Supporting Information

The Role of Ligand Coordination on the Cytotoxicity of Cationic Quantum Dots in HeLa Cells

Yi-Cheun Yeh,[a,#] Krishnendu Saha,[a,#] Bo Yan,[a] Oscar R. Miranda,[a] Xi Yu,[a] and Vincent M. Rotello [a,*]

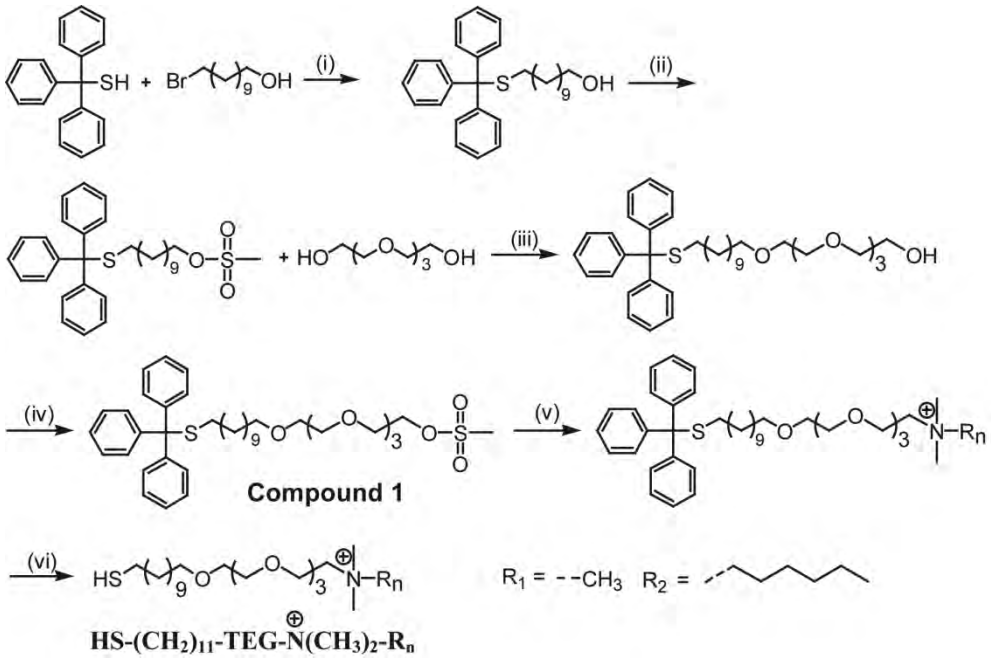
[a] Department of Chemistry, 710 North Pleasant St, University of Massachusetts Amherst, Amherst, Massachusetts, USA.

V. M. R.: E-mail: rotello@chem.umass.edu; Fax: 413-545-4490; Tel: 413-545-2058

[#] These authors contributed equally to this work.

Syntheses of cationic ligands

Synthesis of HS-$(CH_2)_{11}$-TEG-N$(CH_3)_2$-R_n ligand



Scheme S1. Synthetic route of the HS-$(CH_2)_{11}$-TEG-N$(CH_3)_2$-R_n. Reagents and conditions: (i) NaOH, EtOH/toluene, 70 ^oC, 6 h; (ii) MsCl, NEt_3, DCM, 0 ^oC to r.t., 24 h; (iii) NaOH, H_2O/DCM, 90- 100 ^oC, 24 h (iv) MsCl, NEt_3, DCM, 0 ^oC to r.t., 24 h; (v) N$(CH_3)_2$-R_n/EtOH, 35 ^oC, 48 h; (vi) TFA, $(iPr)_3$Si-H, DCM, r.t.

