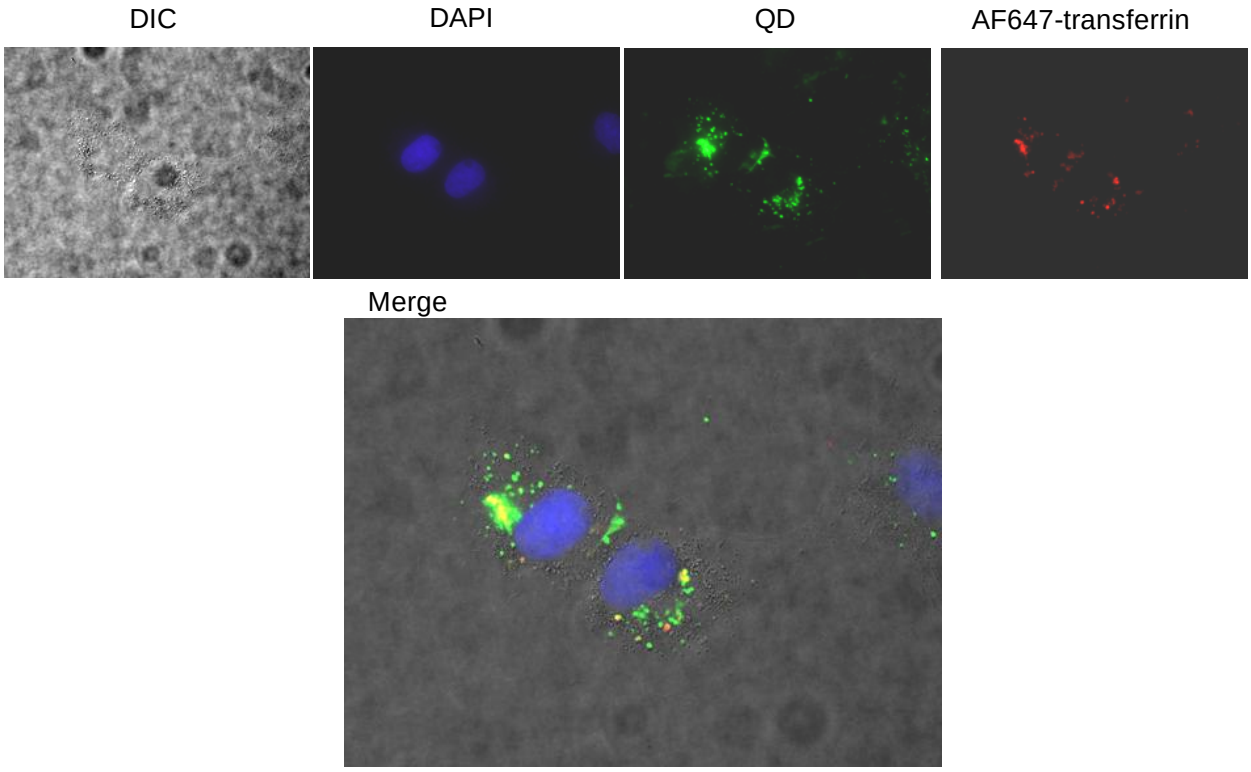
