Supporting Information

An Ultra-efficient Cap-Exchange Protocol to Compact Biofunctional Quantum Dots for Sensitive Ratiometric Biosensing and Cell Imaging

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Experimental Section

A) Materials

The CdSe/ZnS core/shell QD (λ_{EM} = ~600 nm) was purchased commercially from PlasmaChem GmbH (Berlin, Germany). The QD was supplied as dry powders capped with mixed ligands of trioctylphosphine oxide (TOPO), hexadecylamine and oleic acid. Three different colored CdSe/ZnSe/ZnS core/shell/shell QDs in toluene (CANdots, λ_{EM}s = ~610, 575 and 525 nm, nominal quantum yield >30-40%) were purchased from STREM Chemicals UK ltd (Cambridge, UK) as a QD kit. Their stock molar concentrations were provided by the supplier. Hoechst 33342 was purchased from ThermoFisher. Poly(ethylene glycol methyl ether) (Average MW: 750; Acros organics), polyethylene glycol (PEG) with an average molecular weight of 600 (containing an average of ~13 PEG units, denoted as PEG_{13}); N,N-dimethyl-1,3-propanediamine (>99%), 1,3-propanesultone (> 99%), triphenyl-phosphine (>98.5%), dicyclohexylcarbodiimide (DCC, >99%), dimethylaminopyridine (DMAP, >99%), lipoic acid (LA, > 99%), tris(2-carboxyethyl)phosphine hydrochloride (TCEP.HCl, >98%), triethylamine (>99%), chloroform (> 99.8%), magnesium sulfate (>99%), methanol (>99.9%), potassium hydroxide, ethyl acetate (>99.0%), methylene chloride (>98%), sodium bicarbonate (>99.5%), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, typsin-EDTA, Dulbecco's phosphate buffered saline (PBS) and other chemicals were all purchased from Sigma-Aldrich (Dorset, UK) and used as received without further purification unless stated otherwise. Solvents were obtained from Fisher Scientific (Loughborough, UK) and used as received. Ultra-pure water (resistance >18.2 MΩ.cm) purified by an ELGA Purelab classic UVF system, was used for all experiments and making buffers.

B) Instrument and Methods

All moisture-sensitive reactions were performed under nitrogen atmosphere using oven-dried glassware. Dry solvents were obtained through an innovative technology solvent drying system. Evaporations were performed under reduced pressure on a rotary evaporator. The synthesis was monitored by TLC on silica gel 60 F254 plates on aluminum and stained by iodine. Flash column chromatography was performed on silica gel 60 A (Merck grade 9385). All ^{1}H and ^{13}C NMR spectra were recorded on Brucker DPX300 (500 MHz for ^{1}H, 125 MHz for ^{13}C) in $CDCl_3$). All chemical shifts were reported in parts per million (ppm) and the coupling constants were given in Hz.

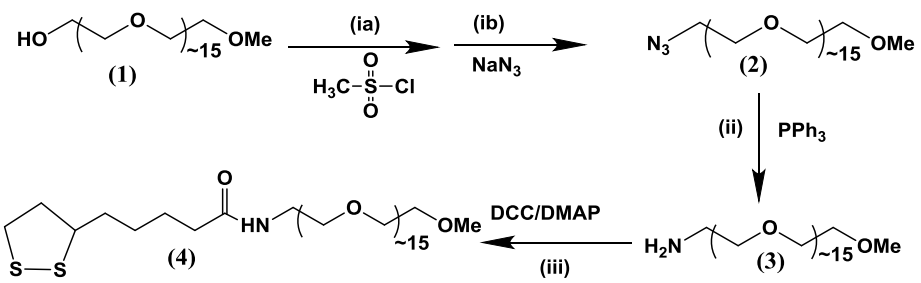
High resolution mass spectra (HR-MS) were obtained on a Bruker Daltonics MicroTOF mass spectrometer. Infra-red spectra were recorded on a PerkinElmer FT-IR spectrometer. UV-vis absorption spectra were recorded on a Varian Cary 50 bio UV-Visible Spectrophotometer over 200-800 nm using 1 mL quartz cuvette with an optical path of 1 cm or on a Nanodrop 2000 spectrophotometer (Thermo scientific) over the range of 200-800 nm using 1 drop of the solution with an optical path length of 1 mm. All centrifugations were carried out on a Thermo Scientific Heraeus Fresco 21 microcentrifuge using 1.5 mL microcentrifuge tubes at room temperature (unless stated otherwise). The QD purification was performed by Amicon ultra-centrifugal filter tubes with a cut-off MW of 30,000. Dynamic light scattering (DLS) was measured using Zetasizer Nano (Malvern) using the volume size distribution function. Atomic absorption spectra were recorded on a Perkin-Elmer Atomic Absorption Spectrometer AAnalyst 400, operating with an air-acetylene flame. Samples were prepared by dissolving the CdSe/ZnS QD prepared at 500 and 10000 LQMR in 1 M of HCl. Their Zn and Cd contents were obtained by using the corresponding absorbance and the standard calibration curves obtained with the $ZnCl_2$ or $CdCl_2$ solutions.

All fluorescence spectra were measured on a Spex Fluoro Max-3 Spectrofluorometer using a 0.70 mL quartz cuvette under a fixed excitation wavelength (λ_{EX}) of 450 nm. This wavelength corresponds to the absorption mimimum of the acceptor dye, minimizing the direct excitation of the dye acceptor.[1] An excitation and emission bandwidths of 5 nm and a scan rate of 120 nm/min over 480-800 nm range were used.

Confocal fluorescence imaging was recorded on Zeiss LSM-510 inverted laser scanning confocal microscope, (Germany) using at 488 nm excitation and collecting emission at above 570 nm. Flow cytometry was recorded on a BD LSRFortessa cytometer. The samples were excited at 488 nm and the emission was collected in the 570-585/42 nm band and the results were analysed using the FlowJo v10 software.

C) Experimental Procedures

C1) Synthesis of Lipoic acid-PEG750-OMe (LA-PEG750-Ome) ligand.[1, 2]



Scheme S1. The synthetic route to DHLA-PEG750-OMe. The reaction conditions are: (**ia**) MsCl, Et_3N, THF; (**ib**) NaN_3, $NaHCO_3$, H_2O; (**ii**) PPh_3, EtOAc and 1 M HCl; (**iii**) thioctic acid, DCC/DMAP, CH_2Cl_2.

Step (i): Synthesis of MeO-PEG$_{750}$-N$_3$ (2)

Mono methoxy-polyetheylene glycol with an average molecular weight of 750 (MeO-PEG750, 37.5 g, 50 mmol), THF (150 mL) and methanesulfonyl chloride (11.45 g, 100 mmol) were added in a 500 ml two-necked round-bottomed flask equipped with an addition funnel, septa and a magnetic stirring bar. Triethylamine (15 mL, 111 mmol) was added to the addition funnel. The reaction mixture was purged with nitrogen and cooled to 0 °C in an ice bath. Triethylamine was then added dropwisly to the reaction mixture through the addition funnel over a course of ~30 min. After that, the reaction mixture was warmed up gradually to room temperature (~20 °C) and stirred overnight. The reaction was monitored by silica gel TLC using $CHCl_3$:MeOH =10:1 (vol/vol) as eluting solvent till the reaction was complete, $R_{f(MsO–PEG750–OMe)}$ = 0.65, $R_{f\ (HO–PEG750–OMe)}$ = 0.35. The reaction mixture was then diluted with H_2O (50 ml) followed by addition of $NaHCO_3$ (3.125 g, 37 mmol). The resulting mixture was transferred to a separator funnel and extracted with $CHCl_3$ (60 mL × 3). The combined organic phase was evaporated by dryness on a rotary evaporator, giving the desired product as slightly yellowish oil, 40g (48.3 mmol, 96.6% yield).

The product (40 g), sodium azide (7.75 g, 120 mmol), THF (50 mL), H_2O (50 mL) and $NaHCO_3$ (3.125 g, 37 mmol) were added to 500 ml two-necked round-bottomed flask equipped with a distilling head connected to a round-bottomed flask as a solvent trap. The solvent trap was cooled with an ice-bath. The biphasic reaction mixture was heated under N_2 to distill off the THF. The reaction mixture was then refluxed for ~8 hrs (or overnight). After the reaction mixture was cooled to room temperature, it was transferred to a separation funnel. The product was extracted with $CHCl_3$ (100 mL × 5) and checked by TCL using $CHCl_3$:MeOH =10:1 (vol/vol) as eluting solvent, $R_{f\ (N3-PEG750-OMe)}$ = 0.75. The combined organic layer was dried over Mg_2SO_4 (~20 g, for ~30 min). After Mg_2SO_4 was filtered off, the organic layer was evaporated to dryness on a rotary evaporator to give the desired compoud as a pale brown oil (24.93g, 66.7% yield). ^{1}H NMR (500 MHz, $CDCl_3$): δ_H (ppm), 3.35 (m, 5H, -OC*H*$_3$ and N$_3$-C*H*$_2$-); 3.6-3.9 (m, 70H, C*H*$_2$ in repeated PEG groups).

