Cellular Uptake Properties of the Complex Derived from Quantum Dots and G8 Molecular Transporter

Jungkyun Im, Kaustabh K. Maiti, Wanil Kim,[†] Kyong-Tai Kim,[†] and Sung-Kee Chung^{*}

Department of Chemistry, Pohang University of Science and Technology, Pohang 790-784, Korea

^{*}E-mail: skchung@postech.ac.kr

[†]Department of Life Science, Pohang University of Science and Technology, Pohang 790-784, Korea

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The biotin-attached G8 molecular transporter (**5**) was synthesized and used together with quantum dots in preparing the complexes (**QD-MT**). The **QD-MT** complexes were studied in terms of the cellular uptake and the internalization mechanism in live HeLa cells with the aid of various known endocytosis inhibitors. It has been concluded that the **QD-MT** complex is internalized largely by macropinocytosis. The mouse tissue distribution of the **QD-MT** complex by *i.p.* and *i.v.* routes showed some organ selectivity and a good ability to cross the BBB.

Key Words : Quantum dot, Molecular transporter, Internalization, Macropinocytosis, Blood-brain barrier

Introduction

Quantum dots (QDs) are fluorescent semiconductor nanocrystals.¹ Compared to conventional fluorescent dyes, QDs exhibit the following characteristics: 1) Broad absorption spectra combined with narrow and symmetric emission peaks, enable multicolor analysis with a single excitation line and without spectral crosstalk between different detection channels. 2) High quantum yield and excellent photostability make QDs especially suitable for ultrasensitive detection of biomolecules, possibly at the single molecule level. 3) QDs can be detected by electron microscopy, providing their ultrastructural localization in a biological environment.² These advantages make QDs superior to the conventionally used fluorophores such as small organic dyes or fluorescent proteins. Thus as a new class of fluorescent probe, QDs appear attractive for molecular, cellular, and *in vivo* imaging applications such as real-time imaging of cellular signaling pathways.³

However, one of the major problems in using QD nanoparticles for *in vivo* applications is poor cellular uptake characteristics. While some organic dyes are able to permeate cell membranes by passive diffusion, the size and surface properties of QDs hinder the translocation of QDs across the cellular membranes. Entry of QDs into a cell may be achieved by using a variety of approaches, including electroporation,⁴ and microinjection.⁵ Electroporation temporarily generates hydrophilic pores in the plasma membrane by applying an electric field pulse. The pores allow the passive transport of DNA and QDs into the cell. However, the disadvantages are the low cell viability and the need of special equipments. Microinjection enables the delivery of QDs to the cell's interior in a monodisperse form. But it is a labor intensive process, as individual cells are injected at a time. Besides these physical delivery methods, QDs may be made cell membrane permeable and functionalized by

conjugation with surface modifying agents such as lipofectamine,⁶ small peptides, proteins, and nucleic acids.⁷ For example, cell-penetrating peptides (CPPs) such as HIV-I TAT peptide and oligoarginine have been used to facilitate the delivery of QDs into cells.⁸ Uptake of QD by a cell can occur in principle by a single or multiple endocytic pathways such as phagocytosis and pinocytosis. Phagocytosis in mammals is typically confined to specialized cells, including macrophages, monocytes, and neutrophils. They can remove large pathogens or large debris such as the fragments of dead cells, arterial deposits of fat, and so on.⁹ Pinocytosis is a kind of fluid-phase uptake and occurs in all cells by four basic mechanisms: macropinocytosis, clathrin-mediated endocytosis, caveolae-mediated endocytosis, and clathrin- and caveolae-independent endocytosis.¹⁰⁻¹⁴ However, the detailed mechanisms of these endocytic processes remain poorly understood.

The cell membrane permeability of QDs largely depends on the surface coatings or the conjugated materials. Previously we developed the G8 (eight guanidine residue containing) molecular transporter built on the sorbitol scaffold, which showed excellent cellular uptake, an affinity toward mitochondria, and high efficiency in crossing the blood-brain barrier (BBB).¹⁵ This particular G8 molecular transporter has been successfully demonstrated as a viable delivery vector for various clinically used drugs through the covalent conjugation method.¹⁶ Now we have investigated the cellular uptake characteristics of the complex (**QD-MT**) derived from the biotin-attached G8 sorbitol-based molecular transporter (**5**) and two QDs (QD565 and QD655), and the results are described herein.

Experimental

I. Synthesis

General Methods. Column chromatography was performed

ed on Merck 60 silica gel [70-230 or 230-400 mesh (flash)]. All NMR spectra were recorded on a Bruker DPX 300 instrument operating at 300 MHz for ^1H , 75 MHz for ^{13}C , unless otherwise stated. MALDI-TOF-MS was obtained on a Micromass M@DI at the Biomolecular Diversity Core Facility (POSTECH). Analytical HPLC was performed on Agilent 1100-HPLC Chemstation with an analytical column ZORBAX C8-monomeric (BU-300, 5 μm , 300 $\text{\AA}$, 4.6×250 mm), and preparative HPLC with a semi-preparative column GRACEVYDAC C18 (5 μm , 300 $\text{\AA}$, 10×250 mm).

