Chapter 23

Cellular Internalization of Quantum Dots

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Abstract

Cell-penetrating peptides (CPPs) can facilitate uptake of quantum dots (QDs) for a variety of basic and applied sciences. Here we describe a method that utilizes simple noncovalent interactions to complex QDs and CPPs. We further describe methods to study uptake mechanisms of the QD/CPP complex. The inhibitor study coupled with the RNA interference (RNAi) technique provides a comprehensive approach to elucidate cellular entry of the QD/CPP complex.

Key words Quantum dots, Cell-penetrating peptides, Inhibitors, siRNA, Clathrin, Caveolin, Lipid raft, Macropinocytosis

1 Introduction

Nanomaterials have found numerous biomedical applications in recent years. The applications include, but not limited to, drug delivery, disease staging and therapeutic planning, sentinel lymph node mapping and removal, cellular receptor trafficking monitoring, and nanoelectronic biosensing. Among nanomaterials, fluorescent semiconductor quantum dots (QDs) have been widely used to deliver and monitor biologically active molecules into cells (1–3). QDs have unique physical and chemical properties such as photostability, high quantum yield, narrow emission peak, resistance to degradation, and broad size-dependent photoluminescence (4). These properties enable QDs for long-term multiplexing imaging. QDs are extremely slow in entering the cell. One solution to overcome the slow uptake is to conjugate with cell-penetrating peptides (CPPs) (5–7). We have developed protocols to (1) enhance efficiency of cellular uptake of QDs mediated by cell-penetrating peptides (8, 9) and (2) study molecular mechanisms of action of QD internalization.

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2 Materials

2.1 General Components

1. Quantum dots (QDs): Carboxyl-functionalized QDs with a CdSe/ZnS core-shell have emission and excitation peak wavelengths at 520 and 505 nm, respectively. Store at 4°C.
2. Synthetic nona-arginines (SR9): SR9 are synthesized by solid-phase peptide synthesis and then purified by high performance liquid chromatography (HPLC) using a reverse phase column. The desired purity of SR9 should be ≥90%. Store at −20°C.
3. Cell culture medium: Ham's F-12 modified medium (Cellgro, VA, USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Utah, USA) and 1% penicillin–streptomycin. Add 50 mL FBS and 5 mL penicillin–streptomycin into 445 mL Ham's F-12 modified medium. Store at 4°C.
4. Phosphate buffered saline (PBS): Dissolve 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, and 0.24 g KH_2PO_4 in 800 mL distilled H_2O. Adjust pH to 7.4 and make up to 1 L with distilled H_2O. Sterilize by autoclaving. Store at 4°C.
5. Trypsin–EDTA: 0.25% (w/v) trypsin supplemented with 0.53 mM EDTA in PBS. Mix 5 mL trypsin (5×) and 50 μL of 53 mM EDTA together. Make up to 50 mL with PBS. Store at −20°C.
6. Tris-acetate–EDTA (50× TAE) buffer: Weigh 242 g Tris and then transfer into a graduated cylinder containing 500 mL double distilled water (ddH_2O). Add 57.1 mL glacial acetic acid and 100 mL of 0.5 M EDTA. Make up to 1 L with ddH_2O. Store at 4°C.

2.2 Components of Gel Retardation

1. 0.6% agarose gel: Dissolve 3 g agarose in a 100-mL graduated cylinder containing 50 mL TAE buffer (1×). Heat agarose solution in a microwave until agarose is completely dissolved. Cool down the agarose solution to 60°C. Insert the comb onto the glass plate and then pour the agarose liquid into glass plate. Remove the air bubbles under or between the teeth of the comb. Wait the agarose gel to become solid before use.
2. UV transilluminator.

2.3 Components of Fluorescence Image Studies

1. Medium for fluorescence imaging: Ham's F-12 modified medium supplemented with 1% FBS. Add 5 mL FBS into 495 mL Ham's F-12 modified medium. Store at 4°C.
2. Dish for fluorescence imaging: 35-mm glass-bottom tissue culture plates (MatTek, Massachusetts, USA).
3. Phenol red-free medium (Invitrogen, California, USA).
4. Epifluorescent microscopy or confocal microscopy.