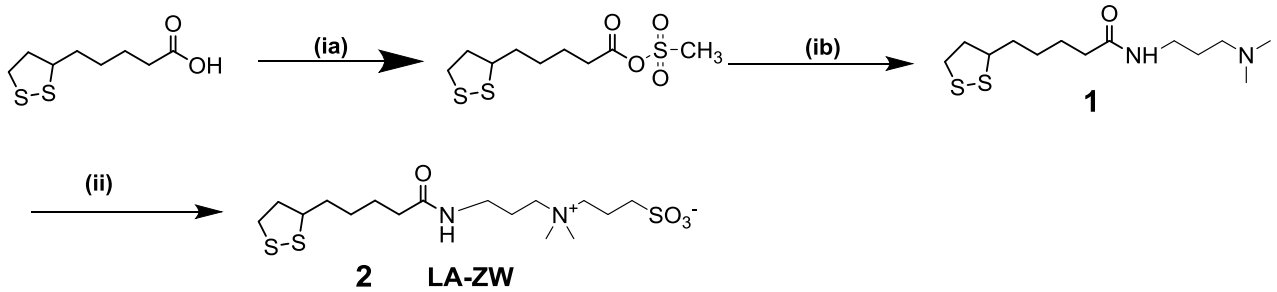
Step (ii): Synthesis of OMe-PEG750-NH$_2$ (compound 3)

N$_3$-PEG$_{750}$-OMe (8.0 g, ~10 mmol), EtOAc (150 mL) and 1 M HCl (25 ml, 25 mmol) were added to a 500 mL two-necked round-bottom flask. The reaction mixture was purged with N_2 and cooled to 0 °C by an ice-bath. Then triphenylphosphine (2.8 g, ~10 mmol) in 100 ml EtOAc was added dropwisely into the reaction mixture *via* an addition funnel under N_2. The temperature was maintained to <5 °C during the addition. Once addition was complete, the reaction mixture was allowed to gradually warmed up to room temperature and stirred under N_2 overnight. The reaction mixture was transferred to a separation funnel and the biphasic solution was separated. The aqueous layer was collected and washed with EtOAc (100 mL×2) to remove any unreacted triphenylphosphine and formed triphenylphosphine oxide byproduct. The aqueous layer was transferred to a round-bottomed flask and cooled in an ice bath, into which KOH (13.5 g) was then added slowly. The mixture was stirred magnetically till all KOH was fully dissolved. The aqueous solution was then transferred to a separation funnel and extracted with $CHCl_3$ (60 mL × 5). The combined organic phase was dried over $MgSO_4$ (20 g, ~20 min) under stirring. After filting off $MgSO_4$, the solvent was evaporated on a rotary evaporator, giving the desired compound as a light yellow oil in 59.2% yield (4.58 g). R_fs ($CHCl_3$:MeOH = 10:1, vol/vol) for $R_{f\ (N3-PEG750-OMe)}$ = 0.75, $R_{f\ (NH2-PEG750-OMe)}$ = 0.25. ^{1}H NMR (500 MHz, $CDCl_3$), δ (ppm), 2.87 (2H, -N*H*$_2$), 3.35 (s, 3H, -OC*H*$_3$), 3.6-3.9 (70H, m, *CH*$_2$s in repeat PEG unit).

Step 3: Synthesis of LA-PEG$_{750}$-OMe (Compound 4)

NH_2-PEG_{750}-OMe (3.75 g, ~5.0 mmol), 4-(N,N-dimethylamino)pyridine (0.12 g, ~1.0 mmol), N,N'-dicyclohexyl-carbodiimide (1.06 g, ~5.0 mmol) and CH_2Cl_2 (80 mL) were added into a 250 mL round-bottomed flask equipped with a magnetic stirring bar and an addition funnel. The mixture was stirred and cooled to 0 °C by an ice bath. Thioctic acid (1.03 g, ~5.0 mmol) in 30 mL of CH_2Cl_2 was then added dropwisely through the addition funnel over 30 min under N_2. After the addition was complete, the reaction mixture was allowed to warm up to room temperature gradually and stirred overnight. A white precipitate was formed and filtered off through celite and the celite plug was rinsed with $CHCl_3$. The combined solution was evaporated on a rotary evaporator. The residue was mixed with saturated $NaHCO_3$ and extracted with ether (100 mL × 2). The aqueous layer further extracted with CH_2Cl_2 (100 mL × 2). The combined organic layers were dried over $MgSO_4$. After filtration, the solvent was evaporated to yield the desired product as a yellow solid, weight 2.30 g in 48.4% yield. TLC ($CHCl_3$:MeOH=10: 1 (vol/vol)), $R_{f(TA\text{-}PEG750\text{-}OMe)}$ = ~0.6, $R_{f\ (NH2\text{-}PEG750\text{-}OMe)}$ = 0.25. ^{1}H NMR (500 MHz, $CDCl_3$), δ (ppm): 1.45 (2H, m), 1.60 (m, 4H), 2.10 (t, 2H); 2.50 (m, 2H), 3.08-3.30 (m, 3H), 3.40 (m, 5H, -O*CH$_3$* + (CO)-NH-*CH$_2$*-), 3.6-3.9 (m, 70H, *CH$_2$*s of the PEG unit).

C2) Synthesis of lipoic acid-zwitterion (LA-ZW) ligand[3]



Scheme S2. Synthetic route to LA-ZW. Reaction conditions are: (**ia**) Et_3N, CH_2Cl_2, MsCl; (**ib**) N,N-dimethyl-1,3-propanediamine; (**ii**) $CHCl_3$, 1,3-propanesultone.

Step (i): Synthesis of LA-N, N-Dimethyl-1, 3-propanediamine.

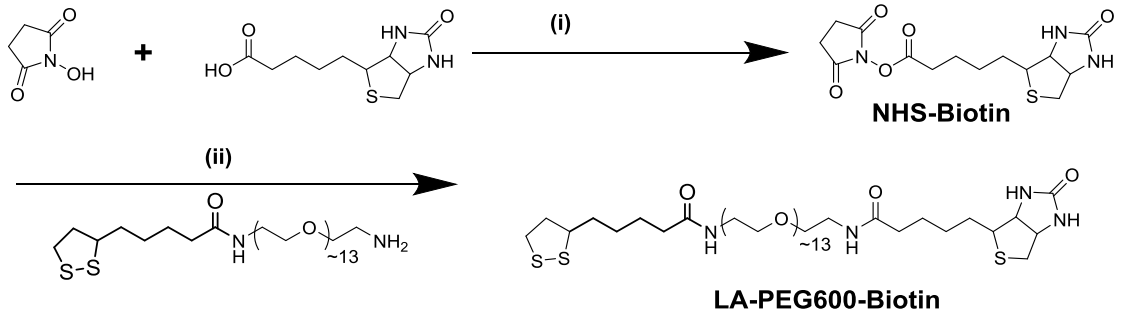
Lipoic acid (3.0 g, ~15 mmol), triethylamine (1.47 g, ~15 mmol) and CH_2Cl_2 (30 mL) were added to three-necked round bottom flask (250 mL) and stirred at 0°C for 30 min under a stream of N_2. Methanesulfonyl chloride (1.67 g, ~15 mmol) was then added dropwisely through a syringe. The reaction mixture was allowed to slowly warm up to room temperature and left stirring for 5 hrs. After that, N,N-dimethyl-1,3-propanediamine (1.24, ~12 mmol) and triethylamine (0.61 g, ~6 mmol) in 20 mL CH_2Cl_2 was slowly added to the reaction mixture and stirred at RT overnight under N_2. The reaction mixture was transferred to a seperation funnel, washed with water (30 mL×2) and then saturated Na_2CO_3 solution (100 ml). The organic layer was dried over Na_2SO_4 and filtered. After evaporation of the solvent, the desired compound (**1**) was obtained as a yellow oil in 34.7% yield (1.48 g). ^{1}H NMR (500 MHz, $CDCl_3$): δ (ppm): 5.45 (s, 1H), 3.50-3.59 (m, 1H), 3.30 (m, 2H), 3.05-3.20 (m, 2H), 2.38-2.49 (m, 1H), 2.38 (t, 2H, J = 6 Hz), 2.20 (s, 6H), 2.17 (t, 2H, J=6 Hz), 1.83-1.94 (m, 1H), 1.57-1.72 (m, 6H), 1.38-1.52 (m, 2H).

Step (ii): Synthesis of LA-ZW.

Compound **1** (1.48 g, ~5.2 mmol) was dissolve in 20 mL dry tetrahydrofuran (THF) and purged with N_2 for 30 mins,

then 1,3-propanesultone (1.0 g, ~8 mmol) dissolved in 4 mL dry THF was slowly added and stirred for 3 days. A turbidity was formed instantly as the 1,3-propanesultone solution was added into the reaction mixture, an indication of forming LA-zwitterion which has low solubility in THF. Once the reaction was complete, the solvent was evaporated to give a crude product as a pale yellow solid. The crude product was washed with $CHCl_3$ (20 mL× 3) and further purified by HPLC to give the pure LA-ZW ligand in 23% yield. ^{1}H NMR (300 MHz, D_2O): δ (ppm) 3.60-3.70 (m,1H), 3.40-3.50 (m, 2H), 3.28-3.35 (m, 2H), 3.20-3.28 (m, 2H), 3.10-3.20 (m, 2H), 3.10 (s, 6H), 2.90 (t, 2H), 2.40-2.50 (m, 1H), 2.20 (t, 2H), 2.15 (m, 2H), 1.93-2.0 (m, 2H), 1.70 (m, 1H), 1.50-1.60 (m, 4H), 1.35-1.40 (m, 2H).

C3) Synthesis of LA-PEG600-Biotin ligand[1]



Scheme S3. The synthetic route to LA-PEG600-biotin. Reaction conditions were: (**i**) N,N'-dicyclohexylcarbodiimide/Dimehtyl aminopyridine, DMF; (**ii**) TA-PEG600-NH_2, DMF, Et_3N.

Step (i) Synthesis of NHS-biotin.