1-O-Trityl-2,3,4,5-tetra-O-(N-{bis-[3-(N',N''-bis-Boc-guanidino)-propyl]}-6-amino-octanoyl)-D-sorbitol (1) was prepared according to a literature procedure.^{15(a)}

1-O-(N-Benzyloxycarbonyl-6-amino-hexanoyl)-2,3,4,5-tetra-O-(N-{bis-[3-(N',N''-bis-Boc-guanidino)-propyl]}-6-amino-octanoyl)-6-O-trityl-D-sorbitol (2): To a solution of **1** (75.70 mg, 22.36 μmol) in CH_2Cl_2 (3 mL) at rt, were added Cbz-protected amino-hexanoic acid (20.77 mg, 78.29 μmol), EDC (10.72 mg, 55.92 μmol), and DMAP (1.40 mg, 11.18 μmol). After stirring for 24 hr, the solution was concentrated under reduced pressure. The residue was diluted with EtOAc and then washed with aq. NaHCO_3 and brine. The organic layer was dried over Na_2SO_4 , filtered and condensed in vacuo to give the crude product, which was purified by column chromatography on silica gel to give **2** (60 mg, 80%) as colorless foamy solid. R_f : 0.38 (CH_2Cl_2 : MeOH = 9:1); ^1H NMR (CDCl_3 , δ) 1.25-1.72 (m, 206H), 2.29-2.51 (m, 34H), 3.41-3.53 (m, 20H, aromatic protons), 4.00-4.40 (m, 4H), 5.08 (s, 2H, CH_2Ph), 5.20 (brs, 1H), 5.48 (brs, 1H), 7.25-7.39 (m, 20H), 8.48 (brs, 8H), 11.49 (brs, 8H); ^{13}C NMR (CDCl_3 , δ) 25.11, 26.42, 27.85, 28.70, 29.69, 30.05, 34.32, 39.90, 51.89, 53.81, 66.85, 79.49, 83.24, 127.52, 127.64, 128.21, 128.85, 129.05, 143.74, 153.46, 156.47, 163.99, 172.29, 172.78, 173.12.

1-O-(Amino-hexanoyl)-2,3,4,5-tetra-O-(N-{bis-[3-(N',N''-bis-Boc-guanidino)-propyl]}-6-amino-octanoyl)-6-O-trityl-D-sorbitol (3): A mixture of **2** (49.9 mg, 13.74 μmol) and Pd/C 10% (80 mg) in CH_2Cl_2 /MeOH (1:9, 8 mL) was stirred under H_2 (g) (50 psi) for 12 hr. The reaction mixture was filtered through a short pad of celite and washed with MeOH. Evaporation of the filtrate gave the amino compound **3** (47 mg, 98%) as colorless foamy solid. R_f : 0.10 (CH_2Cl_2 : MeOH = 10:1); ^1H NMR (MeOD, δ) 1.22-1.58 (m, 206H), 1.78-2.21 (m, 18H), 2.33-2.39 (m, 4H), 2.88-3.22 (m, 16H), 3.47 (brs, 16H), 3.57-3.68 (m, 2H), 4.01-4.21 (m, 2H), 4.66-4.70 (m, 2H), 5.22 (brs, 1H), 5.58 (brs, 1H), 7.27-7.39 (m, 15H, trityl protons).

1-O-(N-Biotin-6-amino-hexanoyl)-2,3,4,5-tetra-O-(N-{bis-[3-(N',N''-bis-Boc-guanidino)-propyl]}-6-amino-octanoyl)-6-O-trityl-D-sorbitol (4): To a solution of **3** (35 mg, 10.01 μmol) in DMF (1.5 mL), were added biotin (12 mg, 49.12 μmol), EDC (4.77 mg, 24.88 μmol), *N*-hydroxybenzotriazole (1.5 mg, 11.10 μmol) and DMAP (1 mg, 8.19 μmol) at rt. After stirring for 24 hr at rt, the solution was concentrated several times at 45 $^\circ\text{C}$ under reduced pressure by azeotropic distillation with toluene. The residue was diluted with aq. LiCl (5%) and then extracted with EtOAc three times. The

organic layer was washed with aq. NaHCO_3 , dried over Na_2SO_4 , filtered and condensed in vacuo to give the crude product, which was purified by column chromatography on silica gel to give **4** (26.1 mg, 70%) as colorless foamy solid. R_f : 0.45 (CH_2Cl_2 : MeOH = 10:1); ^1H NMR (CDCl_3 , δ) 1.25-1.69 (m, 206H), 2.29-2.49 (m, 34H), 3.45-3.48 (m, 20H), 3.63-3.76 (m, 16H), 4.29 (brs, 1H), 4.48 (brs, 1H), 4.89 (brs, 3H), 5.11 (brs, 2H), 5.32 (brs, 1H), 5.48 (brs, 1H), 7.27-7.39 (m, 15H, trityl protons), 8.51 (brs, 8H), 11.48 (brs, 8H); ^{13}C NMR (CDCl_3 , δ) 25.12, 25.71, 26.33, 26.75, 28.17, 28.46, 28.71, 28.96, 29.63, 30.09, 31.83, 34.34, 36.88, 39.54, 39.88, 51.24, 55.73, 60.46, 62.22, 64.15, 72.69, 79.59, 83.37, 126.30, 127.57, 128.25, 129.08, 143.68, 153.47, 156.55, 162.93, 163.96, 172.79, 172.81, 173.21.