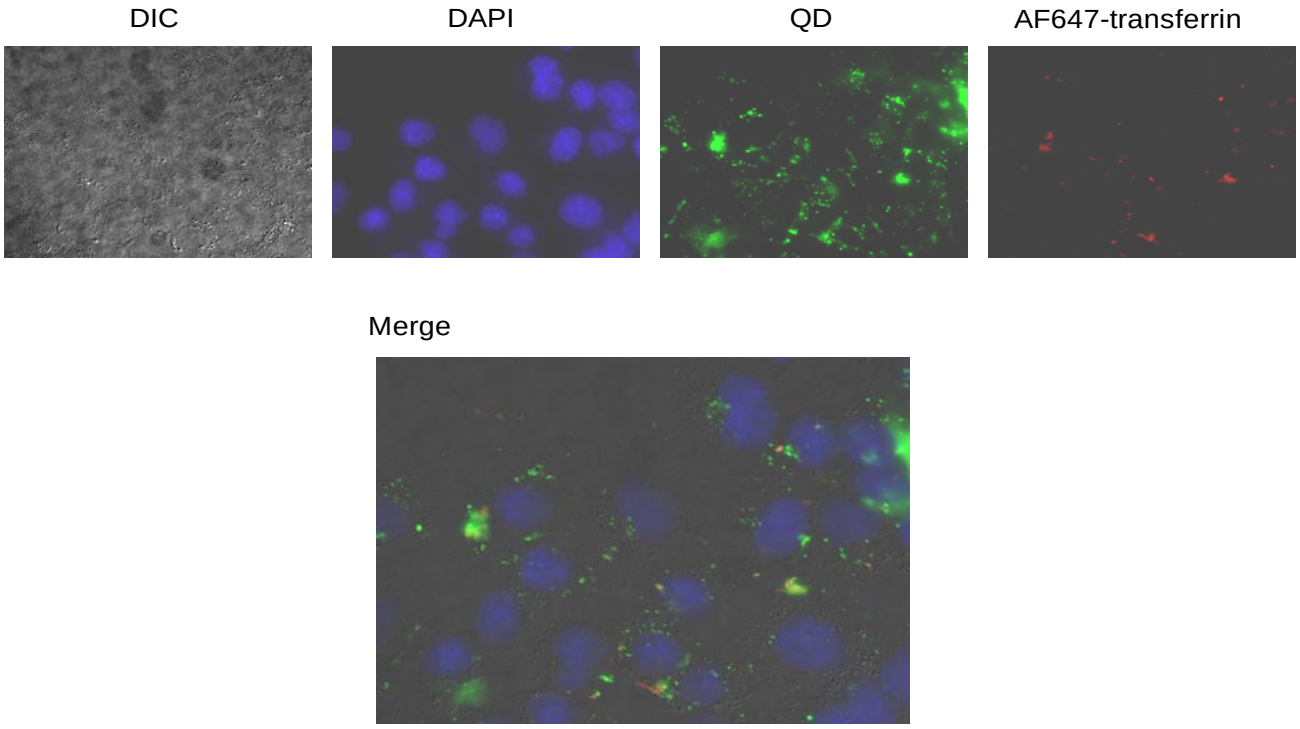