General procedure[1]: HS-$(CH_2)_{11}$-TEG-N$(CH_3)_2$-R_n ligand were synthesized by the reaction of **Compound 1** with corresponding alkylated N,N-dimethylamines for 48 h at 35 ^oC. The resulting compound was then deprotected in an excess of trifluoroacetic acid (TFA, 20 equivalents) and triisopropylsilane (TIPS, 1.2 equivalents) in dichloromethane

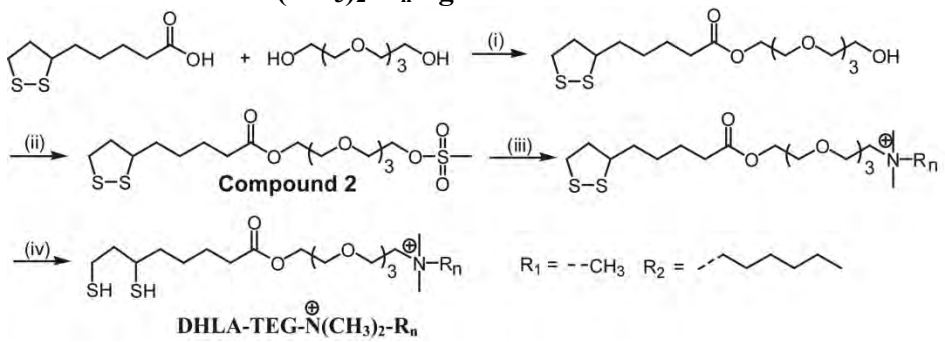
(DCM) under inner atmosphere. The reaction mixture was evaporated under reduced pressure to remove TFA and DCM, and further washed several times with hexanes and subjected to dry in high vacuum. The ligand structure was confirmed by ^{1}H-NMR and ESI-MS.

^{1}H NMR and ESI-MS of HS-$(CH_2)_{11}$-TEG-N$(CH_3)_2$-R_n

HS-$(CH_2)_{11}$-TEG-N$(CH_3)_2$-R_1: ^{1}H NMR (400MHz, $CDCl_3$): 3.98 (br, 2H, -OC$\underline{H}_2$-CH_2N-), 3.84-3.77 (br, 2H, -C$\underline{H}_2$N-), 3.68-3.53 (m, 12H, -CH_2-*TEG*-), 3.47-3.40 (t, 2H, -C$\underline{H}_2$O-), 3.35 (s, 9H, -N(C$\underline{H}_3$)$_3$), 2.80 (s, 3H, C$\underline{H}_3SO_3^-$-), 2.52 (q, 2H, -C$\underline{H}_2$S-), 1.70-1.51 (m, 6H, -NC$\underline{H}_2$-CH_2- + -SC$\underline{H}_2$-CH_2 + -CH_2-C$\underline{H}_2$O)-), 1.42-1.21 (m, 21H, -S$\underline{H}$ + -NC$\underline{H}_2$C$\underline{H}_2$-CH_2- + -C$\underline{H}_2$-). MS (ESI-MS) calculated for $C_{22}H_{48}NO_4S^+$ 422.3, found 422.1 [M^+].

HS-$(CH_2)_{11}$-TEG-N$(CH_3)_2$-R_2: ^{1}H NMR (400MHz, $CDCl_3$): 3.95 (br, 2H, -OC$\underline{H}_2$-CH_2N-), 3.70-3.54 (m, 14H, -CH_2-*TEG*- + -C$\underline{H}_2$N-), 3.47-3.41 (t, 2H, -C$\underline{H}_2$O-), 3.40-3.35 (m, 2H,-NC$\underline{H}_2$-), 3.20 (s, 6H, -N-(C$\underline{H}_3$)$_2$-CH_2), 2.86 (s, 3H, C$\underline{H}_3SO_3^-$-), 2.51 (q, 2H, -C$\underline{H}_2$S-), 1.80-1.51 (m, 6H, -NC$\underline{H}_2$-CH_2-) + -SC$\underline{H}_2$-CH_2 + -CH_2-C$\underline{H}_2$O-), 1.43-1.22 (m, 21H, -S$\underline{H}$ + -NC$\underline{H}_2$C$\underline{H}_2$-CH_2- + -C$\underline{H}_2$-), 0.89 (t, 3H, - C$\underline{H}_3$-). MS (ESI-MS) calculated for $C_{27}H_{58}NO_5S^+$ 492.4, found 492.3 [M^+].