1-O-(N-Biotin-6-amino-hexanoyl)-2,3,4,5-tetra-O-(N-{bis-(3-guanidinopropyl)}-6-amino-octanoyl)-D-sorbitol-8HCl (5): Compound **4** (26 mg, 6.96 μmol) was added to a solution of EtOAc saturated with gaseous HCl (3 mL) at rt, and the solution was stirred for 2 days. After evaporation of solvent, the residue was washed several times with diethyl ether. The precipitate was dissolved in deionized water, filtered through a polytetrafluoroethylene (PTFE) syringe filter, and lyophilized to give the crude product, which was purified by preparative RP-HPLC (GRACEVYDAC, C18), (2.5 mL min^{-1} , 75% CH_3CN in H_2O , 220 nm) to yield **5** (10.1 mg, 67%) as white sticky solid. ^1H NMR (D_2O , 500 MHz, δ) 1.11-1.35 (m, 29H), 1.39-1.70 (m, 23H), 1.78-2.05 (m, 16H), 2.11-2.48 (m, 11H), 2.65-2.71 (m, 1H), 2.78-2.90 (m, 1H), 3.01-3.45 (m, 40H), 3.62-3.88 (m, 4H), 4.15-4.21 (m, 2H), 4.32 (brs, 1H), 4.55 (brs, 1H), 5.32 (brs, 1H), 5.48 (brs, 1H); ^{13}C NMR (D_2O , δ) 22.43, 22.53, 22.70, 23.66, 23.94, 24.94, 25.12, 27.18, 27.26, 27.36, 27.49, 31.61, 33.14, 33.31, 33.46, 35.09, 37.50, 37.73, 49.80, 52.52, 52.71, 59.82, 61.15, 61.65, 80.94, 121.59, 129.79, 152.57, 156.39, 162.50, 169.29, 170.21, 174.34, 175.08, 177.55, 180.06; MALDI-TOF-MS $[\text{M}+\text{H}]^+$ calcd for $\text{C}_{86}\text{H}_{172}\text{N}_{31}\text{O}_{13}\text{S}$ m/z 1879.3472, found 1879.3036; analytical HPLC (BU-300, at 220 nm, 1 mL min^{-1} , 70% CH_3CN in H_2O , t_R = 2.15 min), purity 99%.

II. Bioassays

Materials. Streptavidin conjugated CdSe/ZnS QDs [emission peak at 565 nm (QD565) or 655 nm (QD655)] were purchased from Invitrogen Inc. Chlorpromazine, sucrose, wortmannin, 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA), methyl- β -cyclodextrin, nystatin, and nocodazole were purchased from Sigma-Aldrich. Hoechst33342, ERtracker green, Mitotracker, LysoTracker, transferrin Texas red conjugate, cholera toxin B Alexa488 conjugate, and dextran Rohdamine B conjugate (MW 10,000) were obtained from Molecular Probes Inc. High glucose Dulbecco's modified Eagle's medium (DMEM), Dulbecco's phosphate buffered saline (PBS, pH 7.4), fetal bovine serum (FBS), and trypsin/EDTA were obtained from Invitrogen. Milli-Q purified water (18.2 M Ω) was used to prepare all aqueous solutions.

Preparation of QD-MT Complex. The QD-MT complex was prepared by incubating the streptavidin conjugated QD (QD-SA, 400 nM) with biotin-attached molecular trans-

porter at 1:40 ratio. The mixture was allowed to react for 1 hr at rt, and then kept at 4 °C as the stock solution for the **QD-MT** complex. The excess amount of the molecular transporter is very small, and thus not separated out of the product complex.

Transmission Electron Microscopy (TEM). The samples for electron microscopy were prepared on 300 mesh carbon-coated copper grid. A solution of **QD565-MT** (5 μ L, 30 nM) was placed on the grid for 5 min, and was gently blotted by Kimwipe. The sample was viewed by TEM (JEM-1011, JEOL, 50 kV). In order to view streptavidins attached to QDs, an indirect method, i.e. negative staining, was used; 0.5% (w/w) of uranyl acetate (5 μ L) was dropped to the prepared specimen, and after 5 min it was dried with Kimwipe.

Cell Culture. HeLa cells were cultured at 37 °C in a humidified 5% CO₂ containing air environment in DMEM and 10% (v/v) FBS with antibiotics. The subculture was conducted every 2-3 days using the cells grown to subconfluence.

Cellular Uptake and Localization Experiments. HeLa cells (1×10^4 cells per well) were plated into an 8-well chambered cover glass (Nalgene Nunc International) and cultured for 48 hr. After removing the medium, HeLa cells were washed with PBS (X1). The working concentration of the **QD-MT** was typically 10 nM, and the total volume of the incubation solution in each well was 300 μ L. For the time-course investigation, live HeLa cells were incubated with **QD655-MT** for 1, 2, 4, and 24 hr. QD655 alone was also incubated for 24 hr as a control.