Biotin (2.0 g, ~8.2 mmol), N-hydroxysuccinimide (NHS, 0.94 g, ~8.2 mmol) were dissolved in 100 mL DMF in a 250 mL round-bottomed flask. The reaction mixture was flushed with N_2 under stirring for 30 mins, and then N,N'-dicyclohexylcarbodiimide (2.47 g, ~12 mmol in 10 mL DMF) was added. The resulting solution was stirred overnight at room temperature under N_2. A white precipitate was formed and filtered off. The filtrate was evaporated to dryness on a rotary evaporator to give a white powder. After further washing with diethyl ether and drying under vacuum, the desired product was obtained as a white powder, weight 1.22 g (43.9 % yield). HPLC-MS: found m/z 342.2, calculated m/z for $[M + H]^+$ = 342.4.

Step (ii) Synthesis of LA-PEG600-biotin

LA-PEG600-NH_2 (0.29 g, ~0.37 mmol, containing ~13 PEG units synthesized previously), NHS-biotin (0.122 g, ~0.36 mmol) and DMF (8.0 mL) were placed in a 20 mL round-bottomed flask. The reaction vessel was purged with N_2 and cooled to 0 °C in an ice-bath under stirring. Then triethylamine (0.50 mmol) was added dropwisely through a syringe under N_2. Once the addition was completed, the reaction mixture was allowed to gradually warm up to room temperature and stirred under N_2 overnight. The reaction mixture was filtered and the solvent was evaporated. The residue was purified by HPLC and the pure LA-PEG600-biotin fractions were collected. After evaporation of solvent, LA-PEG600-biotin was obtained as a waxy solid, weight 0.185 g (51.3% yield). ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 6.79-6.98 (m,1H), 6.43-6.65 (m, 1H), 5.65-5.85 (m, 1H), 4.92-5.12 (m, 1H), 4.47-4.63 (m, 1H), 4.33 (m, 1H), 3.5-3.9 (m), 3.40-3.50 (m, 2H), 3.10-3.20 (m, 3H), 2.80-2.98 (m, 1H), 2.75 (d, 1H), 2.40-2.60 (m, 1H), 2.10-2.30 (m, 4H), 1.80-1.96 (m, 1H), 1.60-1.80 (m, 8H), 1.40-1.60 (m, 4H). LC-MS found a series of peaks that were

separated by 44 m/z units, corresponding to different PEG chain lengths in the mixed length PEG600 linker, *e.g.* 1048.0, 1004.8, 959.6, 915.7, 871.7. The calculated corresponding $[M+H]^+$ peaks for LA-PEG_n-Biotin for n = 13, 12, 11, 10 and 9 are 1048, 1004, 959, 915 and 871, respectively. The double charge peaks were stronger with m/z values of 568.4, 546.4, 524.4, 502.4, 480.4, 458.4, 436.4 and 414.4, corresponding to the $([M + 2H]^{2+}$ with n = 15, 14, 13, 12, 11, 10, 9, 8, and 7, respectively.

D) Preparation, characterisation and cell based studies of biocompatible QDs

D1) Preparation of DHLA-zwitterion ligand capped QDs (QD-ZW)

A typical ligand exchange procedure for preparing QD-ZW is as follows: commercial hydrophobic CdSe/ZnS or CdSe/ZnSe/ZnS QD (1 nmol, 20 μL in hexane or tolune) was precipitated by adding 500 μL EtOH followed by centrifugation to remove any unbound free ligands. The QD pellet was then dispersed in 50 μL $CHCl_3$ and then added with 20 μL EtOH to make solution **A**. LA-ZW (0.10 M, 2 μL in H_2O) was reduced to DHLA-ZW by mixing with TCEP.HCl (0.10 M, 2 μL in H_2O) for 10 mins. After which NaOH (0.10 M in EtOH, 12 μL) was added to fully deprottonate the DHLA thiol-groups and to neutralise the acid groups in TCEP.HCl (each containing 4 acid groups) to make solution **B**. Solutions **A** and **B** were mixed in a new Eppendorf tube for 1-3 mins with occasional shaking by hand, after which H_2O (50 μL) was added to the reaction mixture. The QD was found to rise to the top aqueous phase, leaving the bottom $CHCl_3$ layer effectively colorless, indicating full QD water-dispersion. The top aqueous layer was then carefully separated from the bottom $CHCl_3$ layer and transferred to an Amicon ultra-centrifugal tube with a 30,000 MW cut-off filter membrane and centrifugated for 1 min at 3000 rpm. The residue was washed with H_2O (200 μL) and followed by a brief centrifugation. The process was repeated three times to remove any unbound free ligands, yielding a stable QD stock water-dispersion. The stock QD concetration was determined by using their first exciton peak absorbance and respective extinction coefficient (*e.g.* 2.5×10^5 $M^{-1}.cm^{-1}$ at ~590 nm for the 600 nm emitting CdSe/ZnS QD) using our previously established procedures.[2, 4]

D2) Preparation of DHLA-PEG750 capped QD

QD (1 nmol, 20 μL in hexane) was first precipitated by adding 500 μL EtOH followed by centrifugation to remove unbound free TOPO ligands. The resulting QD pellet was then dispersed in 30 μL hexane and then added with 10 μL EtOH to make solution **A**. TA-PEG750-OMe (0.189 M in EtOH, 1.06 μL) was reduced by 1 molar equivalent of TCEP (0.10 M, 2 μL in H_2O) for ~10 mins, and then NaOH (0.10 M in EtOH, 12 μL) was added to make solution **B**. After that, solutions **A** and **B** were mixed for 1-3 mins under agitation, then H_2O (20 μL) was added. The QD was found to transfer to lower aqueous layer, leaving the top organic layer colorless. The aqueous layer was transferred to an Amicon ultra-centrifugal tube equiped with a 30,000 MW cut-off filter membrane. After centrifugation and washing with H_2O three times to remove any unbound free ligands, a stable, biocompatible QD-PEG750 aqueous stock was obtained. The stock QD concentration was determined using the same method as above.[2]

D3) Protein preparation and prufication

The target protein, yeast SUMO (SUMO) protein and the anti-SUMO Affimers were expressed in BL21 (DE3) cells using isopropyl β-D-1-thiogalactopyranoside (IPTG) induction and purified by Ni-NTA resin (Qiagen) affinity

chromatography according to the manufacturer's instructions. The detailed experimental procedures were described in our recent publication.[5]

D4) Protein labeling[6]

The anti-SUMO Affimer (5 mg/mL, 12.5 µL in PBS, MW = 13,267) in a microcentrifuge tube was first mixed with Alexa 647 NHS-ester (50 µg in 2 µL DMSO) and then 5 µL $NaHCO_3$ (0.5 M, pH = 8.3) and 7.5 µL PBS (10 mM phosphate, 150 mM NaCl, pH 7.4) were added and thoroughly mixed at room temperature for 2 h (dye:protein molar ratio ≈ 8:1). After that the reaction mixture was loaded on a small G25 gel fitration column using PBS as eluting solution on natural flow. The first eluted blue band (corresponding to the labeled anti-SUMO Affimer) was collected and its absorption spectrum was recorded. Using the excitnction coefficients of the Alexa-647 dye (ε_{650nm} = 239,000 $M^{-1}cm^{-1}$) and Affimer (ε_{280nm} =7904 $M^{-1}cm^{-1}$) and the CF_{280nm} of 0.03 for the Alexa-647 dye, the average dye labeling ratio on per Affimer was calcualted as 1.08. The stock protein concentration was 86 µM.[6]

Simiarily, the SUMO protein was labelled with Alexa-647 NHS ester using the same procedures under a dye: protein molar ratio of 6.4. After purification by using a G25 column as above, the average number of dyes labeled on each protein was determined as 0.80. The labeled protein stock concentration was 32.7 µM.

Neutravidin was also labelled with Alexa-647 NHS ester using the same procedures under a dye:protein molar ratio of 7. After purification by using a G25 column, the average Alexa-647 dye label per protein was determined as 1.67.

D5) Gel electrophresis analysis[6]

2 or 5 µL of a DHLA-ZW capped CdSe/ZnS core/shell QD (QD-ZW, λ_{EM} ~600 nm) prepared at a LQMR of 200, 500 or 1000 respectively was mixed with 18 or 15 µL of 60% glycerol in H_2O. A QD-Affimer assembly was also prepared by mixing the QD-ZW prepared at a LQMR of 500 with 10 molar equivalent of the His_8-tagged Affimer. The resulting QD-Affimer conjugate was treated the same way as the above QD-ZW samples. Then 20 µL of each sample was loaded onto a 0.75% agrose gel in TAE buffer pH 8.3. Gel was run at 100 mV for ~30 min and QD was visualized under a UV illumination (λ = 365 nm).

D6) Cell culture and cell based studies[7]

HeLa adherent epithelial cells derived from human cervical carcinoma were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and 100 U mL^{-1} penicillin. The HeLa cells were trypsinized using trypsin-EDTA and maintained in a humidified incubator with 5% CO_2 at 37 °C.

Laser scanning confocal microscopy

2 mL of HeLa cells (1.5×10^5 cells mL^{-1}) were cultured for 24 h followed by the treatment with 1 mL of the serum free DMEM with or without of the QD samples (50 nM). After incubation for 4 h, the cells were washed three times with Dulbecco's phosphate buffered saline. Hoechst 33342 was added to a final concentration of 5 µg mL^{-1} for nuclei staining. The cells were then imaged by laser scanning confocal microscopy (Zeiss LSM-510 inverted laser scanning confocal microscope, Germany). The QD was excited at 488 nm and the emission above 570 nm was collected.

