Development of a Bioorthogonal and Highly efficient Conjugation Method for Quantum Dots using Tetrazine-Norbornene Cycloaddition

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Supporting information

Experimental Section

Materials: All chemicals were purchased from Sigma Aldrich unless noted and were used as received. Selenium shot, cadmium oxide 99.999%, and n-tetradecylphosphonic acid (TDPA) were purchased from Alfa Aesar (Ward Hill, MA). Trioctylphosphine (TOP) and tributylphospine (TBP) were purchased from Strem Chemicals (Buchs, Switzerland). Epithermal Growth Factor (EGF, Human recombinant) and Alexa Fluor® 594 carboxylic acid, succinimidyl ester was purchased from Invitrogen. (1S,2S,4S)-bicyclo[2.2.1]hept-5-en-2-yl acetic acid was purchased from ChemBridge. All solvents were of reagent grade or higher and were used without further purification.

Tributylphosphine selenide (TBP-Se) was prepared by dissolving 0.15 mmol of selenium shot in 100 mL of TBP under inert atmosphere and stirring vigorously overnight, forming a 1.5 M TBP-Se solution. All air sensitive materials were handled in an Omni-Lab VAC glove box under dry nitrogen atmosphere with oxygen levels < 0.2 ppm. All solvents were spectrophotometric grade and purchased from EMD Biosciences.

Analytical HPLC and LC/MS were performed on a Waters 2695 HPLC equipped with a 2996 diode array detector, a Micromass ZQ4000 ESI-MS module, and a Grace-Vydac RPC18 column (model 218TP5210) at a flow rate of 0.3 mL/min. Preparative HPLC was performed on a Varian ProStar model 210 instrument equipped with a model 335 diode array detector, a model 701 fraction collector, and a Varian RPC18 column (model A6002250X212) at a flow rate of 21 mL/min. All UV/vis spectra were recorded on an Agilent 8453 diode array UV/vis spectrophotometer.

Photoluminescence and absorbance spectra were recorded with a BioTek Synergy 4 Microplate Reader. The absorbance of all solutions was kept below 0.1 OD to avoid inner-filter effects. Flash column chromatography was performed on a Teledyne Isco CombiFlash Companion. Polymer molecular weights were determined in DMF solutions on an Agilent 1100 series

HPLC/GPC system with three PLgel columns (103, 104, 105 Å) in series against narrow polystyrene standards.

Synthesis of CdSe(CdS): CdSe cores with 480nm first absorption peak were synthesized using a previously reported method[1]. To summarize,0.4 mmol (54.1mg) of CdO, 0.8 mmol (0.2232g) of TDPA, 9.6mmol (3.72g) of TOPO were placed in 25mL round bottom flask. The solution was degassed for 1 hr at 160°C and heated to 300°C under argon until the CdO dissolved and formed a clear homogenous solution. This was followed by putting the solution under vacuum at 160°C to remove evolved water. The solution was reheated to 360°C under argon and a TBP-Se solution (1.5mL of 1.5M TBP-Se in 1.5mL of TOP) was rapidly added to give CdSe cores with the first absorption feature at 468nm. The cores were then grown further at 260°C to produce cores with the desired wavelength for the first absorption feature.

CdS shells were deposited on CdSe cores via modification of previously reported procedures.[2] Cores isolated by repeated precipitations from hexane with acetone were brought to 180 °C in a solvent mixture of oleylamine (3 mL) and octadecene (6 mL). Aliquots of Cd and S precursor solutions were then introduced alternately starting with the metal (Cd) and waiting 15 min between the start of each addition. The Cd precursor consisted of 0.33 mmol Cd-oleate and 0.66 mmol oleylamine in a solvent mixture of octadecene (1.5 mL) and TOP (3 mL). The S precursor consisted of 0.3 mmol hexamethyldisilathiane [$(TMS)_2S$] in 6 mL TOP. The dose of each overcoating precursor aliquot corresponds to adding a single monolayer of atoms to the QD surface. Addition of a total of 4 aliquots each of Cd and S yielded QDs with emission at 570 nm and a quantum yield close to unity when diluted in octane. A similar procedure was performed on larger CdSe cores[3,4] to obtain CdSe(CdS) QDs emitting at 605 nm. The extinction coefficient of CdSe(CdS) was calculated using the extinction coefficient of CdSe cores from the literature[4] and assuming that 100% of the CdSe cores were retained during the overcoating step.