Synthesis of DHLA-TEG-N$(CH_3)_2$-R_n ligand



Scheme S2. Synthetic route of DHLA-TEG-N$(CH_3)_2$-R_n ligand. Reagents and conditions: (i) EDC, HOBt, DIPEA, DCM, r.t., 24 h; (ii) MsCl, NEt_3, DCM, 0 ^{o}C to r.t., 24 h; (iii) N$(CH_3)_2$-R_n/EtOH, 35 ^{o}C, 48 h (iv) $NaBH_4$, EtOH/ H_2O, r.t., 2 h.

General procedure[2] : DHLA-TEG-N$(CH_3)_2$-R_n ligand were synthesized by the reaction of **Compound 2** with corresponding alkylated N,N-dimethylamines for 48 h at 35 ^{o}C. The reaction mixture was evaporated under reduced pressure to remove the solvent, and further washed several times with hexanes and subjected to dry in high vacuum. $NaBH_4$ was added to reduce the disulfide bond. The reaction mixture was diluted with water and extracted with $CHCl_3$ to obtain the final product. The ligand structure was confirmed by ^{1}H-NMR and ESI-MS.

^{1}H NMR and ESI-MS of DHLA-TEG-N$(CH_3)_2$-R_n

DHLA-TEG-N$(CH_3)_2$-R_1:^{1}H NMR (400 MHz, $CDCl_3$) major peaks assigned: δ 4.22 (t, 2H, COO-C$\underline{H}_2$-CH_2), 3.98 (brs, 2H, -OCH_2-C$\underline{H}_2$-CH_2-N$(CH_3)_3$), 3.94 (brm, 2H, COO-CH_2-C$\underline{H}_2$-O), 3.71-3.59 (m, 12H, -CH_2-*TEG*-), 3.42 (s, 9H, -N(C$\underline{H}_3)_3$), 2.35 (t, 2H, CH_2-C$\underline{H}_2$-COO), 1.94-1.86 (m, 2H), 1.8-1.7 (m, 4H), 1.68-1.61 (m, 4H). MS (ESI-MS) calculated for $C_{19}H_{40}NO_5S_2^+$ 426.23, found 426.1 [M^+].

DHLA-TEG-N$(CH_3)_2$-R_2:1H NMR (400 MHz, $CDCl_3$) major peaks assigned δ 4.22 (t, 2H, COO-C$\underline{H}_2$-CH_2), 3.98 (brs, 2H, -OCH_2-C$\underline{H}_2$-CH_2-N$(CH_3)_2$-), 3.91 (brm, 2H, COO-CH_2-C$\underline{H}_2$-O), 3.75-3.57 (m, 12H, -CH_2-*TEG*-), 3.34 (s, 6H, -N(C$\underline{H}_3)_2$-CH_3), 2.34 (t, 2H, CH_2-C$\underline{H}_2$-COO), 1.8-1.69 (m, 3H), 1.68-1.57 (m, 4H), 1.4-1.27 (m, 9H), 0.89 (s, 3H, -N$(CH_3)_2$-C$\underline{H}_3$). MS (ESI-MS) calculated for $C_{24}H_{50}NO_5S_2^+$ 496.3, found 496.2 [M^+].