Various kinds of subcellular markers were incubated with the cells in the presence of **QD-MT**. They include Hoechst33342 (10 μ M, 1 hr, a nucleus marker), ERtracker green (1 μ M, 30 min, an endoplasmic reticulum marker), Mitotracker (100 nM, 30 min, a mitochondria marker), and LysoTracker (200 nM, 30 min, a lysosome marker). After incubation, HeLa cells were washed gently with PBS three times and were immersed in cold PBS for imaging with a confocal microscope.

In order to disrupt microtubules, the cells were preincubated with 15 μ M nocodazole for 30 min at 37 °C. The drug treated HeLa cells were incubated with **QD655-MT** for 24 hr and then coincubated with **FITC-MT** (10 μ M) for 15 min before confocal microscope imaging.

Each experiment was performed at least twice.

Confocal Laser Scanning Microscopy (CLSM). After incubation, the cell medium was removed from each well. HeLa cells were washed three times with cold PBS, replaced with cold PBS, and then imaged directly on the chambered cover glass. CLSM was performed by using an Olympus Fluoview FV1000 equipped with an N.A. 1.30, 40X, plan-Apo, oil immersion lens. Fluorescence of each fluorophore was analyzed and collected using the following excitation and emission bands: Hoechst33342, 405 nm (ex), 425-475 nm (em); FITC and cholera toxin B-Alexa488, 488 nm (ex), 500-530 nm (em); ERtracker green, 488 nm (ex), 500-550 nm (ex); QD565, 488 nm (ex), 530-590 nm (em); QD655,

543 nm (ex), 630-700 nm (em); transferrin-Texas red, 543 nm (ex), 590-650 nm (em); Mitotracker, LysoTracker, and dextran-Rhodamine B, 543 nm (ex), 600-700 nm (em). When **QT-MT** was coincubated with another fluorophore, in order to prevent the crosstalk between different colors of fluorophores, the excitation and the collection of emission were performed sequentially from the individual fluorophores. Merged images and intensity profiles were obtained by the Olympus Fluoview Viewer.

Endocytosis Inhibition Studies. The drugs described below are maintained in the cell medium during incubation with **QD-MT** or fluorophore-labeled compounds. The cell density was 1×10^4 cells per well and incubation solution was typically 300 μ L in the 8-well chambered coverglass. The concentration and incubation time of **QD-MT** was typically 10 nM and 4 hr.

(1) Low Temperature Incubation at 4 °C: HeLa cells in media were preincubated at 4 °C for 30 min. Then, cellular incubations with **QD655-MT** and **FITC-MT** (10 μ M, 30 min) were carried out at 4 °C. The cells were washed with PBS at 4 °C prior to fluorescence analysis.

(2) ATP Depletion Experiment: HeLa cells were preincubated in PBS supplemented with 10 mM NaN₃ and 50 mM 2-deoxy-D-glucose for 30 min at 37 °C, followed by incubation in a solution of **QD655-MT** and **FITC-MT** (10 μ M, 30 min).

(3) Clathrin-mediated Endocytosis Inhibition: HeLa cells were preincubated with 30 μ M chlorpromazine in DMEM for 30 min at 37 °C, followed by incubation with **QD655-MT** or transferrin Texas red conjugate (25 μ g mL⁻¹, 1 hr).

(4) Hypertonic Incubation: HeLa cells were preincubated at 37 °C for 30 min in DMEM supplemented with 0.45 M sucrose. Then, cellular incubations with **QD655-MT** and Transferrin Texas red conjugate were carried out as described above.

(5) Caveolae-mediated Endocytosis Inhibition: HeLa cells were preincubated with 10 mM methyl- β -cyclodextrin or 10 μ g mL⁻¹ nystatin in DMEM for 30 min at 37 °C, followed by incubation with **QD655-MT** or cholera toxin B Alexa488 conjugate (15 μ g mL⁻¹, 1 hr).

(6) Macropinocytosis Inhibition: HeLa cells were preincubated with either 100 nM wortmannin or 100 μ M EIPA in DMEM for 30 min at 37 °C. Incubation was followed with **QD655-MT** or dextran Rhodamine B conjugate (1 mg mL⁻¹, 1 hr).

Mouse Tissue Biodistribution Study. All mouse experiments were performed in the POSTECH animal facility in compliance with the relevant laws and institutional guidelines.

For intraperitoneal (*i.p.*) administration, a solution of **QD655-MT** (0.2 nmol) in PBS (pH 7.4, 500 μ L) was prepared and injected into an eight-week-old mouse (C57BL/6, 22 g). After 24 hr, the treated mouse was perfused with paraformaldehyde (4%) in PBS (pH 7.4), and the major organs (brain, heart, lung, kidney, spleen, and liver) were incubated overnight in a solution of sucrose (0.5 M) in PBS.

Placed in cryoprotectant, they were cut into 15 μm sections with a cryostat and transferred to coated glass slides. After drying, each section was washed with PBS, treated with Triton X-100 (0.3%) for 15 min at rt, and then analyzed with an Axioplan2 fluorescence imaging microscope. As a control, triple distilled water (500 μL) was also injected into the mouse and treated in the same process. Red fluorescence from **QD655-MT** in each tissue was compared with the autofluorescence background from the control.