Preparation of amine-reactive tetrazine (Tz-NHS): 2,5-dioxopyrrolidin-1-yl 5-(4-(1,2,4,5-tetrazin-3-yl)benzylamino)-5-oxopentanoate (Tz-NHS) was prepared from 3-(4-benzylamino)-1,2,4,5-tetrazine (Tz-benzylamine) that was synthesized as previously described[5]. Tz-benzylamine (10 mg) was added to a solution of methylene chloride containing 6 mg glutaric anhydride. The solution was stirred overnight at 50°C. The methylene chloride was removed by rotary evaporation and the crude mixture purified by column chromatography resulting in 5-(4-(1,2,4,5-tetrazin-3-yl)benzylamino)-5-oxopentanoic acid (Tz-acid) in quantitative yield. This acid was then immediately introduced to an acetonitrile (2 mL) solution of N, N'-disuccinimidyl carbonate (68 mg) and triethylamine (30 mg) and allowed to stir at room temperature until reaction reached completion (monitored by TLC). The acetonitrile was removed by rotary evaporation and the crude mixture purified by column chromatography yielding 17 mg (80% yield) of the desired Tz-NHS. ^{1}HNMR (400 MHz $CDCl_3$): δ 10.3-10.2 (s, 1H), 8.7-8.5 (d, 2H),

7.6-7.4 (d, 2H), 6.6-6.2 (br, 1H), 4.7-4.4 (m, 2H), 3.1-2 (m, 10H). LR-MS [M+H]+ calc mass 399.1 found mass 399.2.

Synthesis of norbornene conjugated polymeric imidazole ligands (NB-PIL): Poly(amino-$PEG_{11})_{20\%}$-PIL was synthesized using a previously reported method[2]. (1S,2S,4S)-bicyclo[2.2.1]hept-5-en-2-yl acetic acid (norbornene) was activated by reacting 0.05mols of (1S,2S,4S)-bicyclo[2.2.1]hept-5-en-2-yl acetic acid with 0.06mols n-hydroxysuccinimide (NHS) and 0.06mol N,N'-Dicyclohexylcarbodiimide (DCC) in anhydrous tetrahydrofuran (THF) for 2 hours at room temperature. NHS activated norbornene was reacted with Poly(amino-$PEG_{11})_{20\%}$-PIL in dry THF overnight (2 times excess of norbornene was added to the number of the amine groups in the polymer). After the reaction was completed THF was removed by vacuum and the reaction mixture was redissolved in ethylacetate to precipitate the byproducts. Precipitates were filtered out using a 0.2 μM PTFE syringe filter. This workup was repeated several times until no precipitate was observed after removal of the solvent.

Fluorescamine Assay of Amine Reactivity PILs. Stock solutions of amine-containing PIL polymers were made at 20 mg/mL concentration. A serial dilution was made using 1, 2, and 4 μL of polymer stock into 240 μL of PBS buffer, followed by addition of 10 μL of a 30 mg/mL solution of fluorescamine in acetone. This mixture was vortexed and incubated at room temperature for 1 hour before fluorescence analysis on a BioTek plate reader with excitation at 380 nm and detection at 480 nm. The recorded fluorescence intensity signals were calibrated against solutions of known concentrations of methoxyPEG_{11}-NH_2.

Preparation of norbornene coated water soluble QDs: Ligand exchange of native QDs with NB-PIL was performed as described in the literature.[2] To summarize, QDs (1 nmol) were precipitated using hexanes (30 μL), $CHCl_3$ (30 μL) and EtOH(200 μL) and brought into 50 μL of $CHCl_3$. The QD stock solution was mixed with a solution of NB-PIL (4 mg) in $CHCl_3$ (30 μL), and stirred for 20 min at RT, after which 30 μL of MeOH was added followed by stirring for an additional 20 min. QD samples were precipitated by the addition of EtOH (30 μL), $CHCl_3$ (30 μL), and excess hexanes. The sample was centrifuged at 4,000 g for 2 minutes. The clear supernatant was discarded, and the pellet dried *in vacuo*, followed by the addition of PBS (500 μL, pH 7.4). The aqueous sample was then filtered through a 0.2 μm syringe filter before use. Prior to any conjugation chemistry or cell studies, free ligand was removed by three cycles of dilution/concentration through an Amicon Ultra Ultracel 50,000 Da MW cutoff filter (Millipore). The quantum yield of norbornene coated QDs was about 60%. (Quantum yield of natively capped QDs was ~100%).