Figure S9. Normalized absorption spectra of 550 nm emitting QD (blue line) and Lissamine Rhodamine B ethylenediamine (orange line), together with the fluorescence spectra of the QD (green line) and the rhodamine B dye (red line). The spectral overlap integral between the QD fluorescence and the rhodamine dye absorption was calculated to be 5.30 x 10^{-13} $M^{-1}cm^3$.[3,9] For the 550 nm emitting QD of which fluorescence quantum yield is 15 %, Förster distance, corresponding to 50 % energy transfer efficiency, was estimated to be 4.9 nm.

(A) CL1-QD-CPP

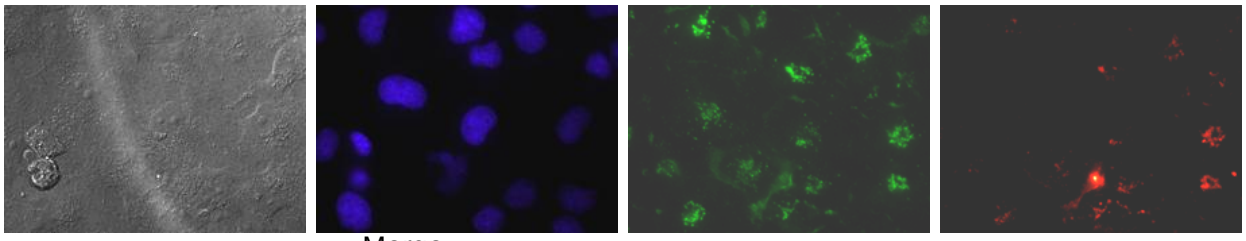


(B) CL2-QD-CPP

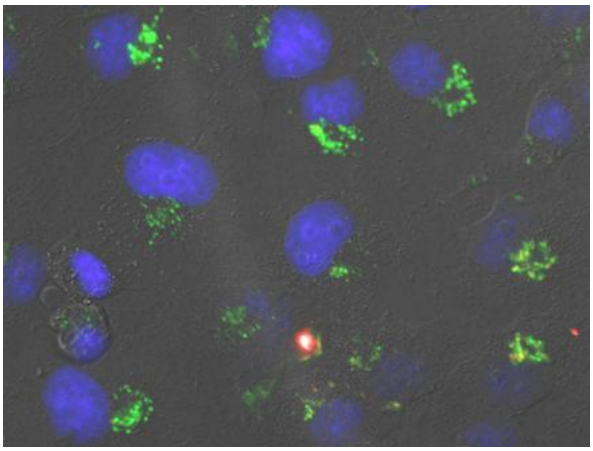


(C) CL3-QD-CPP

DIC DAPI QD AF647-transferrin

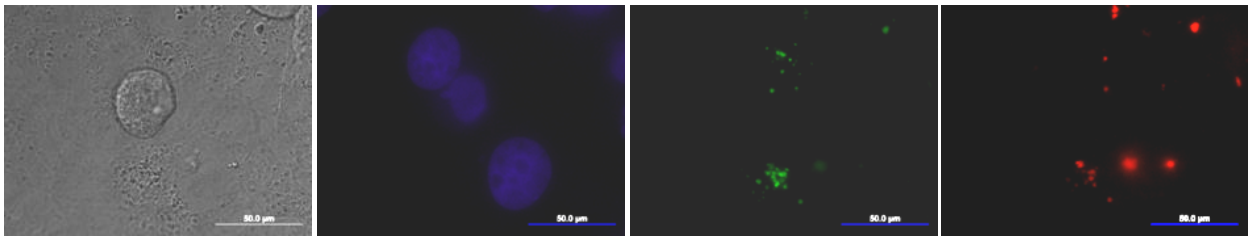


Merge



(D) CL4-QD-CPP

DIC DAPI QD AF647-transferrin



Merge

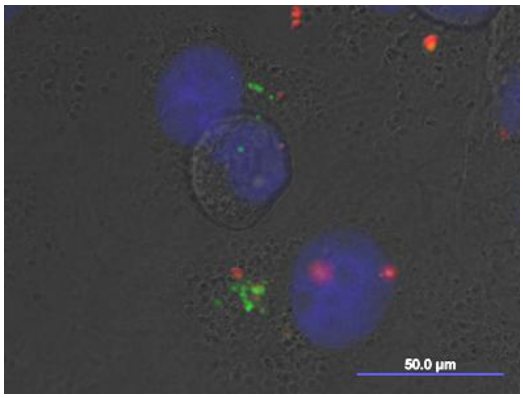


Figure S10. Cellular internalization and colocalization within endosomes of **CL**-QD-CPP assemblies: (A) **CL1**-QD, (B) **CL2**-QD, (C) **CL3**-QD, and (D) **CL4**-QD. COS-1 cells were coincubated with 550 nm emitting **CL**-QD-CPP assemblies (**CL**-QD:CPP ratio 1:50) at QD concentrations of 100 nM and 30 μg/mL AlexaFluor647-transferrin for 1-2 h. Nuclei were also stained with DAPI. For each series, the corresponding differential interference contrast (DIC) image, DAPI, 550 nm emitting QD, AlexaFluor647-transferrin and merged fluorescence images are shown.