Experimental section

Materials

All chemicals were purchased from Sigma-Aldrich unless otherwise stated. The organic solvents were bought from Fisher and used as received while dichloromethane (DCM) was distilled in the presence of calcium hydride. Flash column chromatography was performed for purification using silica gel (SiO_2, particle size 40-63 μm). Low glucose Dulbecco's Modified Eagle's Medium (DMEM) (Sigma, D5523) and fetal bovine serum (FBS) (Fisher Scientific, SH3007103) were used in cell culture. The reagent necessary for the MALDI-MS analysis of the QDs included HPLC grade acetonitrile (Fisher Scientific Co LLC, Fair Lawn, NJ), trifluoroacetic acid (Sigma-Aldrich, St. Louis, MO), and α-cyano-4-hydroxycinnamic acid (α-CHCA). Milli-Q water from a Millipore Simplicity 185 system (Millipore Corp., Billerica, MA) was also used to prepare samples.

Synthesis of hydrophobic QDs

CdSe/ZnS core-shell QDs were prepared according to the reported procedure.[3] Briefly, CdO (0.05 g, 0.4 mmol), tetradecyl phosphonic acid (TDPA) (0.22 g, 0.8 mmol) and trioctylphosphine oxide (TOPO) (3.78 g, 9.77 mmol) were loaded into a 50 ml three-neck flask and heated to 350 ^oC under N_2 flow. After 3 h the solution becomes optically clear and the Se solution (Se (0.04 g, 0.53 mmol) in 2.40 ml trioctyl phosphine (TOP)) was swiftly injected into the hot solution. The CdSe QDs were purified and precipitated with chloroform ($CHCl_3$) and methanol (MeOH), and finally dissolved in $CHCl_3$. For the shell coating, the CdSe core solution was mixed with TOPO (4 g, 10.3 mmol) and

hexadecylamine (HDA) (1.5 g, 6.2 mmol), and then heated to 150 ^{o}C for 1 h. Diethylzinc ($ZnEt_2$) (1.6 ml, 1.6 mmol) in 2.4 ml TOP and hexamethyl-disilathiane $(TMS)_2S$ (0.28 ml, 1.3 mmol) in 5.25 ml TOP were used as shell solution. After injecting the shell precursor solution, the QD mixture was reacted for 1 h at 100 ^{o}C. The resulting CdSe/ZnS QDs were purified and precipitated with $CHCl_3$ and MeOH, and finally stored in toluene.

Preparation of cationic QDs

The preparation of the monothiol-functionalized QDs was through ligand exchange process. Monothiolate ligands have placed in MeOH under ligand to QD ratio by weight (10: 1), and the solution was stirred at 40 ^{o}C for 4 days. These critical conditions can help the monothiol ligands replace most of the hydrophobic TOPO/TOP ligands on the surface of QDs and transfer QDs into aqueous solution. The preparation of dithiol-functionalized QD was through two steps ligand exchange reaction using the strategy described previously.[2] In first step, TOPO/TOP coated QDs (10 mg) were mixed with HS-$(CH_2)_5$-TEG-OH ligands (30 mg) in MeOH, and the reaction mixture was stirred at 35 ^{o}C for 24 h. Next step involved the purification of QDs with hexane and the addition of dithiol cationic ligands (30 mg) to the amphiphilic QDs in MeOH. As a result, dithiol ligands slowly substitute monothiol ligands from QD surface due to its better chelating capability compared to monothiol analogues.[4] After 24 h of stirring, MeOH was evaporated and QDs were dispersed in water. Both of the aqueous QD sample, including monothiol- and dithiol-functionalized QDs, were purified by dialysis. Mass spectra analysis revealed the characteristic peaks of the quaternary ammonium derivatives on QD surface, which was showed in supporting information. MALDI-MS analyses of QDs were modified based on previous method.[7]

DLS and zeta potential

DLS experiments and zeta potential measurements were performed using a Malvern Zetasizer (Nano series, Malvern Instruments Inc, USA). Samples were sonicated and filtered with 0.2 μm syringe filter before measurement.

Thermal gravimetric analysis (TGA)

TGA was performed using a TGA 2950 high-resolution thermo-gravimetric analyzer (TA Instruments, Inc., New Castle, DE), which was equipped with an open platinum pan and an automatically programmed temperature controller. TGA data were obtained as

follows: about 2.0 mg of QDs was placed in the TGA pan and heated in a nitrogen atmosphere at a rate of 5 °C / min up to 800 °C.