Synthesis of 3-(4-benzylamino)-1,2,4,5-tetrazine conjugated Alexa 594: Alexa Fluor® 594 carboxylic acid, succinimidyl ester (Alexa 594) was reactivated with n-hydroxysuccinimide by adding 1.2equiv of NHS and 1.2equiv of DCC in dry DMF and reacted for 2 hours at room temperature. 1 equiv of 3-(4-benzylamino)-1,2,4,5-tetrazine was added to the solution and reacted overnight at room temperature. Completion of the reaction was confirmed using ninhydrin staining.

Synthesis of EGF-BAT: Amine reactive tetrazine (4mg/mL) was reactivated with n-hydroxysuccinimide by adding 1.2equiv of NHS and 1.2equiv of DCC in dry DMF and reacted for 2 hours at room temperature. 50μg of EGF was dissolved in 200μL 1X PBS and 1.2 equiv of NHS activated tetrazine was added to the solution and reacted overnight at room temperature. The conjugates were dialyzed three times with an Amicon Ultra Ultracel 3,000 Da Mw cutoff filter (Millipore) to remove excess NHS, DCC and byproducts.

Synthesis of QD-Alexa594 conjugates: 200μL of ~1μM norbornene coated QDs were mixed with different concentrations of Alexa594 tetrazine in 1X PBS and reacted for 4 hrs at 37°C. Excess reagents were removed by gel filtration chromatography and dialyze three times with 1X PBS using Amicon Ultra Ultracel 50,000 Da MW cutoff filter. The control experiments were performed using Poly(amino-PEG$_{11}$)$_{20\%}$-PIL coated QDs. Final materials were analyzed by UV-Vis absorption to determine the number of dyes on the QD surface. Concentrations of QDs and Alexa 594 were measured based on ε_{dye} = 90,000 cm^{-1}M^{-1} at 590nm for Alexa 594, and ε_{QD} = 2,630,000 cm^{-1}M^{-1} at 350nm for QD570.

Synthesis of QD-EGF: 0.2nmol of norbornene coated QDs and 0.4 nmol of EGF-tetrazine were mixed in 1X PBS with a final concentration of 1μM for QDs and incubated for 2 hrs at 37°C. Unreacted EGF was removed by three cycles of dilution/concentration through an Amicon Ultra Ultracel 50,000 Da MW cutoff filter (Millipore).

Quantum yield (QY) of QDs: The QY of QD570 was measured relative to Rhodamine 610 (QY 68% in ethanol) with excitation at 505 nm and the QY of QD605 was measured relative to Rhodamine 640 (QY 100% in ethanol with excitation at 535nm). Solutions of QDs in octane (native CdSe/CdS QDs) or PBS (QDs after ligand exchange with either Poly(amino-PEG$_{11}$)$_{20\%}$-PIL or NB-PIL) and dye in ethanol were optically matched at the excitation wavelength. Fluorescence spectra of QD and dye were taken under identical spectrometer conditions in quadruplicate and averaged. The optical density was kept below 0.1 at the λ_{max}, and the

integrated intensities of the emission spectra, corrected for differences in index of refraction and concentration, were used to calculate the quantum yields using the expression $QY_{QD} = (Absorbance)_{dye}/(Absorbance)_{QD} \times (Peak\ Area)_{QD}/(Peak\ Area)_{Dye} \times (n_{QD\ solvent})^2/(n_{Dye\ solvent})^2 \times QY_{Dye}$.

Gel Filtration Chromatography (GFC): GFC was performed using an ÄKTAprime Plus chromatography system from Amersham Biosciences equipped with a self-packed Superdex 200 10/100 column. PBS (pH 7.4) was used as the mobile phase with a flow rate of 1.0 mL/min. Detection was achieved by measuring the absorption at 280 nm.

Transmission Electron Microscopy: The inorganic size of CdSe(CdS) QDs was determined using a JEOL 200CX TEM operating at 200 kV. One drop of a dilute sample of QDs in hexane precipitated two times using acetone was placed onto a Formvar coated copper grid, allowed to settle for 20 seconds, and wicked away using an absorbent tissue. Size analysis was performed on captured digital images using ImageJ 1.34s.