2.4 Components of Energy-Dependent Cellular Uptake

1. Temperature treatment: Cells are treated at either 37°C or 4°C.
2. A combination of metabolic inhibitors: 0.15% sodium azide, 15 mM sodium fluoride, and 2 μg/mL antimycin A.

2.5 Components of Pathway-Specific Inhibitors

1. Inhibitors of clathrin-dependent pathway: Chlorpromazine stabilizes the nascent clathrin-coated vesicles while monodansylcadaverine (MDC) inhibits relocation of clathrin and adaptor protein complex-2 (AP-2) from the plasma membrane to the endosomal membrane. The mechanism of hypertonic sucrose (0.45 M) involves the dispersion of clathrin lattices on the plasma membrane. Mix chlorpromazine or MDC stock solution in Ham's F-12 modified medium supplemented with 10% FBS and 1% penicillin–streptomycin at final concentrations of 10 μM and 25 μg/mL, respectively. For hypertonic studies, dissolve 0.154 mg sucrose in 1 mL Ham's F-12 modified medium supplied with 10% FBS and 1% penicillin–streptomycin.
2. Inhibitors of caveolin-dependent pathway: Filipin and nystatin are used to deplete cholesterol. Mix the filipin or nystatin stock solution in Ham's F-12 modified medium supplemented with 10% FBS and 1% penicillin–streptomycin at final concentrations of 3 and 20 μg/mL, respectively.
3. Inhibitors of macropinocytosis: 5-(*N*-ethyl-*N*-isopropyl) amiloride (EIPA) and cytochalasin D (CytD) are used to inhibit Na+/H+exchange or cap the barbed, fast-growing ends of actin filaments, respectively. Mix the EIPA or CytD stock solution in Ham's F-12 modified medium supplemented with 10% FBS and 1% penicillin–streptomycin at final concentrations of 30 μM or 1 μg/mL, respectively.

2.6 Components of siRNA Experiments

1. Small interfering RNA (siRNA): The sequences of siRNAs for clathrin heavy chain and caveolin-1 are as follows:

   Clathrin heavy chain:

   5′-CCCUAAACACCUCAACGAU[dT][dT]-3′ (sense)

   5′-AUCGUUGAGGUGUUUAGGG[dT][dT]-3′ (antisense)

   Caveolin-1:

   5′-CAUUAUGACCGGGCUCAUA[dT][dT]-3′ (sense)

   5′-UAUGAGCCCGGUCAUAAUG[dT][dT]-3′ (antisense)
2. Lipofectamine 2000: Dilute lipofectamine stock solution in the OPTI-MEM I-reduced serum medium according to the manufacturer's instructions (Invitrogen).
3. Treatment medium: Desired siRNA is premixed with OPTI-MEM I-reduced serum medium (Invitrogen) before complexing with lipofectamine.