For intravenous (*i.v.*) injection *via* tail vein, an eleven-week-old mouse (C57BL/6, 34 g) was briefly anesthetized by diethyl ether, and was held in a restraining device. A solution of **QD655-MT** (0.2 nmol) in PBS (pH 7.4, 500 μL) was prepared and slowly injected into the tail vein (5 cm from distal end) using a plastic 0.5 mL syringe. Afterwards, some PBS (*ca.* 30 μL) was post-injected to flush the tail vein. After 24 hr, the administered mouse was perfused with paraformaldehyde (4%) in PBS (pH 7.4), and the rest protocol for specimen preparation and fluorescence imaging was same as described above.

Results and Discussion

Synthesis of Biotin-attached Sorbitol G8 Molecular Transporter. The *N*-Cbz-protected aminohexanoic acid was coupled to compound **1** under the EDC coupling conditions. The *N*-Cbz protecting group in **2** was removed by hydrogenolysis over Pd/C to give **3** in 98% yield. The resulting amino group was coupled with biotin with EDC, DMAP and HOBT in DMF to furnish **4**. Compound **4** was converted to compound **5** by treatment with ethyl acetate saturated with HCl gas to remove all the *N*-Boc and trityl protecting groups. The target compound **5** was purified by preparative RP-HPLC on a C18 column, and thoroughly characterized by NMR, HPLC, and Maldi-TOF mass spectral analyses (Scheme 1).

Preparation of the Quantum Dot-molecular Transporter Complex (QD-MT) and its Size Determination. The **QD-MTs** were prepared by incubating QD-streptavidin conjugates (QD-SA) with **5** at the molar ratio of 1 to 40. Since each QD contains 5-10 streptavidins on its surface, and each streptavidin has 4 binding sites available for biotin, about 40 molecules of **5** would be needed to fully occupy all the binding sites of a single QD-SA. The **QD-MTs** were also prepared by mixing QD-SA and the molecular transporter at lower ratio (1:20). However, this version showed little difference from the higher ratio version except the slightly weaker fluorescence in the cells. Thus, the mixing ratio was kept at 1:40.

The **QD565-MT** complexes were observed by TEM (JEM-1011, JEOL, 50 kV). The dark granules indicate the core nanocrystal, and they were well dispersed in PBS without forming larger clusters (Fig. 1(a)). The core size was estimated to be 5 nm. Negative staining of the sample by 0.5% uranyl acetate did not stain the inside part of streptavidins, thus revealing the periphery of streptavidins (Fig. 1(b)). The dark granules are surrounded by a white disk

Scheme 1. (a) $\text{HOOC}-(\text{CH}_2)_5-\text{NHCbz}$, EDC, DMAP, CH_2Cl_2 , 24 hr, 80%; (b) H_2 (g) (50 psi), Pd/C, $\text{MeOH}-\text{CH}_2\text{Cl}_2$ (9:1), 12 hr, 98%; (c) biotin, EDC, DMAP, HOBT, DMF, rt, 36 hr, 70%; (d) HCl (g) in EtOAc, rt, 2 days, 67%.

which is unstained streptavidins. By using this technique, both the QD and the streptavidins could be distinguished, and the size of a single **QD565-MT** was estimated to be in the range of 18-20 nm (Fig. 1(c)). The **QD655-MT** complex displayed similar patterns on the TEM analyses.

Cellular Uptake and Intracellular Localization of QD-MT. Internalization of **QD655-MT** in live HeLa cells was observed by confocal laser scanning microscopy (Olympus Fluoview FV1000, N.A. 1.30, 40 X, oil immersion lens). Figure 2 shows time-dependent fluorescence images of live HeLa cells when incubated with **QD655-MT**. After incubation of HeLa cells with **QD655-MT** (10 nM) for 1, 2, 4, and 24 hr at 37 $^\circ\text{C}$, each dish of cells was washed with PBS (pH 7.4) and directly observed by confocal microscope. Relatively high fluorescence intensity reached after 1 hr incubation, and the fluorescence appeared exclusively around the plasma membrane. Entry of **QD655-MT** into HeLa cells occur relatively slowly compared to **FITC-MT** itself, which was completely taken up within 15 min.^{15(a)} However, when the incubation time was 2 hr, punctate figures began to appear inside the cell, suggesting increased internalization of **QD655-MT**. Up to 4 hr incubation time, more and more **QD655-MTs** were internalized and accumulated in the intracellular region, with fewer and fewer **QD655-MTs** on the cell surface. After 24 hr incubation, most **QD655-MTs**

Figure 1. TEM images of **QD565-MT** on a carbon-coated copper grid. (a) Only the QD part appears as dark granules, (b) Negative staining by 0.5% uranyl acetate enables to observe both the QD and the streptavidins, (c) Further magnification of the inset in (b) reveals the approximate size of **QD565-MT**. [scale bar: (a) 5 nm, (b) and (c) 20 nm]

were localized in the perinuclear region (Fig. 2(d)). At 10 nM concentration of **QD655-MT**, the required incubation time to obtain reasonable fluorescence intensity inside the cell by confocal microscope is about 4 hr. Hence, 4 hr incubation condition was used throughout the study. When **QD655** alone was incubated in HeLa cells in the same

Figure 2. Time dependent internalization of **QD655-MT** (10 nM) in live HeLa cells.

manner, no fluorescence was observed inside the cells, confirming that internalization of QD is only possible with suitable surface modifications (Fig. 1. in supporting information).