Cell culture and labeling: A-431 human epidermoid carcinoma cells were grown in DMEM (Invitrogen) with 10% Fetal Bovine Serum (Invitrogen), 50 μg /mL penicillin and 50 μg /mL streptomycin (Invitrogen). When labeling cells with preformed QD-EGF conjugates (Figure 2B), cells were rinsed with 4°C 1% Bovine Serum Albumin (BSA) in PBS and incubated with 50 nM QD-EGF conjugates at 4°C for 30 minutes. For *in situ* click conjugation between tetrazine and norbornene on cells (Figure 2C), cells were rinsed with 4°C 1% BSA in PBS, incubated with 200 nM EGF-BAT at 4°C for 30 minutes, and then rinsed three times with 1% BSA in PBS to block non-specific binding. Subsequently, norbornene coated QDs at varying concentrations were added to the cells and incubated for 30 minutes at 37°C. The cells were then washed three times with 25°C PBS to remove excess QDs.

Fluorescence imaging: Cells were imaged with an epifluorescence microscope (Nikon) with a 60x water-immersion objective and Princeton instruments MicroMax Camera with a 1.5x magnification tube lens. Bright field images were collected using differential interference contrast with an exposure time of 100 ms and fluorescence images were collected by exciting with a 488 nm Argon-ion laser line combined with a D605/30M emission filter. Exposure times for fluorescence imaging were 200 ms for QD blinking time-lapse imaging and 500 ms for all others. All fluorescence image frames were background corrected using Matlab.

Copper quenching : To verify that copper (I) ions quench the QD fluorescence irreversibly, 2×10^{-10} mol of CdSe/ZnS (5 monolayers of ZnS), $CdSe/Cd_{0.3}Zn_{0.7}S$ (5 monolayers of

$Cd_{0.3}Zn_{0.7}S$) and CdSe/CdS (4 monolayers of CdS) coated with poly (PEG_{12})-PIL in 0.1×PBS were incubated with varying concentrations of Cu(I). Cu(I) was generated in situ by reducing Cu(II) with sodium ascorbate.

100 mmol of $CuSO_4$ and 500 mmol of sodium ascorbate were dissolved in 1mL of 0.1× PBS (Solution A). 1 μL, 3 μL, 10μL, 30μL, and 100 μL of solution A was added to 2×10^{-10} mmol of QDs and final volume was adjusted to 200 μL (0.1× PBS). Absorption and emission spectra of the samples were measured at 0min, 20min, 40min, 60min, and 80min after the copper addition.