Flow cytometry

1 mL of HeLa cells (3 ×10^5 cells/mL) were cultured in 6 well plates for 24 h followed by treatment with 1 mL of serum-free DMEM with or without QD (50 nm). After 4h incubation, the cells were washed three times with Dulbecco's Phosphate Buffered Saline (D-PBS). After cell detachment using 0.5 mL of Trypsin-EDTA, 0.5 mL of serum-free DMEM was added to each well and the samples were centrifuged in 2 mL microcentrifuge tubes for 5 minutes at 1000 rpm. The supernatant was discarded and replaced with 0.5 mL of serum-free DMEM. The samples were filtered using Flowmi™ cell strainers (40 µm) and analysed in 5 mL Falcon plastic tubes using BD LSRFortessa cytometer. The samples were excited at 488 nm and the emission was collected in the 570 - 585/42 nm band. The results were analysed using FlowJo v10 software.

Free biotin competition. 1 mL of HeLa cells (3 ×10^5 cells/mL) were cultured in 6 well plates for 24 h, followed by 1 h treatment of one set of triplicates with 10 mM free biotin in serum-free DMEM. 1 mL of serum-free DMEM with QD (50 nM) with or without free biotin (10 mM) were then added to the wells which had been pre-treated with free biotin or incubated normally, respectively. After a 4 h incubation, the cells were washed three times with Dulbecco's Phosphate Buffered Saline (D-PBS). After cell detachment using 0.5 mL of Trypsin-EDTA, 0.5 mL of serum-free DMEM was added to each well and the samples were centrifuged in 2 mL microcentrifuge tubes for 5 minutes at 1000 rpm. The supernatant was discarded and replaced with 0.5 mL of serum-free DMEM. The samples were filtered using Flowmi™ cell strainers (40 µm) and analysed in 5 mL Falcon plastic tubes using BD LSRFortessa cytometer. The samples were excited at 488 nm and the emission was collected in the 586/16 nm band. The results were analysed using FlowJo v10 software.

D7) Atomic adsorption analysis of the QD Zn^{2+} and Cd^{2+} content

Two CdSe/ZnS QD samples after cap-exchange with the DHLA-zwitterion ligand at a LQMR of 500 and 10000 were prepared. They were then washed three times by pure water using a 30000 M.W. cut off filter tube to remove any unbound free ligand and etched Zn^{2+} ions. The QDs were then dissolved in 1M HCl and then diluted with water before measurement. The Zn^{2+} and Cd^{2+} calibration curves were obtained by using the known concentration of the $ZnCl_2$ (M.W. 136.23 g/mol) and $CdCl_2$ (M.W. 183.32 g/mol) aquesous solution as standard. The Zn^{2+} and Cd^{2+} absorbances of the two QD samples were measured. All measurements were done in triplicate with error bars obtained from the standard deviations).

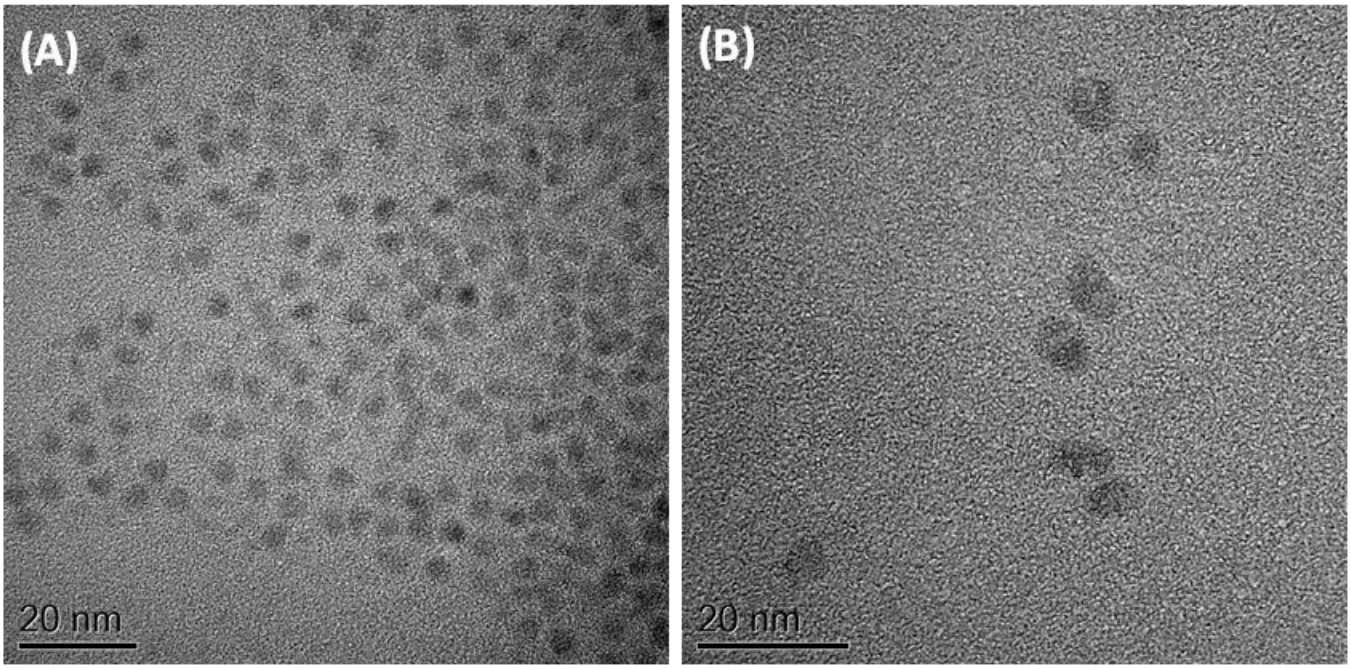


Figure S1. (**A**) TEM image of the TOP-TOPO capped CdSe/ZnS core/shell QD (λ_{EM} ~ 600 nm) used in this study prior to cap-exchange. It has an average core crystal diameter of ~4.5 nm. (**B**) TEM image of the CdSe/ZnSe/ZnS core/shell/shell QD after ligand exchange with the DHLA-ZW at a LQMR of 600. This QD appears to be ellipsoidal, with long and short axis of ~8.2 and ~6.0 nm, respectively.

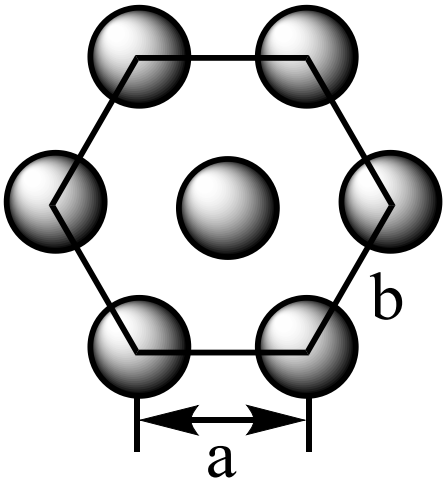


Figure S2: Schematic of a hexagonally packed Zn^{2+} outer layer (assuming ZnS being in its most stable Wurtzite structure) where $a = b$ = 382 pm = 0.382 nm.

Within the hexagon area, there are three Zn^{2+} ions (spheres, 1 in the middle + 6 × (1/3) one the corners).

The total area of the hexagon = $6 \times (1/2)\ a^2 \times \sin 60^o = 3 \times 0.382^2 \sin 60^o = 0.379\ nm^2$

Therefore the surface area occupied by each $Zn^{2+} = 0.379/3 = 0.126\ nm^2$

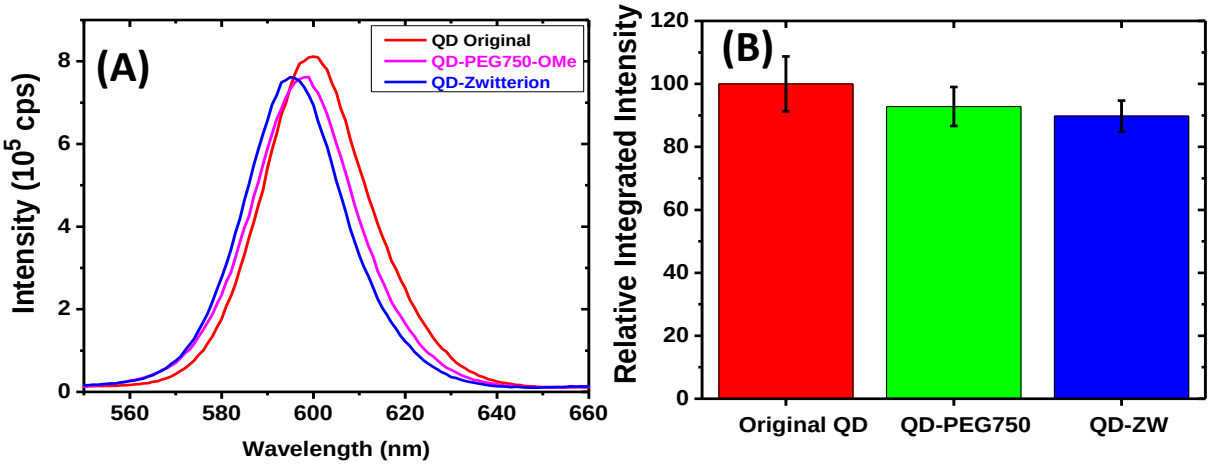


Figure S3. (A) Fluorescence spectra of a CdSe/ZnS core/shell QD (λ_{EM} ~ 600 nm) before (red) and after cap-exchange with the DHLA-ZW (blue) or DHLA-PEG750 (pink) ligand at a LQMR of 200. (**B**) Comparison of the integrated fluorescence intensity for the above three QDs (the original QD in $CHCl_3$ was normalized to 100).