4. Lysis buffer: 150 mM NaCl, 1% NP-40, and 50 mM Tris (pH 8.0) supplemented freshly with 1% protease inhibitor cocktail. Weigh 0.876 g NaCl and then transfer to a 100-mL cylinder containing 50 mL ddH_2O. Add 1 mL NP-40 and 5 mL of 1 M Tris solution (pH 8.0). Make up to 100 mL with ddH_2O. Store at −20°C. Add 1% protease inhibitor cocktail before use.
5. Bio-Rad protein assay (1×): Dilute 1 mL of Bio-Rad protein assay (5×) (Bio-Rad, California, USA) with 4 mL ddH_2O.
6. Standard solutions: Prepare bovine serum albumin in a series of five dilutions. The range depends on predicted concentrations in samples.
7. Resolving buffer: 1.5 M Tris–HCl (pH 8.8). Add 100 mL ddH_2O to a graduated cylinder. Weigh 181.7 g Tris and 4 g sodium dodecyl sulfate (SDS), and then transfer them into the cylinder. Make up to 900 mL with ddH_2O. Mix and adjust pH to 8.8 with HCl. Make up to 1 L with ddH_2O. Store at 4°C.
8. Stacking gel buffer: 0.5 M Tris–HCl (pH 6.8). Weigh 60.6 g Tris and 4 g SDS. Prepare a 1-L solution as in the previous step. Store at 4°C.
9. 30% acrylamide/bisacrylamide solution: Weigh 29.2 g acrylamide monomer and 0.8 g *N*,*N*′-methylenebisacrylamide. Transfer them to a graduated cylinder containing 80 mL ddH_2O. Make up to 100 mL ddH_2O and filter through a 0.45-μm filter (Corning, Massachusetts, USA). Store at 4°C in the darkness.
10. 10% ammonium persulfate solution: Dissolve 0.1 g ammonium persulfate in 1 mL ddH_2O. Store at 4°C in the darkness.
11. *N*,*N*,*N*′,*N*′-tetramethyl-ethylenediamine (TEMED): Store at 4°C.
12. SDS-PAGE buffer (5×): 0.125 M Tris–HCl (pH 8.3), 0.96 M glycine, 0.5% SDS. Weigh 15.1 g Tris, 72 g glycine, and 5 g SDS. Transfer all of them into a 1-L cylinder containing 700 mL ddH_2O. Adjust pH to 8.3 with HCl. Make up to 1 L with ddH_2O. Store at 4°C.
13. SDS loading buffer (5×): 300 mM Tris–HCl (pH 6.8), 10% SDS, 0.05% bromophenol blue, and 40% glycerol. Prepare 1 mL of 3 M Tris–HCl (pH 6.8), 1 g SDS, and 0.5 g bromophenol blue. Transfer them to 5 mL ddH_2O. Make up to 9 mL with ddH_2O. Store at 4°C. Mix 100 μL mercaptoethanol and 900 μL SDS loading buffer before use.
14. Nitrocellulose membranes (Bio-Rad).
15. Western blot transfer buffer: 0.025 M Tris, 0.192 M glycine, and 20% methanol. Weigh 14.4 g glycine and 3 g Tris, and then transfer them to 200 mL methanol. Make up to 1 L with ddH_2O.

16. Phosphate buffered saline-Tween 20 (PBS-T): Dissolve 8 g NaCl, 0.2 g KCl , 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, and 1 mL Tween 20 in 800 mL distilled H_2O. Adjust pH to 7.4 and make up to 1 L with distilled H_2O. Sterilize by autoclaving. Store at 4°C.
17. Blocking solution: 5% milk in PBS. Dissolve 2.5 mg nonfat dry milk in PBS and then make up to 50 mL with PBS. Store at 4°C.
18. Diluent solution: 5% milk in PBS-T. Dissolve 2.5 mg nonfat dry milk in PBS and then make up to 50 mL with PBS-T. Store at 4°C.
19. Mini PROTEAN® 3 System glass plates: Bio-Rad (catalogue #1653311).
20. Pierce ECL western blotting substrate (catalogue #32106).

3 Methods

3.1 Formation of QDs and SR9 Noncovalent Binding

To test whether SR9 peptides complex with QDs, QDs are mixed with SR9 at various molar ratios (1:10, 1:20, 1:30, and 1:60) followed by separation with a 0.6% agarose gel. That the complexes mobility decrease as the amount of SR9 increases indicates the formation of noncovalent QD/SR9 complexes.

3.1.1 QDs/SR9 Noncovalent Binding

1. The concentrations of QDs and SR9 stock solutions are 10 and 625 μM, respectively.
2. Mix 5 μL of QDs stock solution with SR9 stock solution to reach various molecular ratios; make up to a final volume of 40 μL with PBS.
3. Incubate the QDs/SR9 mixture at room temperature for 20 min before use.

3.1.2 Gel Retardation Assay

1. Prepare the QDs/SR9 mixture of various molecular ratios.
2. Prepare 0.6% agarose gel. After gel becomes solid, fill the electrophoresis tank with TAE buffer (1×).
3. Load QDs/SR9 mixture into wells.
4. Turn on the power supply. Set the voltage at 130 V and then perform the electrophoresis for 60 min.
5. After 60 min, stop the electrophoresis and then capture the image by a UV transilluminator.