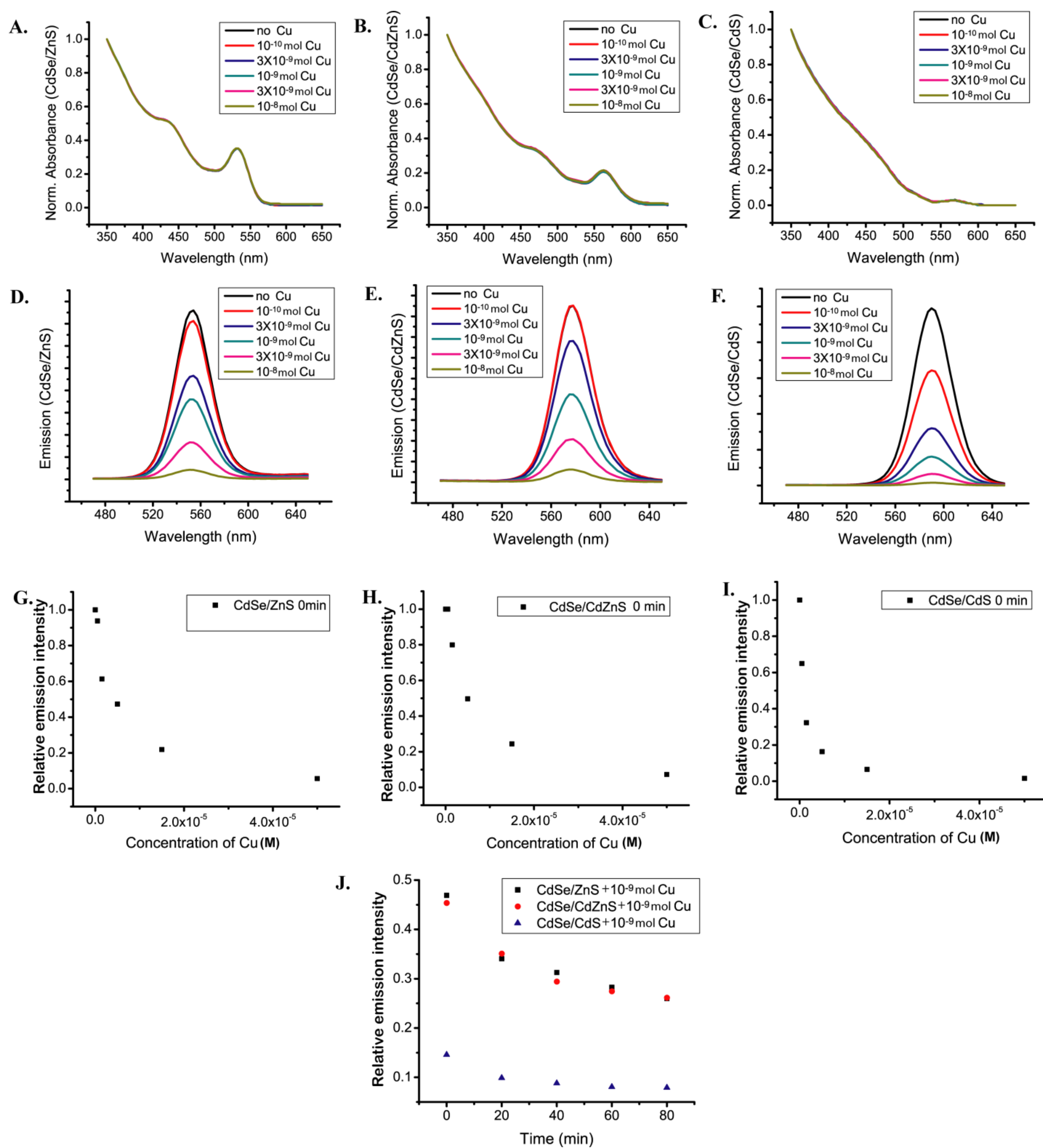

Figure S1. Absorption (A-C) and emission (D-F) spectra of 1μM of QDs (in 200μL 0.1× PBS) after adding varying concentrations of Cu(I) (0 min). (G-I) Relative emission intensity of the samples with varying concentration of Cu(I) to the control sample without Cu(I) (0min). (J) Drop of the relative emission intensity of the QD samples with 1×10^{-9} mol of Cu(I) over time. (A, D, and G) are for CdSe/ZnS (5 monolayers of ZnS), (B, E, and H) are for CdSe/$Cd_{0.3}Zn_{0.7}S$ (5 monolayers of $Cd_{0.3}Zn_{0.7}S$) and (C, F, and I) are for CdSe/CdS(4 monolayers of CdS). The experimental data shows CdS coated CdSe is most prone to quenching by Cu(I). For all samples,

an immediate drop of fluorescence is followed by a gradual decay of the emission intensity after the copper addition and the quenching is irreversible. In general procedures [6-9] for "click" chemistry on nanoparticles, >10^{-6} mol of Cu(I) is used as a catalyst for ~ 10^{-10} mol of nanoparticles. With 10^{-6} mol of Cu(I), we observed almost no emission from CdSe/ZnS, CdSe/$Cd_{0.3}Zn_{0.7}S$, or CdSe/CdS.