To investigate the intracellular localization of **QD655-MT**, subcellular markers were coincubated. **QD655-MT** was first incubated for 24 hr, and then Hoechst33342 (Molecular Probes Inc., 10 μ M, incubated for the last 1 hr) as nucleus marker and ERtracker green (Molecular Probes Inc., 1 μ M, for the last 30 min) as endoplasmic reticulum (ER) marker, were added to the culture media toward the end of the incubation. After washing with PBS (pH 7.4), three different fluorescence signals were recorded by confocal microscope. As shown in Figure 3, **QD655-MT** has accumulated in the perinuclear region, but not inside the nucleus. As ER is present near the nucleus, ER tracker green also appears in the perinuclear region. The intensity profile along the arrow in the merged image clearly shows no discernible colocalization between **QD655-MT** and ERtracker green. The data suggest that the major fraction of internalized QD does not traffic either to the ER or to nucleus.

Figure 3. The intracellular localization of **QD655-MT** (red, 10 nM, 24 hr incubation) with subcellular markers: Hoechst33342 (blue color, 10 μ M, 1 hr incubation) and ERtracker green (green color, 1 μ M, 30 min incubation). The intensity profile along the arrow in the merged image is shown in the graph.

Figure 4. Confocal microscopy images of live HeLa cells. **QD565-MT** (green) was coincubated with subcellular markers (red). The intensity profiles of the fluorescence signals along the arrows in the merged images are shown on the right. (a) **QD565-MT** (10 nM, 4 hr incubation) and Mitotracker (100 nM, 30 min); (b) **QD565-MT** (10 nM, 4 hr incubation) and Lysotracker (200 nM, 30 min); (c) **QD565-MT** (10 nM, 24 hr incubation) and Lysotracker (200 nM, 30 min).

The intracellular localization of the **QD-MT** complex was examined further with Mitotracker and Lysotracker. Since these markers emit the red color fluorescence, **QD655-MT**

was replaced with **QD565-MT**, in which the excitation and emission ranges do not overlap with those of markers. When HeLa cells were incubated with **QD565-MT** (10 nM) for 4

Figure 5. Uptake and intracellular localization of **FITC-MT** (green, 10 μ M, 30 min incubation) and **QD655-MT** [red, 10 nM, 4 hr except (a)] in live HeLa cells (a) coinubation of **FITC-MT** and **QD655-MT** (10 nM, 24 hr incubation) under the normal conditions; (b) microtubule disruption by pretreatment with nocodazole (15 μ M, 30 min incubation); (c) incubation at 4 $^{\circ}$ C, and (d) ATP depletion by pretreatment with NaN_3 and 2-deoxy-D-glucose.

hr and then with Mitotracker (100 nM) for another 30 min, confocal microscope did not exhibit any colocalization (Fig. 4(a)). In contrast, when Lysotracker (200 nM) was similarly coinubated for 30 min, a significant colocalization was observed (Fig. 4(b)). When **QD565-MT** was increased for 24 hr and then Lysotracker (200 nM) was coinubated for the final 30 min, the merged image and the intensity profile clearly indicate an efficient colocalization (Fig. 4(c)), which in turn suggests that the internalized **QD565-MT** are trapped in lysosomes.

Comparison of Internalization between FITC-MT and QD-MT. The intracellular localization of **QD655-MT** was compared with **FITC-MT**, which contains FITC instead of QD as the probe. The HeLa cells were incubated with **QD655-MT** (10 nM) for 24 hr, and then with **FITC-MT** (10 μ M) for the last 30 min. After washing with PBS (pH 7.4), the live HeLa cells were examined by confocal microscope. As previously reported, **FITC-MT** was well dispersed in the cytoplasm,¹⁵ whereas **QD655-MT** was largely localized in the perinuclear region. This observation indicates that the intracellular localization patterns are changed depending on the nature of the probe, i.e., small FITC vs. very large QD (Fig. 5(a)).

Nocodazole is a cytoskeleton-disrupting drug commonly

used to depolymerize microtubules. In order to see its effect on the uptake and intracellular localization, it was preincubated with HeLa cells for 30 min prior to the treatment with **QD655-MT** (10 nM, 4 hr incubation) and **FITC-MT** (10 μ M, 30 min incubation). Interestingly, **FITC-MT** was found to be taken up nicely and localize in the nucleus, while the internalization of **QD655-MT** was totally blocked (Fig. 5(b) and Figure 2 in supporting information). This result suggests that microtubules are important for the **QD655-MT** internalization and the localization in the perinuclear region is most likely caused by microtubule-dependent transport.