3.2 Dependent Uptake of Qds/SR9

We select the final concentration of QDs at 150 nM as this concentration is below the cytotoxic level. To determine the optimal molar ratio of cellular uptake, cells are incubated with QDs and SR9 at different ratios. The ratio is determined by two criteria: the kinetics of uptake and the imaging quality. For instance, QD uptake increases as the molar ratio of SR9 increases from 1:10 to 1:30; the

uptake reaches the highest level at 1:30. However, good imaging quality can be obtained at 1:20. Thus this ratio is chosen. The following procedure describes time-dependent uptake:

1. Seed 1.2×10^5 cells into 35-mm glass-bottom dishes and then allow attaching for 48 h.
2. To achieve a molar ratio of 1:20 (QD:SR9). Prepare stock solutions of QD (10 μM) and SR9 (625 μM). Mix 15 μL QD and 4.8 μL SR9 in 980.2 μL Ham's F-12 modified medium supplemented with 1% FBS.
3. Incubate the QDs/SR9 mixture at room temperature for 20 min before use.
4. Discard the old medium.
5. Wash cells with 1 mL PBS three times.
6. Add 1 mL phenol red-free medium into the dish and then detect fluorescence intensity using fluorescent microscopy.

3.3 Energy-Dependent Endocystosis

To determine whether uptake of QDs/SR9 is energy dependent, cells are incubated with QDs/SR9 under varying metabolic conditions. In temperature studies, cells are treated at either 37°C or 4°C. In metabolic inhibition experiments, cells are incubated in the absence or presence of a mixture of metabolic inhibitors.

3.3.1 Temperature Treatment

1. Seed 1.2×10^5 cells into 35-mm glass-bottom dishes and then allow cells to attach for 48 h.
2. Preincubate cells at 4°C or 37°C for 1 h.
3. To achieve a molar ratio of 1:20 (QD:SR9). Prepare stock solutions of QD (10 μM) and SR9 (625 μM). Mix 15 μL QD and 4.8 μL SR9 in 980.2 μL Ham's F-12 modified medium supplemented with 1% FBS.
4. Incubate the QDs/SR9 mixture at room temperature for 20 min before use.
5. Discard the old medium.
6. Add 1 mL of the QDs/SR9 mixture into the dish and place the dish at either 4°C or 37°C for another hour.
7. Wash cells with 1 mL PBS three times.
8. Add 1 mL phenol red-free medium into the dish and then detect fluorescence intensity using fluorescent microscopy.

3.3.2 Metabolic Inhibition Studies

1. Seed 1.2×10^5 cells into 35-mm glass-bottom dishes and then allow cells to attach for 48 h.
2. Preincubate cells with a combination of metabolic inhibitors (0.15% sodium azide, 15 mM sodium fluoride, and 2 μg/mL antimycin A.) for 1 h at 37°C.

3. Discard the old medium.
4. To achieve a molar ratio of 1:20 (QD:SR9). Prepare stock solutions of QD (10 μM) and SR9 (625 μM). Mix 15 μL QD and 4.8 μL SR9 in 980.2 μL Ham's F-12 modified medium supplemented with 1% FBS.
5. Incubate the QDs/SR9 mixture at room temperature for 20 min before use.
6. Add 1 mL QDs/SR9 mixture into the 35-mm glass-bottom dish and place the dish at 37°C for another hour.
7. Wash cells with 1 mL PBS three times.
8. Add 1 mL phenol red-free medium into the dish and then detect fluorescence intensity using fluorescent microscopy.

3.4 Pathway-Specific Inhibitor Experiments

To investigate the uptake mechanism of QDs/SR9, inhibitors are used to disrupt three major pathways of endocytosis: clathrin-dependent, caveolin-dependent, and macropinocytosis.

1. Seed 1.2×10^5 cells into 35-mm glass-bottom dishes and then allow cells to attach for 48 h.
2. To achieve a molar ratio of 1:20 (QD:SR9). Prepare stock solutions of QD (10 μM) and SR9 (625 μM). Mix 15 μL QD and 4.8 μL SR9 in 980.2 μL Ham's F-12 modified medium supplemented with 1% FBS.
3. Incubate the QDs/SR9 mixture at room temperature for 20 min before use.
4. Mix a desired inhibitor in Ham's F-12 medium supplemented with 10% FBS.
5. Discard old culture medium. Incubate cells with a specific inhibitor for 30 min at 37°C. See Subheading 2.5 for final concentrations of inhibitors. Add QDs/SR9 mixture into the cells to incubate for another hour.
6. Wash cells with 1 mL PBS three times.
7. Add 1 mL phenol red-free medium into the dish and then detect fluorescence intensity using fluorescent microscopy.