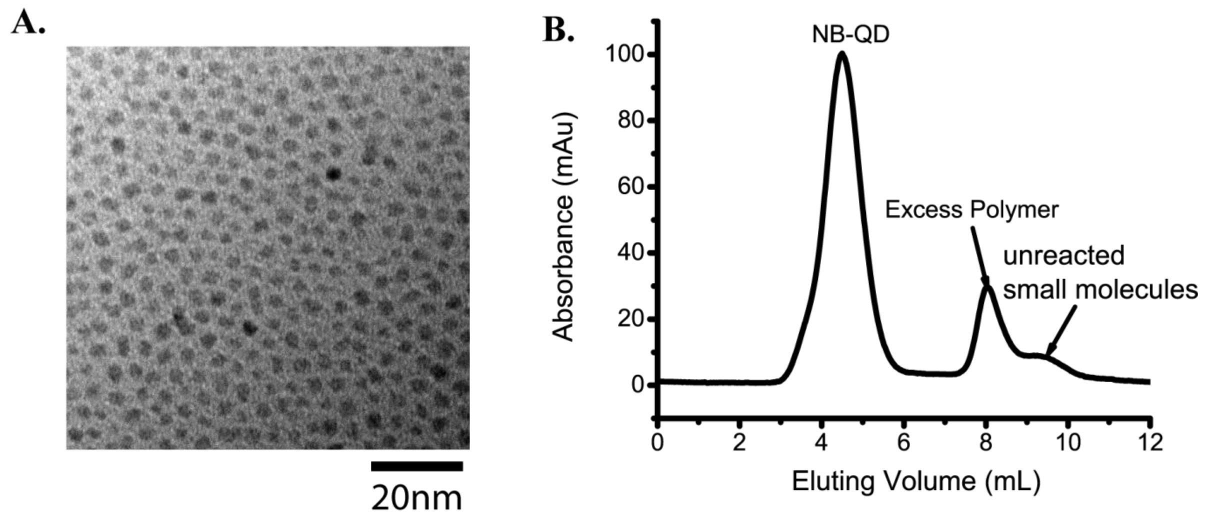

Figure S2. (A) TEM of CdSe(CdS) (λ_{em}=570nm) with inorganic size ~4.6 nm. (B) GFC of QD-Alexa 594 before purification with a retention time of 4.5 minutes corresponding to a hydrodynamic radius of ~11 nm (the peak at 8 min corresponds to free NB-PIL ligand and the peak at 9.4 min corresponds to unreacted Alexa).

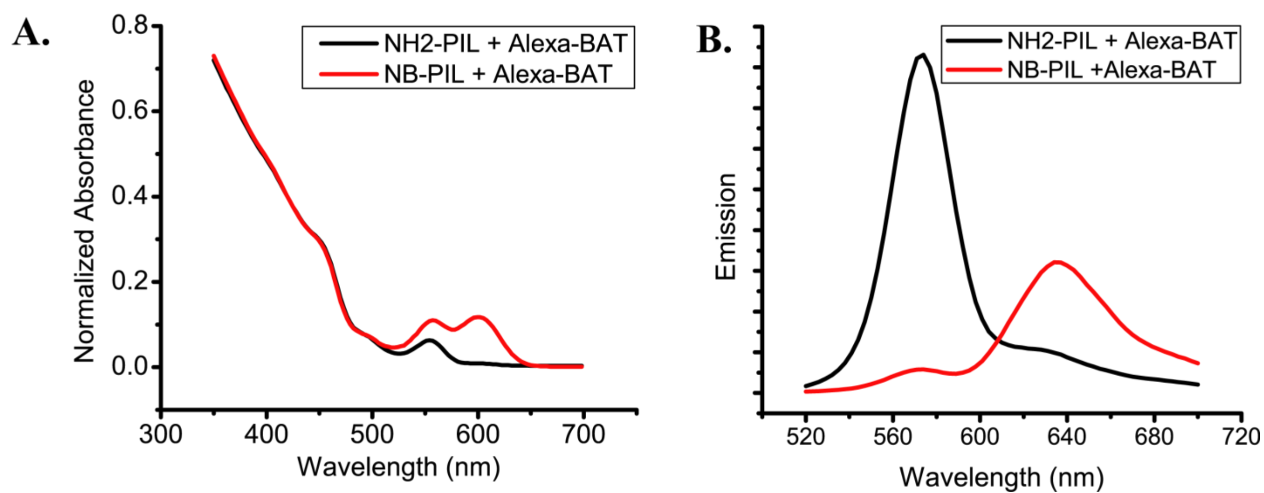

Figure S3 (A) Emission and (B) absorption spectra of control and QD-dye conjugates (~4 dye/dot).