Calculation of the ligand coverage for QDs

A stepwise calculation of the ligand coverage of QDs is based on the following equations:

$$ligand\ molar\ amount = \frac{ligand\ weight}{ligand\ molar\ mass} \quad \text{.....(Eq. 1)}$$

$$ligand\ number\ per\ QD = \frac{ligand\ molar\ amount}{QD\ molar\ amount} \quad \text{.....(Eq. 2)}$$

$$ligand\ coverage\ of\ QD = \frac{ligand\ number\ per\ QD}{surface\ area\ of\ QD} \quad \text{.....(Eq. 3)}$$

(Surface area = $4\pi r^2$; r = radius of QD)

In detail, the concentration of QD **1** solution was first measured using UV-Vis spectrometry.[5] QD **1** solution (3.71 μM in 500 μl, 1.86×10^{-9} mol) was lyophilized to obtain the final weight of 4×10^{-4} g and further used in the TGA experiment. According to the TGA data, the weight loss of QD **1** was 62%, indicating the weight of the ligand in QD**1** solution was 2.48×10^{-4} g. The total ligand molar amount on QD surface can be calculated as 5.88×10^{-7} mol based on the molar mass of the ligand (422.69 g/mol) (Eq. 1). Thus, the ligand number per QD can be determined as 316 by dividing molar amount of QD by molar amount of ligand (Eq. 2). The size of QD **1** was 2.9 ± 0.5 nm in diameter based on the TEM image,[6] indicating the radius (*r*) of QD was 1.45 nm. Thus, the ligand coverage can be calculated as 12 nm^{-2} by dividing surface area of QD by the ligand number per QD (Eq. 3).

Similar calculation process was used to calculate the ligand coverage of QD **2**, and it was determined as 8 nm^{-2}.

Electrospray Ionization Mass Spectrometry (ESI-MS)

Mass spectra were acquired at positive mode on a Bruker Esquire-LC (Billerica, MA) quadrupole ion trap mass spectrometer, equipped with an electrospray ionization source. The electrospray needle voltage was set to 3.5 kV, and the capillary temperature was kept as 300 ^{o}C. Usually a voltage of 30 V was applied to skimmer 1 and a voltage of 80 - 90 V was applied to the capillary offset. Samples (~20 μM) were delivered at 200 μL/h using a syringe pump.

Matrix Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI-MS)

The MALDI-MS analyses were modified based on previous method.[7] It was acquired at positive mode on a Bruker Autoflex III MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) (Autoflex III). Operating conditions were as follows: ion source 1 = 19.00 kV, ion source 2 = 16.60 kV, lens voltage = 8.44 kV, reflector voltage = 20.00 kV, reflector voltage 2 = 9.69 kV, pulsed ion extraction time = 10 ns, suppression = 400 Da, and positive reflectron mode. Laser strength was optimized to around 50μJ/pulse. A saturated α-CHCA stock solution was prepared in 70% acetonitrile, 30% H_2O, and 0.1% trifluoroacetic acid, and to this stock solution was added an equal volume of a 2 μM solution of the QDs. 1 μL of this mixture was applied to target, and after allowing it to dry, the MALDI-MS analysis was performed.

Cell culture

HeLa cells were cultured at 37 °C under a humidified atmosphere of 5 % CO_2.The cells were grown in low glucose Dulbecco's Modified Eagle's Medium (DMEM, 1.0 g/L glucose) containing 10 % fetal bovine serum (FBS) and 1% antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). The cells were maintained in the above medium and subcultured once in a four days.