3.5 siRNA Experiments

RNAi technique is applied to complement the use of pharmacological inhibitors. Clathrin heavy chain and caveolin-1 siRNAs depress expression of critical components of the clathrin and caveolar pathways.

3.5.1 siRNA Treatment and Imaging Experiment

1. Seed 1.0×10^5 cells into 35-mm glass-bottom dishes and then allow cells to attach for 48 h.
2. Desired siRNA is complexed with Lipofectamine 2000 reagent in OPTI-MEM-reduced serum medium for 30 min according to the manufacturer's instruction.

3. Discard old culture medium.
4. Add 1 mL siRNA-lipofectamine mixture into the dish, and then incubate for 4 h at 37°C.
5. After 4 h, add FBS containing Ham's F-12 modified medium to cells to achieve a final concentration of 5%.
6. After another 68 h, mix 15 μL QDs stock solution (10 μM) and 4.8 μL SR9 stock solution (625 μM) in 980.2 μL Ham's F-12 modified medium supplemented with 1% FBS.
7. Incubate the QDs/SR9 mixture at room temperature for 20 min before use.
8. Add 1 mL QDs/SR9 mixture into the dish for 1 h.
9. Wash cells with 1 mL PBS three times.
10. Add 1 mL phenol red-free medium into the dish and then detect fluorescence intensity using fluorescent microscopy.

3.5.2 siRNA-Mediated Protein Downregulation Experiment

1. Seed the 3.0×10^5 cells into 60-mm dishes and then allow cells to attach for 48 h.
2. Desired siRNA is complexed with Lipofectamine 2000 reagent in OPTI-MEM I-reduced serum medium for 30 min according to the manufacturer's instruction. A nonspecific-targeting siRNA is employed as a negative control.
3. Discard old culture medium.
4. Add 5 mL siRNA-lipofectamine mixture into the 60-mm dish for 4 h at 37°C.
5. After 4 h, add FBS containing Ham's F-12 modified medium to cells to achieve a final concentration of 5%.
6. After another 68 h, follow the instruction in Subheading 3.6.3.

3.5.3 Western Blot Analysis

1. The analysis is conducted 3 days after transfection with siRNA.
2. Discard old medium.
3. Wash the cells with 2 mL PBS three times and then place the dish on ice.
4. Add ice-cold 200 μL lysis buffer into the dish.
5. Scrape cells off the dish using an ice-cold plastic cell scraper, and then transfer the cell lysate into 1.5 mL Eppendorf tubes.
6. Set the 1.5 mL Eppendorf tubes on ice for 30 min.
7. After 30 min, centrifuge 1.5 mL Eppendorf tubes at 4°C, 12,000 rpm for 30 min.
8. Transfer the supernatant to another 1.5 mL Eppendorf tubes. Store at 4°C for fresh use. Store the rest of samples at –20°C for future use.
9. Measure the protein concentration of the sample according to the Bio-Rad protein assay.

10. Mix the 10 μL SDS loading buffer (5×) with 40 μL samples. Boil the mixture at 95°C for 5 min. Then set the samples on ice for at least 10 min.
11. Make 10% ammonium persulfate in ddH_2O.
12. Assemble the gel casting apparatus, making sure that the sandwich of glass plates and spacers seal properly.
13. Prepare the separating gel solution according to the acrylamide concentration needed. Vortex before use.
14. Various percentages of separating gels

Final acrylamide conc	5%	6%	7%	8%	9%	10%	12%	13%	15%
30% acryl/0.8% bisacryl (mL)	2.5	3.0	3.5	4.0	4.5	5.0	6.0	6.5	7.5
H_2O (mL)	8.8	8.3	7.8	7.3	6.8	6.3	5.3	4.8	3.8
4× Tris–HCl/SDS pH 8.8 (mL)	3.7	3.7	3.7	3.7	3.7	3.7	3.7	3.7	3.7
10% ammonium persulfate (μL)	200	200	200	200	200	200	200	200	200
TEMED (μL)	10	10	10	10	10	10	10	10	10