Further investigations for the internalization of **QD655-MT** were carried out by employing other inhibition conditions. Endocytosis is an energy-dependent process that may be hindered when incubated at low temperature (4 $^{\circ}$ C instead of 37 $^{\circ}$ C) or in the ATP depleted environments (e.g. with NaN_3 and 2-deoxy-D-glucose).^{17,18} Thus, incubations of **QD655-MT** and **FITC-MT** in HeLa cells were performed at 4 $^{\circ}$ C and in the cells pretreated with NaN_3 and 2-deoxy-D-glucose. After washing with PBS (pH 7.4), live HeLa cells were observed by confocal microscope maintained with the same instrumental settings (e.g. bandpass, offset, gain, laser power and pinhole size) as before. In contrast to the normal incubation conditions, both **FITC-MT** and **QD655-MT**

Figure 6. Clathrin-mediated endocytosis inhibition in live HeLa cells. Cells were preincubated with either (a) chlorpromazine (30 μ M, 30 min incubation) or (b) sucrose (0.45 M, 30 min incubation) followed by the treatment of transferrin-Texas red conjugate (25 μ g mL⁻¹, 1 hr incubation) and **QD655-MT** (10 nM, 4 hr incubation), respectively.

were not internalized under the either conditions, i.e., low temperature or ATP depletion inhibition conditions (Fig. 5(c) and (d)). It is quite evident that the cellular uptakes of **FITC-MT** and **QD655-MT** are energy-dependent, and that some kind of endocytosis is more likely involved than the passive diffusion.

Internalization Process of QD-MT. To examine the possible role of clathrin in the internalization of **QD655-MT**, incubations were carried out under conditions that are known to disrupt the formation of clathrin-coated pits on the cell membrane, namely pretreating the cells with either sucrose (hypertonic treatment) or the inhibitor, chlorpromazine.^{17(a),18} Transferrin is a well known blood plasma protein that enters cells *via* clathrin-coated vesicles after binding to the transferrin receptor and traffics through early and recycling endosomes,¹² and transferrin Texas red conjugate (Molecular Probes Inc.) is used as a reference compound. Thus, HeLa cells were preincubated with either chlorpromazine (30 μ M) or sucrose (0.45 M) for 30 min at 37 °C prior to the exposure to transferrin Texas red conjugate (25 μ g mL⁻¹, 1 hr incubation) and **QD655-MT** (10 nM, 4 hr incubation). After washing with PBS (pH 7.4), live HeLa cells were observed by confocal microscope. Control experiments without the inhibitor were also performed. As expected, the fluorescent color (red) from the transferrin conjugate is very dim under both inhibition conditions, indicating the concentrations of chlorpromazine and sucrose were high enough for good inhibition (Fig. 6(a)). However, in the case of **QD655-MT**, the inhibitors had no discernible effect on the cellular uptake (Fig. 6(b)). Thus, it seems that there is little or no involvement of the clathrin-mediated endocytosis for the uptake of **QD655-MT**.

Figure 7. Caveolae-mediated endocytosis inhibition in live HeLa cells. Cells were preincubated with either (a) methyl-β-cyclodextrin (3.5 μ M, 30 min) or (b) nystatin (10 μ g mL⁻¹, 30 min) followed by the treatment of cholera toxin B-Alexa488 conjugate (15 μ g mL⁻¹, 1 hr, green) and **QD655-MT** (10 nM, 4 hr, red), respectively.

The possibility of **QD655-MT** uptake through caveolae-mediated endocytosis was also examined. As caveolae is formed in cholesterol and sphingolipid-rich domains within the plasma membrane, the caveolae-mediated endocytosis can be inhibited by incubating the cells with cholesterol-depletion reagents such as methyl-β-cyclodextrin and nystatin.¹⁹ Cholera toxin subunit B is relatively nontoxic and commonly used in molecular cell biology experiments. It binds to the GM1 gangliosides receptor which resides mostly in the caveolae.^{17(a)} As cholera toxin B is delivered into cells *via* caveolae-mediated endocytosis, cholera toxin B-Alexa488 conjugate (Molecular Probes Inc.) is used as a reference compound. Thus, HeLa cells were preincubated with either methyl-β-cyclodextrin (3.5 μ M) or nystatin (10 μ g mL⁻¹) for 30 min at 37 °C prior to the exposure to cholera toxin B Alexa488 conjugate (15 μ g mL⁻¹, 1 hr incubation) and **QD655-MT** (10 nM, 4 hr incubation). After washing with PBS (pH 7.4), live HeLa cells were observed by confocal microscope. Control experiments without inhibitors were performed as well. As expected, the fluorescent color (green) from the cholera toxin B conjugate is very dim in the presence of inhibitors, indicating the concentrations of methyl-β-cyclodextrin and nystatin are high enough for inhibition (Fig. 7(a)). Under the identical inhibition conditions, translocation of **QD655-MT** into the cell is not noticeably diminished, indicating that the caveolae-mediated endocytosis is not a major pathway for **QD655-MT** (Fig. 7(b)).