Preparation of the cellular uptake and ICP-MS measurements

HeLa cells were plated 30000 cells/well in a 24-well plate prior to the experiment. On the following day, cells were washed one time with PBS followed by QD treatment (25 nM/well) and further incubated for 24 h (or 3 h). After incubation, cells were lysed and transferred to 15 ml centrifuge tubes. 0.5 ml of *aqua regia* was added to each sample and then the sample was allowed to react for 1-2 h. *Aqua regia is highly corrosive and must be treated with extreme caution!* The sample solution was then diluted to 10 ml with 5% v/v nitric acid. ICP-MS (Elan 6100, Perkin-Elmer, Shelton, CT, USA) was used to determine the ^{111}Cd in the lysed cells. A series of Cd standard solutions (20, 10, 5, 2, 1, 0.5, 0.2, and 0 ppb) were measured to build the calibration curve before each experiment. Each cell uptake experiment was done in triplicate, and each replicate was measured 5 times by ICP-MS. ICP-MS operating conditions are as below: rf power 1600 W; plasma Ar Flow rate, 15 ml/min, nebulizer Ar flow rate, 0.98 ml/min; dwell time, 45 ms.

Cellular Viability

Cell viability was determined by using Alamar blue assay according to the manufacturer's protocol (Invitrogen Biosource, USA). In a typical experiment, HeLa cells were plated at 15000 cells/well in a 96-well plate 24 h prior to the experiment. On the following day, the old media was removed and cells were washed one time with cold phosphate-buffered saline (PBS) buffer before putting the different concentrations of QDs mixed in the pre-warmed 10% serum containing media. Cells were further incubated for 24 h (or 3 h) at 37 °C under a humidified atmosphere of 5% CO_2. Cells were thoroughly washed with PBS buffer (three times) and were treated with 220 μL of 10% alamar blue in serum containing media. Subsequently, the cells were incubated at 37 °C under a humidified atmosphere of 5% CO_2 for 4 h. After 4 h of incubation, 200 μL of solution from each wells was transferred in a 96-well black microplate. Red fluorescence, resulting from the reduction of Alamar blue solution, was quantified (excitation/emission: 560 nm/590 nm) on a SpectroMax M5 microplate reader (Molecular Device) to determine the cellular viability. Cells without any QDs were considered as 100% viable. Each experiment was done in triplicate.

Effect of antioxidant on cell viability

The experiment was performed as mentioned in earlier reports.[8] In short, HeLa cells were plated at a density 15000 cells/well in a 96-well plate on the day prior to the experiment. Next day, the old media was removed and cells were washed one time with PBS. Cells were then treated with N-acetylcysteine (NAC) with final concentration of 2 mM for 2 h prior to QD addition (200 nM/well) and maintained continuously in the media. After 24 h, the cell viability was measured *via* Alamar blue assay following the above mentioned protocol.

Cellular membrane damage

The extent of membrane damage by cationic QDs was quantified by G6PD release assay kit (Invitrogen, V-23111). HeLa cells were plated (15000 cells/well) one day prior to the experiment. On the following day, cells were washed with PBS one time and treated with 300 nM of QDs for 3 h. The following steps of the assay were performed according to manufacturer's protocol. The fluorescence reading was measured on a SpectroMax M5 microplate reader (Molecular Device) with excitation/emission: 560 nm/ 590 nm. Percentage of G6PD release was calculated taking fully lysed cells as 100% control.

Propidium iodide staining

HeLa cells (50000 cells/ well) were plated in a 24-well plate one day prior to the experiment. Next day, cells were washed one time with PBS and QD **1** and QD **2** (300 nM in 10% serum containing media) were added to the cells. After 3 h of incubation, the cells were washed three times and propidium iodide in PBS (final concentration 3 μM) was added to the cells. After 15 minutes of incubation at 37˚C, the images of the cells were captured using an Olympus IX51 microscope with excitation wavelengths of 535 nm.