15. Load the apparatus with 4.5 mL of the separating gel solution.
16. Top with 100 μL of isoamyl alcohol.
17. After polymerization, pour off the isoamyl alcohol, and rinse with distilled water.
18. Remove any water droplets from the inside of the casting apparatus with a Whatman paper or a paper towel. Insert the comb for the stacking gel.
19. Prepare the stacking gel solution. Add 3 mL ddH_2O, 1.3 mL Tris–HCl/SDS (pH 6.8, 4×), 0.9 mL of 30% acrylamide/0.8% bisacrylamide, 80 μL of 10% ammonium persulfate, and 5 μL TEMED. Vortex mixtures.
20. Load the stacking gel solution; do not introduce air bubbles underneath or between the teeth of comb. Remove air bubbles by pipetting up and down. Allow the stacking gel to polymerize completely (~45 min) before removing comb.
21. Remove the glass and gel sandwich from the casting apparatus.
22. Clip the sandwich to the electrophoresis apparatus. Carefully remove the comb from the gel and fill the top of the apparatus with SDS-PAGE electrophoresis buffer (1×).
23. Flush the wells with buffer. Carefully load samples and 5 μL pre-stained protein molecular weight markers into the bottom

of the wells using a flat-tipped pipette tip. The sample protein loaded into each wells is 30 mg.

24. Fill the bottom of the electrophoresis apparatus with SDS-PAGE electrophoresis buffer (1×) and connect the apparatus to the power supply.
25. Perform the gel at 80 V. After the protein markers pass through the stacking gel, increase the voltage to 120 V.
26. When the dye reaches the bottom of the separating gel, turn off the power supply. Remove the gel sandwich.
27. Remove the stacking gel.
28. Use the Bio-Rad gel blotting sandwiches kit to transfer protein from the gel to nitrocellulose membrane.
29. Set the transfer system and filled with transfer buffer. The ampere is 200 mA and transferring time is 2 h.
30. After 2 h, block the nitrocellulose with 5 mL blocking solution for 1 h.
31. Wash with 5 mL PBS-T for 5 min three times.
32. Prepare 1st antibody with diluent solution. Clathrin heavy chain is detected using a mouse monoclonal antibody at a dilution of 1:200. Mix 10 μL antibody with 2 mL diluent solution and keep on ice. Caveolin-1 protein is detected using a rabbit monoclonal antibody at a dilution of 1:1,000. Mix 2 μL antibody with 2 mL diluent solution and keep on ice.
33. Incubate the nitrocellulose in 1st antibody-containing diluent solution. Keep shaking for 12 h at 4°C.
34. After 12 h, wash with 5 mL PBS-T for 5 min three times.
35. Prepare 2nd antibody with diluent solution. Goat anti-mouse IgG-HRP secondary antibody is used for clathrin 1st antibody at dilution of 1:1,000. Goat anti-rabbit IgG-HRP secondary antibody is used for caveolin 1st antibody at dilution of 1:1,000. Mix 2 μL antibody with 2 mL diluent solution and keep on ice. Incubate the nitrocellulose in 2nd antibody-containing diluent solution. Keep shaking for 1 h at room temperature.
36. After 1 h, wash with 5 mL PBS-T for 5 min three times.
37. The blots are probed with the ECL Western blot detection system.

4 Notes

1. It is important that both inhibitors and siRNAs are used, as they are complementary methods to investigate uptake mechanisms. In general, inhibitors are relatively nonspecific. Thereby, they can inhibit multiple pathways of cellular uptake, which mislead false results.

2. Imaging of cellular uptake can be tricky. An image from a single plane sometimes can be difficult to determine whether QD/CPPs are on the membrane surface or inside the cell. A 3D image would be the most appropriate venue to resolve this issue.
3. The uptake efficiency and kinetics are QDs-, CPPs-, and cell type specific.

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