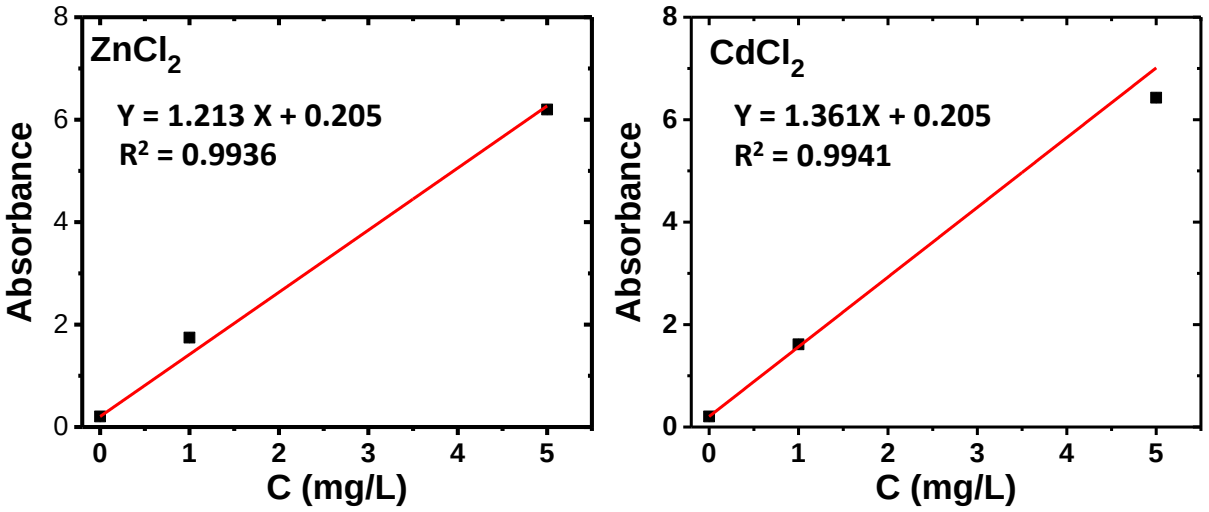


Figure S4. Absorbance *versus* concentration calibration curves for $ZnCl_2$ (left) and $CdCl_2$ (right) measured by atomic adsorption. Good linear calibration curves (R^2 >0.993) were obtained for both $ZnCl_2$ (Y = (1.213±0.069) X + 0.205) and $CdCl_2$ (Y = 1.361X + 0.205). The Cd^{2+} and Zn^{2+} contents of the QD samples were calculated as follows:

For the 500 LQMR QD (SD represents standard deviation of three measurements)

Cd: Y = 3.516 ± 0.024, hence X = 2.433±0.018 mg/L, correponding to C_{Cd2+} = 13.35±0.10 μM

Zn: Y = 4.522 ± 0.017, hence X = 3.559±0.014 mg/mL, correponding to C_{Zn2+} = 26.13± 0.10 μM

For the 10000 LQMR QD sample

Cd: Y = 1.260 ± 0.006, hence X = 0.775±0.004 mg/L, correponding to C_{Cd2+} = 4.228±0.022 μM

Zn: Y = 1.525 ± 0.016, hence X = 1.088±0.013 mg/mL, correponding to C_{Zn2+} = 7.99± 0.10 μM

Therefore for the 500 LQMR QD: the Zn^{2+}/Cd^{2+} molar ratio = 1.96 ± 0.01

For the 10000 LQMR QD: the Zn^{2+}/Cd^{2+} molar ratio = 1.89 ± 0.02

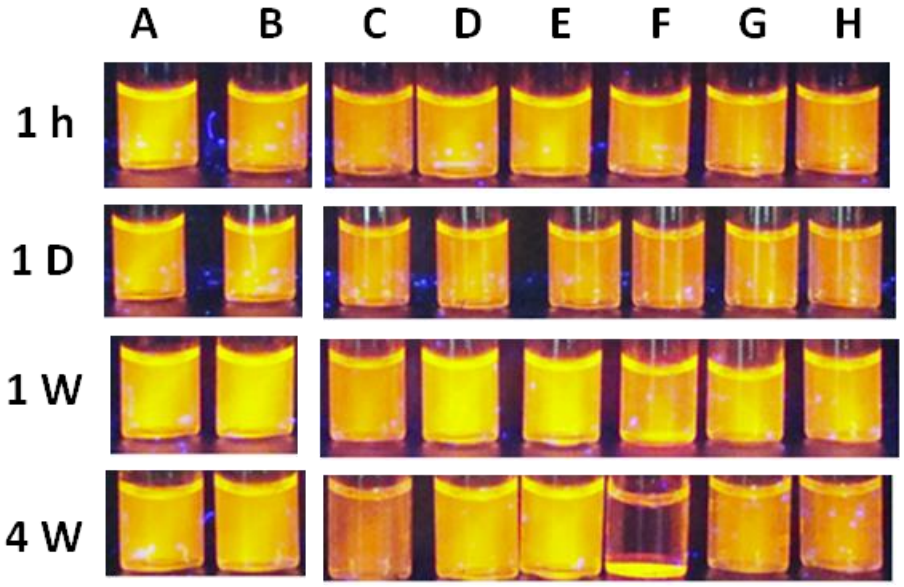


Figure S5: Photographs of the 100 nM CdSe/ZnS core/shell QD-DHLA-ZW (maximum λ_{EM} = ~606 nm) upon exposure to a UV lamp (λ = 350 nm) prepared by the UCEP at a LQMR of 200 at different post-preparation intervals (1 h, 1 day, 1 week and 4 weeks from top to bottom panels). The dispersion conditions are H_2O (**A**), PBS (**B**, 10 mM phosphate, 150 mM NaCl, pH 7.4), PBS with pH adjusted to 4 (**C**), 7 (**D**), 10 (**E**), 13 (**F**), and with NaCl concentration of 1M (**G**) and 2M (**H**), respectively.

The above figure confirmed that the QD-DHLA-ZW prepared at a LQMR of 200 is stable for at least 4 weeks in PBS over pH 4-10 and under high salt conditions of 2 M (NaCl). Its stability over pH 4-10 is comparable to other DHLA-ZW capped QDs prepared by photoligation and other literature methods. Its stability at pH 13 was lower than that prepared by photoligation (stable for 2 months), possibly due to the fact it was slightly aggregated (clustered) as revealed by our DLS and gel electrophoresis data (see Figure 2).

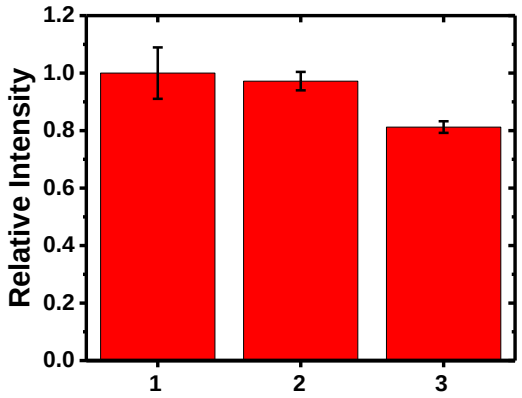


Figure S6: Comparison of the normalised integrated fluorescence intensity for the CdSe/ZnS QD (λ_{EM} ~600 nm) before (**1**, in $CHCl_3$ whose intensity was normalised to 100) and after cap-exchange with the DHLA-ZW ligand at a LQMR of 500 with (**2**, by the normal UCEP procedure with oxidized TCEP) and without (**3**) oxidized TCEP.

Table S1. Hydrodynamic diameters (volume population, D_h) of the CdSe/ZnS QD before and after cap-exchange with DHLA-ZW under different LQMRs. The polydispersity index, PDI, is obtained by PDI = FWHM/D_h.

QD samples	Average D_h (nm)	D_h (nm) and percentage abundance (%)	FWHM (nm)	PDI
QD-TOPO	6.2	4.4±0.1 (41%)	2.1±0.3	0.48
		7.4±0.6 (59%)	4.4±0.8	0.59
QD-ZW (200)	23.2	17.1±0.3 (42%)	8.0±0.9	0.47
		27.5±2.1 (58%)	15.9±2.8	0.58
QD-ZW (300)	17.2	12.5±0.2 (43%)	6.2±0.6	0.50
		20.8±1.6 (57%)	12.5±2.2	0.60
QD-ZW (500)	8.9	8.9±0.1 (100%)	2.7±0.1	0.30
QD-ZW (1000)	8.6	8.6±0.1 (100%)	3.9±0.2	0.45

The original QD and the cap-exchanged QDs at low LQMRs (<500) appear to have bimodal size distributions (*e.g.* containing two species). In this case, the average D_h was calculated by: $D_h = D_h1 \times A1\% + D_h2 \times A2\%$.
Where D_h1 and D_h2 are the D_h values, and A1% and A2% are the percentage areas (abundance %) of the two species obtained from the bimodal Guassian fit. The resulting data are summarised in Table S1.

Assuming 8.6 nm corresponds to the D_h of an isolated individual QD-ZW particle, then its hydrodynamic volume:
$V_h = (4/3)\ \pi\ (D_h/2)^3 = (4/3)\ \pi\ (8.6/2)^3 = 333\ nm^3$.

Using the D_h values obtained from the Guassian fits given in Table S1, the average particle hydrodynamic volume V_h for the QD cap-exchanged with the DHLA-ZW ligand at a LQMR of 200 and 300 is calculated as:
$V_h\ (200) = (4/3)\ \pi\ (23.2/2)^3 = 6535\ nm^3$.
$V_h\ (300) = (4/3)\ \pi\ (17.2/2)^3 = 2663\ nm^3$.
Therefore the V_h (200) and V_h (300) values are ~20 and ~8 times that of an isolated QD-ZW particle, respectively, suggesting that the QD forms small clusters or assemblies each containing a few to ~20 QDs after cap-exchange with the DHLA-ZW ligands under these low LQMRs of <500.