Supporting figures

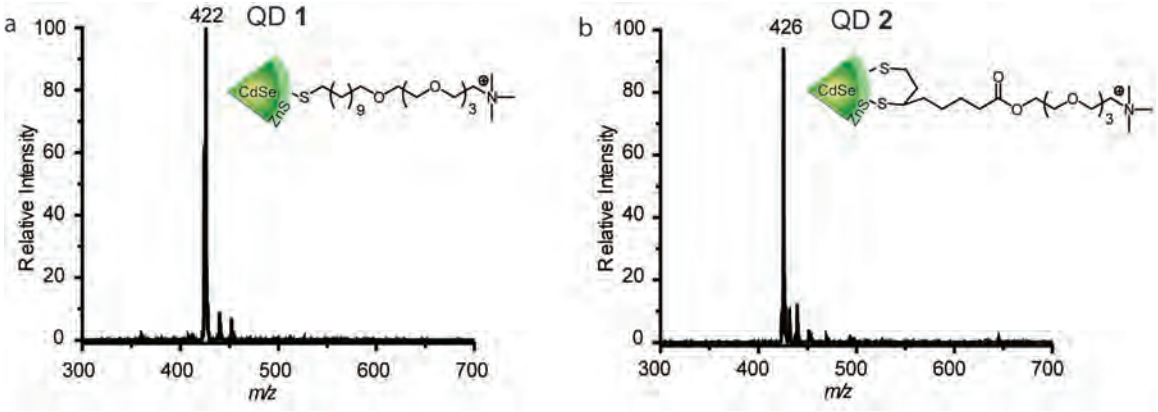


Fig. S1 MALDI-MS spectra of QD **1** and QD **2**.