Finally, the possibility of **QD655-MT** uptake through macropinocytosis pathway was investigated using inhibitors. 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA) is a Na⁺/H⁺ exchange protein inhibitor, and known to inhibit macro-

Figure 8. Macropinocytosis inhibition in live HeLa cells. Cells were preincubated with either (a) wortmannin (100 nM, 30 min incubation) or (b) EIPA (100 μ M, 30 min), followed by the treatment of dextran Rhodamine B conjugate (1 mg mL⁻¹, 1 hr) and **QD655-MT** (10 nM, 4 hr), respectively.

pinocytosis.^{20(a)} Wortmannin inhibits phosphatidylinositol 3-kinase (PI3K), a key kinase for regulating macropinocytosis.^{20(b)} Therefore, the macropinocytosis pathway may be inhibited by these drugs. Macropinocytosis of dextran is well characterized in terms of its fluid phase uptake into early endosomes, through late endosomes, and finally to lysosomes. Dextran Rhodamine B conjugate was used as the reference compound. HeLa cells were preincubated with either wortmannin (100 nM) or EIPA (100 μ M) for 30 min at 37 °C prior to the exposure to the dextran Rhodamine B conjugate (1 mg mL⁻¹, 1 hr incubation) and **QD655-MT** (10 nM, 4 hr incubation). After washing with PBS (pH 7.4), live HeLa cells were observed by confocal microscope. As expected, the emission color (red) from dextran Rhodamine B conjugate is quite dim under both inhibition conditions, indicating the concentrations of wortmannin and EIPA are high enough for inhibition (Fig. 8(a)). Under the same inhibition conditions, the fluorescence of **QD655-MT** appears mostly in the cell boundaries with none inside the cells (Fig. 8(b)), indicating that the internalization of **QD655-MT** is efficiently blocked by both inhibitors. On the basis of these results it may be concluded that macropinocytosis is

Figure 9. Distribution of **QD655-MT** in mice tissues. (a) *i.p.* and (b) *i.v.* Exposure times (ms): brain, 8000; kidney, 3000; lung, 7000; spleen, 3000; heart, 2000; liver, 1000. Tissues isolated from mice 24 hr after *i.p.* and *i.v.* injection. $\lambda_{\text{max}} = 543$ nm (red fluorescence from QD).

the major pathway for the internalization of **QD655-MT**, which is consistent with previous observation with other CPP mediated uptake of QDs.^{8(b), (c)}

Mouse Tissue Distribution of QD-MT. A solution of **QD655-MT** (0.2 nmol) in PBS (pH 7.4, 500 μ L) was prepared, and injected to an eight-week-old mouse (C57BL/6) by *i.p.* route. After 24 hr, the treated mouse was perfused with 4% paraformaldehyde in phosphate buffer solution (PBS; pH 7.4). The organs (brain, heart, spleen, kidney, lung, and liver) were harvested, incubated overnight in sucrose solution in PBS (0.5 M). Placed in cryoprotectant, they were cut into 15 μ m sections with cryostat, and transferred to coated glass slides. After drying at 37 °C, the sections were washed with PBS, treated with 0.3% Triton X-100 for 15 min at rt, and analyzed with an Axioplan2 fluorescence imaging microscope. No significant fluorescence was seen in organs other than liver (Fig. 9(a)). Granular spots are noted in the liver, as possible aggregates of **QD655-MT**. It appears that most of the **QD-MT** was trapped in liver.

Intravenous (*i.v.*) route was also examined for possible variation. Thus, a solution of **QD655-MT** (0.2 nmol) in PBS (pH 7.4, 500 μ L) was prepared, and slowly injected to an eleven-week-old mouse (C57BL/6) *via* tail vein. No unusual behavioral change of treated mice was noticed in both experiments. After 24 hr, the mouse was treated as described for the *i.p.* In the case of the *i.v.* administration, fluorescence was observed in brain, lung, and spleen in addition to liver (Fig. 9(b)). The image of liver appears similar to that of *i.p.* experiment (Fig. 9(b)-x). However, the brain image shows a highly diffused pattern without many granular spots (Fig. 9(b)-n).

Conclusions

Although QD is considered a more promising fluorescent probe than conventional organic dyes, it requires a surface modification to be efficiently taken up by cells. A biotin-attached G8 sorbitol-based molecular transporter (**5**, **MT**), when complexed with quantum dots containing streptavidins, was found to facilitate the internalization into HeLa cells. **QD655-MT** complex appears to physically bind first in the plasma membrane in 1 hr, whereas the **MT** itself (**FITC-MT**) is almost completely internalized into the cytoplasm within 15 min. Perhaps due to the much bigger size of **QD-MT** (ca. 18-20 nm), the cellular uptake of **QD655-MT** is much slower than that of **FITC-MT**. As the incubation time increases to more than 2 hr, **QD655-MT** begins to be internalized by macropinosomes with the help of microtubules, randomly appearing in the cytoplasm and vesicles such as endosomes or lysosomes. In 24 hr, most **QD655-MT** are localized in the perinuclear region, but not inside the nucleus. The observation that **QD655-MT** efficiently crosses the BBB to gain an entry into brain tissue, when given to a mouse by tail vein, is very interesting as QD is generally believed too large to cross the BBB.²¹

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