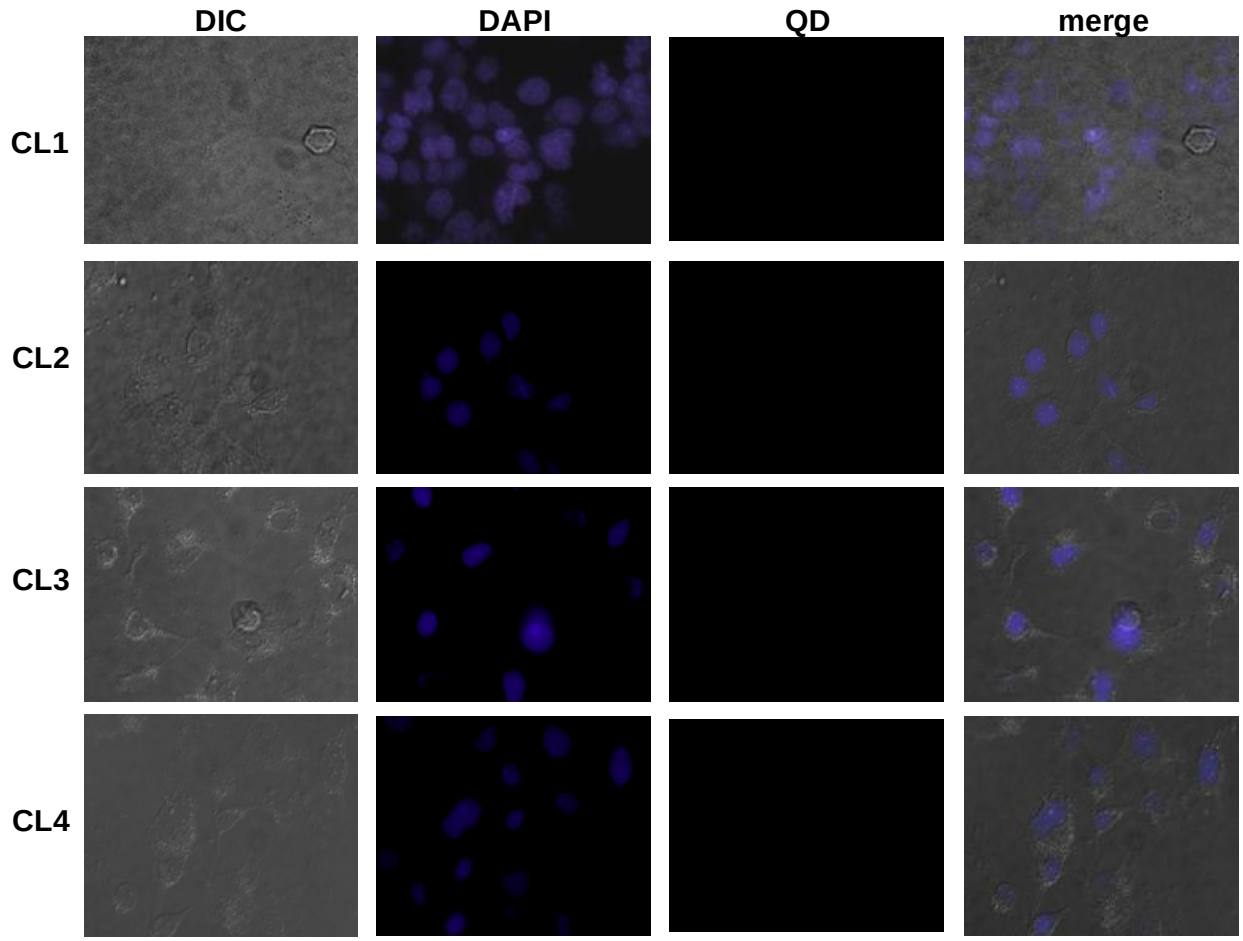


Figure S11. Cellular images after incubation with **CL**-QDs. COS-1 cells were coincubated with 550 nm emitting **CL**-QD-CPP at QD concentrations of 100 nM for 1-2 h. For imaging, cells were washed with phosphate buffered saline (PBS, 137 mM NaCl, 10 mM phosphate, 3 mM KCl, pH 7.4), fixed with 3.7% paraformaldehyde in PBS and cell nuclei were stained with DAPI. For each series, the corresponding differential interference contrast (DIC) image, DAPI, 550 nm emitting QD and merged fluorescence images are shown. Results demonstrate no nonspecific binding of the indicated **CL**-QD in the absence of CPP.