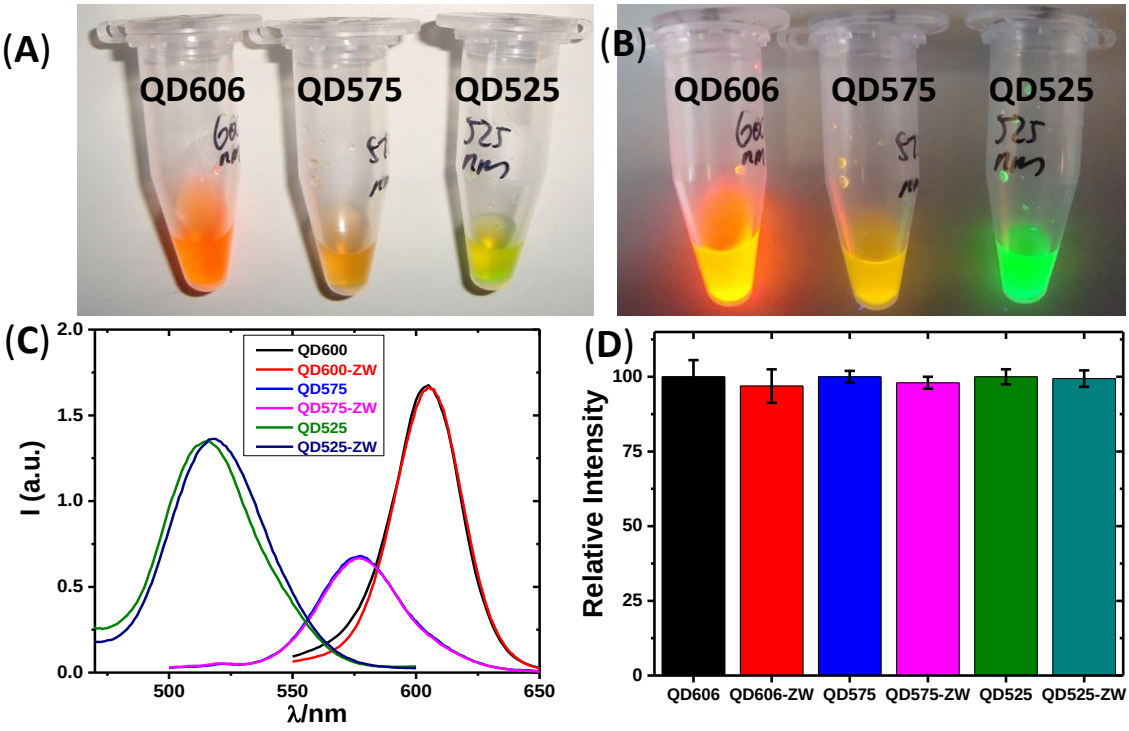


Figure S7: (**A, B**) Photographs of aqueous dispersions of three different colored CdSe/ZnSe/ZnS QDs with λ_{EMs} of 606, 575 and 525 nm respectively after cap-exchange with the DHLA-ZW at a LQMR of 200 under day light (**A**) and a hand-held UV-lamp illumination (λ_{EX} = 365 nm, **B**). (**C**) Fluorescence spectra of the three QDs (10 nM) before (in $CHCl_3$) and after cap-exchange with the DHLA-ZW ligand (in H_2O) at 200 LQMR. The intensity of the 606 nm emitting QD was reduced by 3 fold in order to display in the same graph. (**D**) Comparison of the normalized fluorescence intensity for each QD before and after cap-exchange with the DHLA-ZW ligand (the QD intensity in $CHCl_3$ prior to cap-exchange was set as 100).

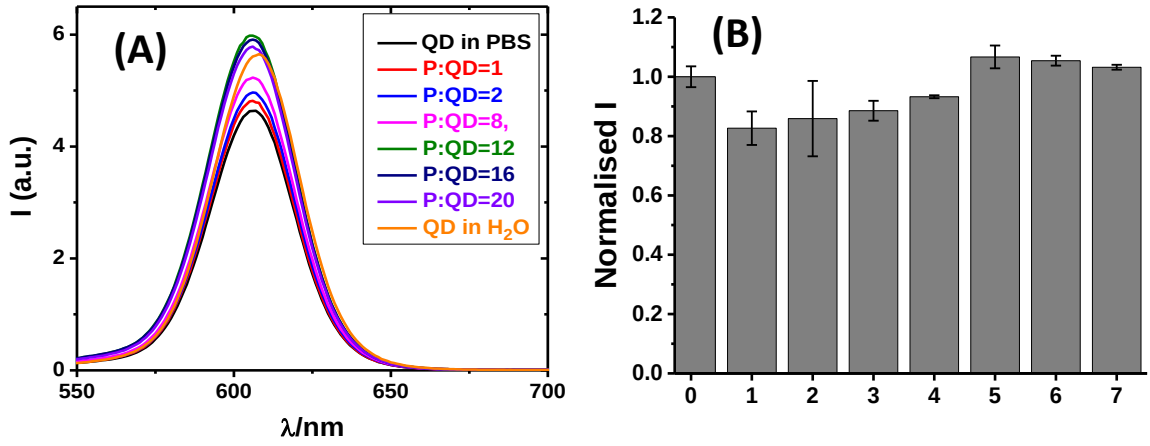


Figure S8: (**A**) Fluorescence spectra of a DHLA-ZW capped CdSe/ZnSe/ZnS QD (λ_{EM} = 605 nm) in pure water and in PBS after mixing with 0, 1, 2, 8, 12, 16 and 20 molar equivalent of a His_8-Affimer. (**B**) Normalised QD fluorescence intensity for the above QD samples, columns 0-7 correspond to QD in pure water and in PBS after mixing with 0, 1, 2, 8, 12, 16, 20 molar equivalents of His_8-Affimer respectively. The QD fluorescence initially increased with the increasing Affimer:QD molar ratio and became saturated at 12:1, suggesting that 12 copies of Affimer may be assembled onto each QD.

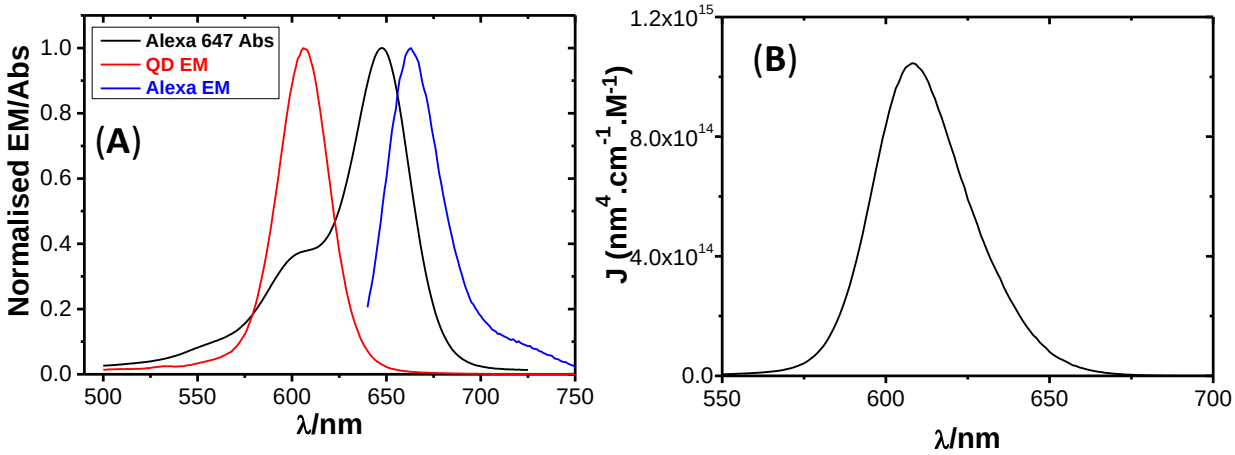


Figure S9. Photophysical properties of the QD donor and the Alexa 647 acceptor. **(A)** Normalized fluorescence emission spectrum of the QD_{605} (red), and the normalized absorption spectrum (black) of the Alexa 647 labeled Adhiron. **(B)** Spectral overlap function of the Alexa 647 and QD_{600} FRET pair.

$$J_{(\lambda)} = \frac{\int PL_{D(\lambda)}\, \varepsilon_{A(\lambda)}\, \lambda^4\, d\lambda}{\int PL_{D(\lambda)} d\lambda}$$

Where $PL_{D(\lambda)}$ is the normalised QD fluorescence intensity at λ; $\varepsilon_{A(\lambda)}$ is the acceptor absorption coefficient at λ.