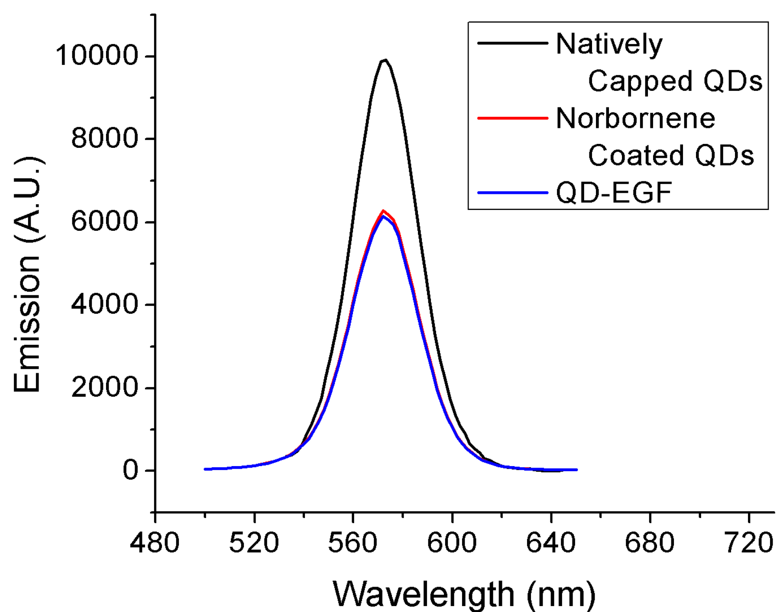

Figure S4. Emission spectra of native QDs (black, QY ~100% in octane) and norbornene coated QDs before (red, QY ~60% in 1×PBS) and after conjugation with EGF *via* norbornene-tetrazine cycloaddition (blue, QY ~60% in 1×PBS). The initial quantum yield of norbornene coated QDs is retained after the norbornene-tetrazine cycloaddition.

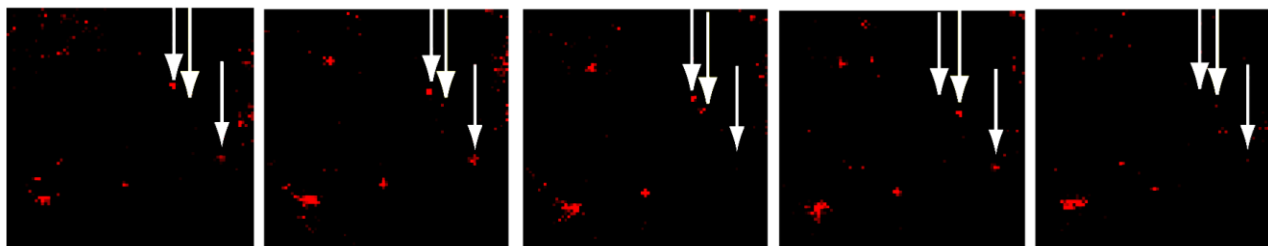

Figure S5. Time series (from left to right) single QDs (arrows) bound to the surface of A431 squamous cancer cells showing blinking. Each frame was acquired with a 200 ms exposure time.

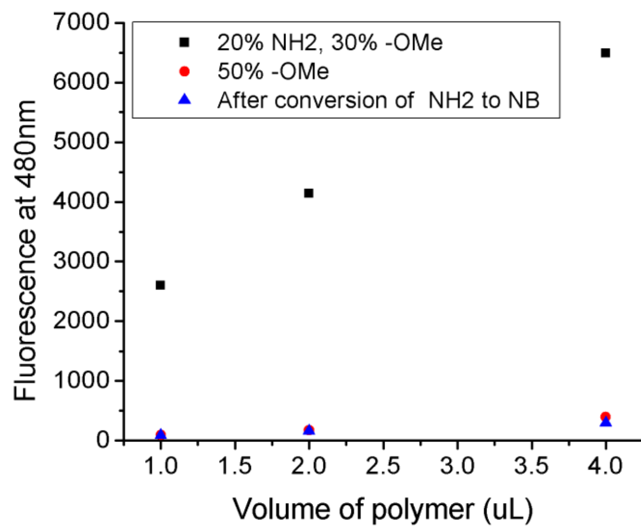

Figure S6. Probing free amines in different polymer samples using fluorescamine. Black square is poly(amino-PEG_{11})$_{20\%}$-PIL, red circle is poly(PEG_{12})-PIL, blue triangle is after converting the amine of poly(amino-PEG_{11})$_{20\%}$-PIL to norbornene (NB-PIL). Fluorescence of NB-PIL being similar level as poly(PEG_{12})-PIL proves the conversion was complete.

Mixed Dye:QD Ratio	QD Abs @ 350	QD Conc (μM)	Dye Abs @ 590	Dye Conc (μM)	Purified Dye:QD Ratio
10	0.251	0.0954	0.035	0.388	4.072
40	0.425	0.1616	0.165	1.83	11.336
100	0.431	0.1639	0.245	2.72	16.609
200	0.442	0.1681	0.232	2.58	15.373

Table S1. QD-Alexa 594 conjugation ratios. Concentrations were measured based on ε_{dye} = 90,000 $cm^{-1}M^{-1}$ at 590nm for Alexa 594, and ε_{QD} = 2,630,000 $cm^{-1}M^{-1}$ at 350nm for QD570.

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