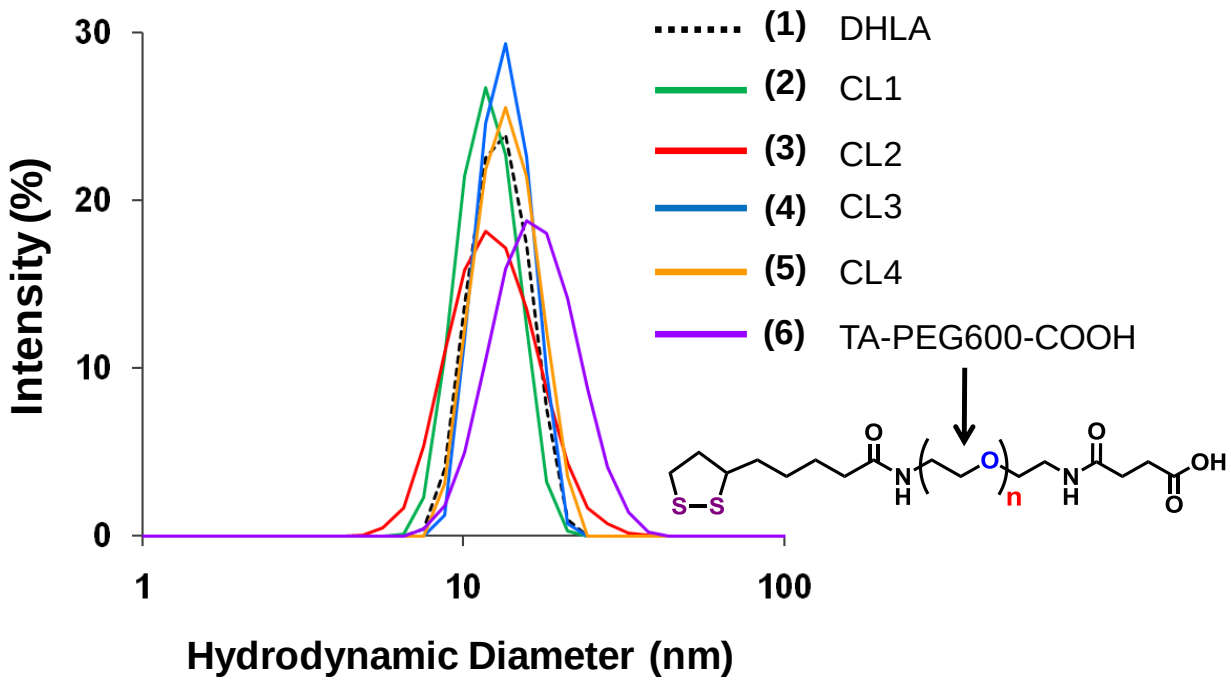


Figure S12. Hydrodynamic size distribution of 5.8 nm AuNPs cap-exchanged with (1) DHLA (13.2 ± 2.65 nm), (2) **CL1** (12.1 ± 2.49 nm), (3) **CL2** (13.0 ± 4.16 nm), (4) **CL3** (13.6 ± 2.46 nm), (5) **CL4** (13.9 ± 2.90 nm), and (6) TA-PEG600-COOH (17.3 ± 5.15 nm) measured by dynamic light scattering. Data is plotted in arbitrary units of scattering intensity. Inset: the chemical structure of TA-PEG600-COOH.[10,11]