The integral of the spectral overlap: $I = 1.32 \times 10^{16}$ ($nm^4.cm^{-1}.M^{-1}$)

The quantum yield of the CdSe/ZnSe/ZnS core/shell/shell QD, Q_{QY}, was determined as 32% using Rhodamine 6G as reference. Assuming a media refractive index of 1.33, and random orientation of the dipoles of the FRET pair ($K^2 = 2/3$), then the Förster radius (R_0, in the unit of Å) of the above QD-dye FRET pair (at 1:1 molar ratio) can be calculated *via* the following equation:[8]

$$R_0 = (8.79 \times 10^{-5}\, n_r^{-4} \times Q_{QY} \times K^2 \times I)^{1/6}$$
$$= (8.79 \times 10^{-5} \times 1.33^{-4} \times 0.32 \times (2/3) \times 1.32 \times 10^{16})^{1/6}$$
$$= 65 \text{ Å}$$
$$= 6.5 \text{ nm}$$

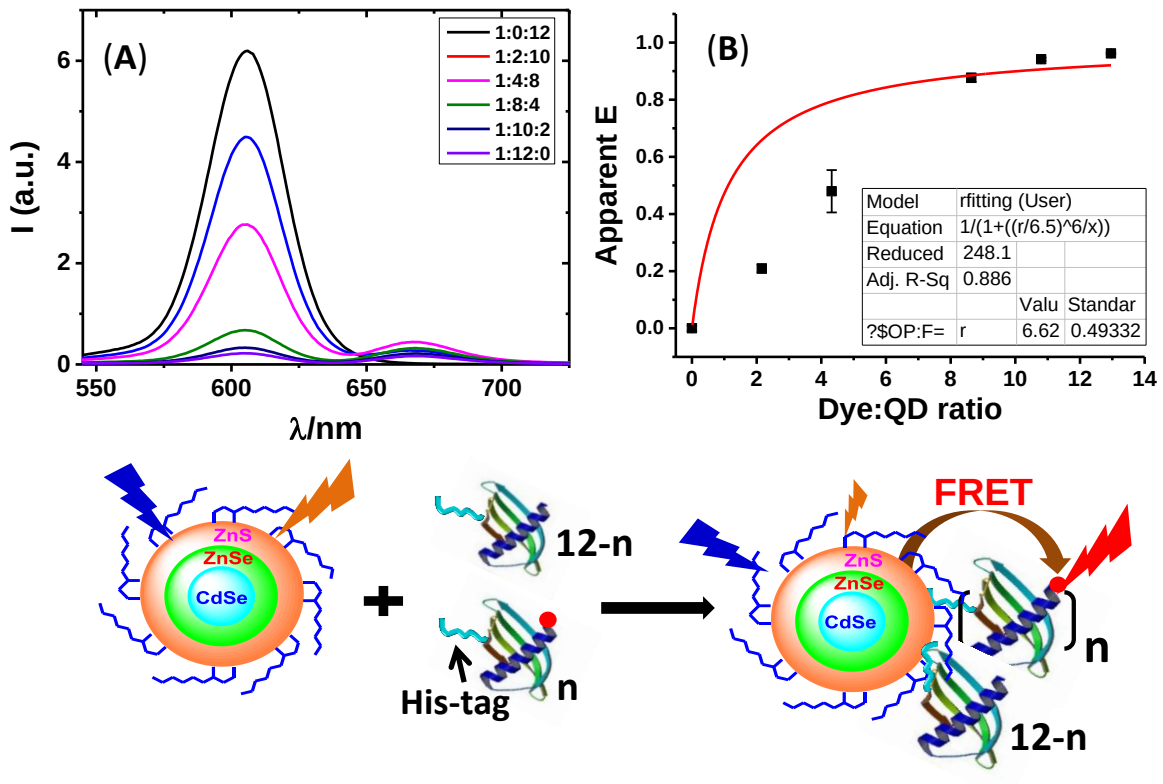


Figure S10. (**A**) Fluorescence spectra of a DHLA-ZW capped CdSe/ZnSe/ZnS core/shell/shell QD (λ_{EM} = 605 nm) after assembly with different molar ratios of the Alexa-647 labeled Adhiron (with the total Affimer:QD molar ratio being fixed at 12). (**B**) A plot of the FRET efficiency *E* (obtained by QD fluorescence quenching) as a function of dye:QD molar ratio (each Affimer was labeled with on average 1.08 dyes). The data are fitted to the single QD donor in FRET interaction with *N* identical acceptor model, $E = 1/[1 + (r/R_0)^6/N]$.[9] Using a R_0 of 6.5 nm, an average donor-acceptor distance *r* of 6.6 ± 0.5 nm was obtained. The scheme dipicted beneath showing the FRET process.

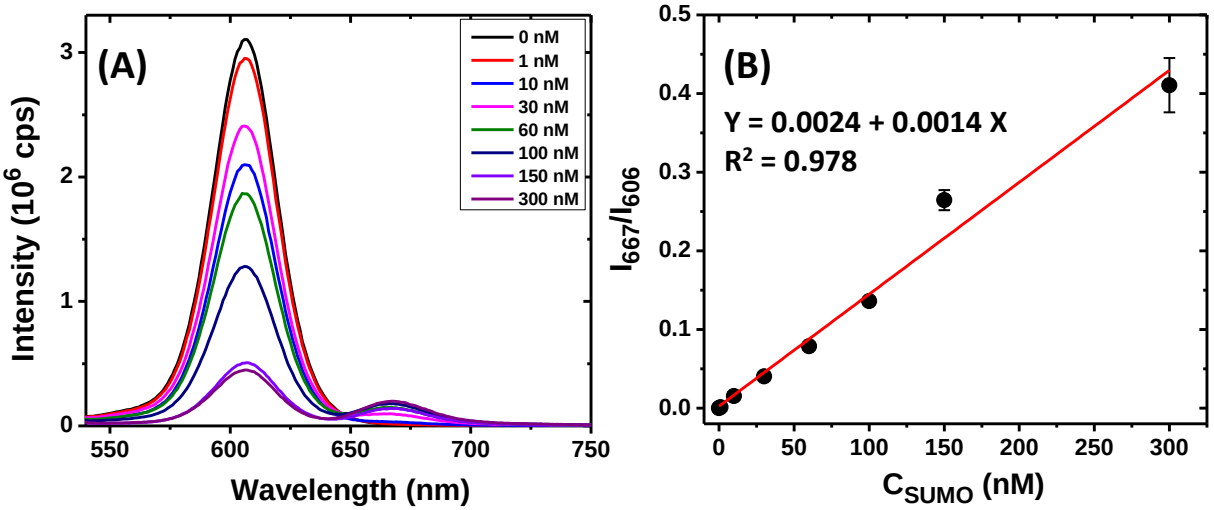


Figure S11. (**A**) Fluorescence spectra of the self-assembled QD-anti-SUMO Affimer conjugate (final C_{QD} = 10 nM) after incubation with different amounts of Alexa 647 labelled SUMO protein target. (**B**) A plot of the I_{667}/I_{606} as a function of SUMO protein concentration, data were fitted to a linear function: Y = 0.0024 + 0.0014X, R^2 = 0.978. The limit of detection here was determined to be ~ 1 nM.

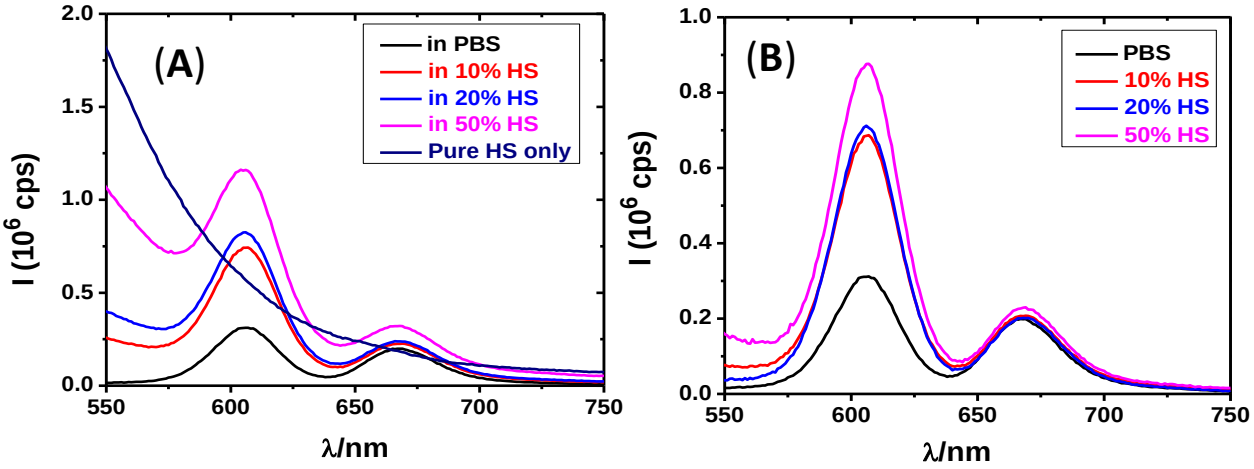


Figure S12. Fluorescence spectra of the self-assembled QD-anti-SUMO Affimer sensor (final C_{QD} = 10 nM) after incubation with the Alexa 647 labeled SUMO protein target (300 nM) in PBS (black), PBS containing 10% (red), 20% (blue) or 50% (pink) of human serum before (**A**) and after (**B**) correction of the human serum fluorescence background.

The above figure clearly shows that the QD-Affimer sensor prepared here is highly robust, it can specifically detect its target protein even under complex, clinically relevant conditions such as 50% human serum (HS).

Table S2. Comparison of the sensing performance of some QD-FRET sensors for direct target detection using fluorescence spectroscopy without target amplification.