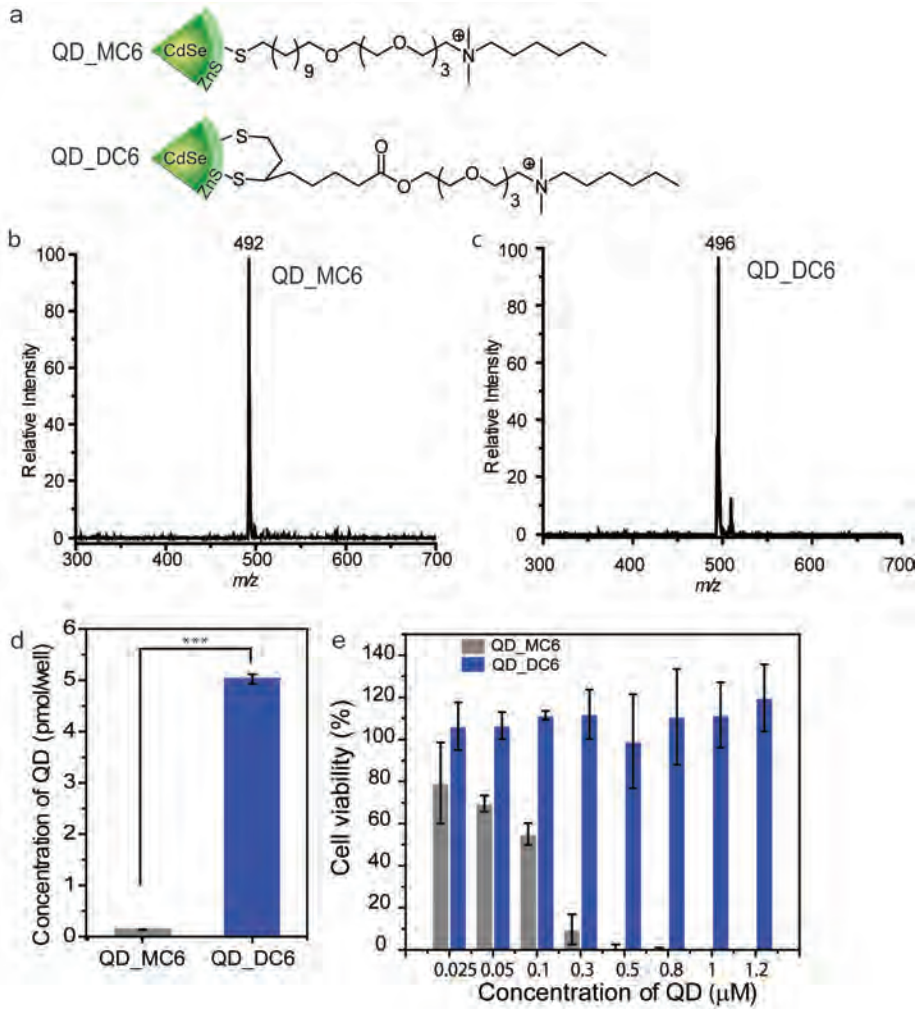


Fig. S2 (a) Molecular structures of analogous QDs featuring dimethylhexyl ammonium terminal functional group (monothiolate QD_MC6 and dithiolate QD_DC6). MALDI mass spectra of (b) QD_MC6 and (c) QD_DC6. (d) Cellular uptake amount of the cationic QDs (25 nM) after incubation with HeLa cells for 24 h. (e) Cell viability of the cationic QDs in HeLa cells at different concentrations after incubation for 24 h. (*** $p \leq 0.001$, one-way, ANOVA).

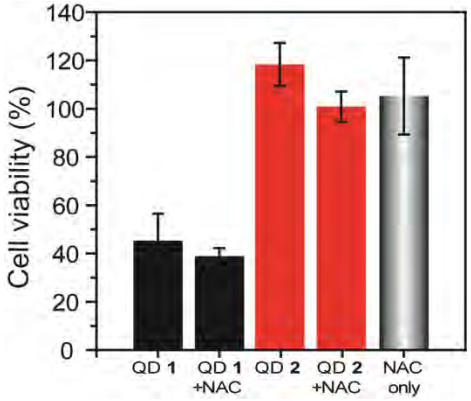


Fig. S3 Cell viability after QD (200 nM) incubation with HeLa cells for 24 h in the absence and presence of NAC.

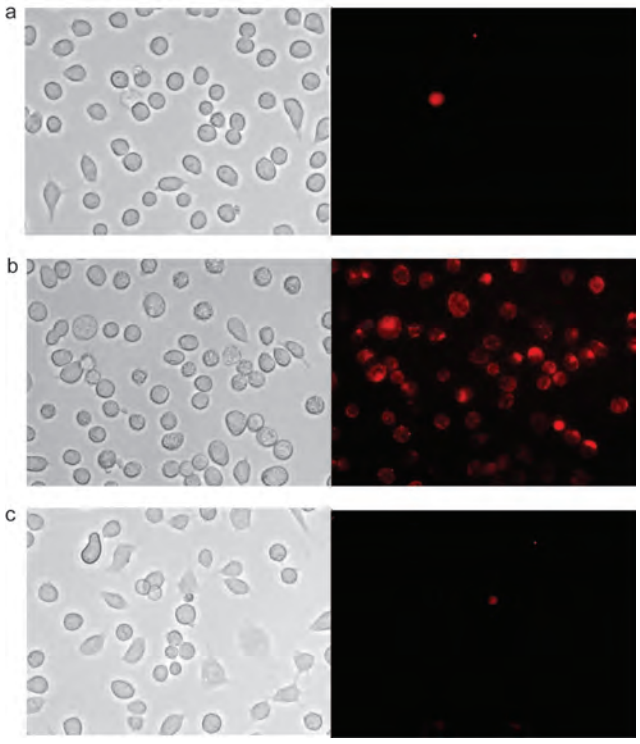


Fig. S4. Necrosis-mediated cell death from QD **1**. HeLa cells were incubated with QDs (300 nM) for 3 h and stained with propidium iodide. (a) HeLa cells only as negative control. HeLa cells were incubated with (b) QD **1** and (c) QD **2**. For each series (left to right) the corresponding contrast and fluorescence images were shown.

Reference:

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