Sensing Mechanism	Target	LOD (nM) limit	Dynamic range (nM)	**Reference**
QD-FRET	Thrombin	10	10-1000	[10]
QD-MB FRET	Thrombin	1	1-500	[11]
ET quenching	Thrombin	10	10-210	[12]
QD-FRET	Mucin-1	250	250-2000	[13]
Electrochemical	Thrombin	~ 1	1-1000	[14]
Tb-to-QD FRET	prostate specific antigen	0.05-0.27	0.05-21	[15]*
QD-FRET	Botulinum neurotoxin	0.02-0.04	0.02-400	[16]
Tb-to-QD FRET	prostate specific antigen	0.02-0.12	0.02-~4	[17]
Tb-to-QD-FRET	EGFR	0.12	~0.12-~5	[18]
Tb-to-QD-FRET	EGFR	0.01-0.11	~0.01-~10	[19]*
Tb-to-QD-FRET	HER2	0.04-0.15	~0.01-~10	[19]*
QD-FRET	SUMO protein	1.0	1-150	This work $^{\phi}$
QD-FRET	SUMO protein (labeled)	0.010	0.005-0.5	This work $^{\phi}$
QD-FRET	Neutravidin (labeled)	0.005	0.005-50	This work $^{\phi}$

* The LOD is dependent on the target binder sizes (antibody, antibody fragment or nanobody).

$^{\phi}$ The LOD is determined from background + 3σ, and the dynamic range is determined by the linear fit.

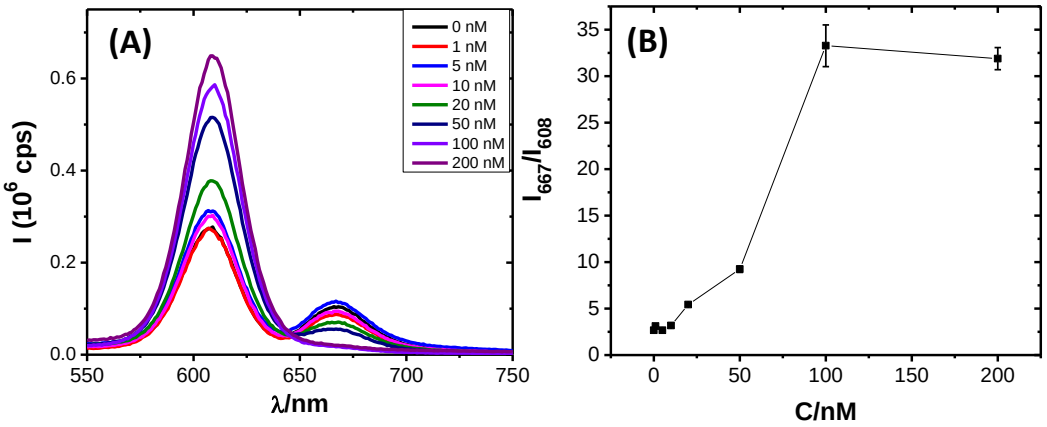


Figure S13. (**A**) Fluorescence spectra of QD-biotin100 (0.5 nM) + Alexa-647 labelled NAV (50 nM) in the presence of different amounts of free biotin. (**B**) A plot of the corresponding I_{608}/I_{667} ratio *versus* free biotin concentration.10 nM free biotin produced a distinguishable signal from the background, suggesting that the QD-biotin/NAV system is suitable for label-free detection of low nM level of free biotin.

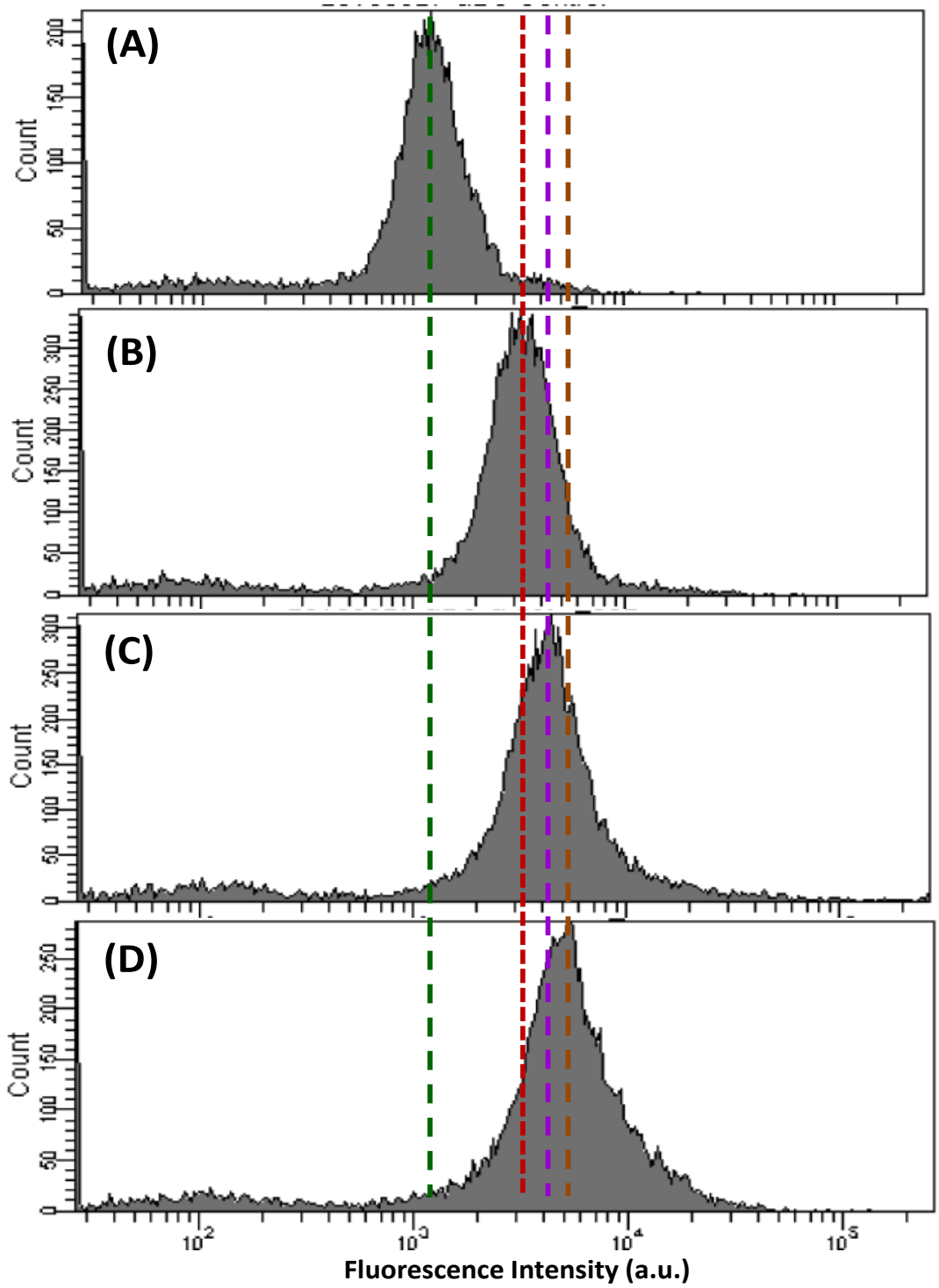


Figure S14: Typical cell fluorescence distribution histograms of HeLa cells measured by flow cytometry after 4 h incubation with the DMEM medium only (**A**) and DMEM medium supplemented with 50 nM of the control QD (**B**), QD-biotin$_{70}$ (**C**) or QD-biotin$_{150}$ (**D**). The media cell fluorescence intensities (a.u.) were 1218, 3255, 4194, and 5071, for **A, B, C** and **D,** respectively. A significant increase of cell median fluorescence (by *ca.* 56%, marked by broken lines for each treatment) was observed for cells treated by QD-biotin$_{150}$ over the control QD capped with DHLA-PEG750 only (note the logarithmic scale of the intensity). This result was consistent with the higher fluorescence intensities observed from the confocal fluorescence image shown in Figure 6.

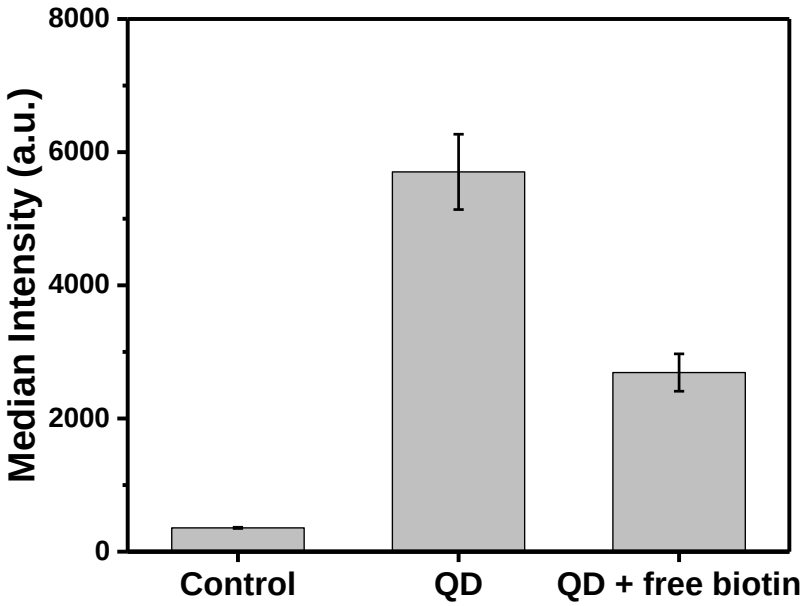


Figure S15. Comparison of the median fluorescence intensity of HeLa cells after incubation with the DMEM medium only (**Control**); 1 h incubation with EMEM medium followed by 4 h incubation with EMEM medium contianing 50 nM of QD-Biotin$_{150}$ (**QD**); and 1h incubation with DMEM medium suplemented with 10 mM free biotin followed by 4 h incubation with DMEM medium containing 50 nM of QD-Biotin150 and 10 mM free biotin (**QD + free biotin**). A signficant decrease of *ca.* 53% of cell median fluorescnece intensity (from 5700±560 to 2690±280) was obtainted by the presence of free biotin.

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