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Cellular Internalization of Quantum Dots Noncovalently Conjugated with Arginine-Rich Cell-Penetrating Peptides

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Protein transduction domains comprised of basic amino acid-rich peptides, can efficiently deliver covalently fused macromolecules into cells. Quantum dots (QDs) are luminescent semiconductor nanocrystals that are finding increasing application in biological imaging. Previous studies showed that protein transduction domains mediate the internalization of covalently attached QDs. In this study, we demonstrate that arginine-rich intracellular delivery peptides (cell-penetrating peptides; CPPs), analogs of naturally-occuring protein transduction domains, deliver noncovalently associated QDs into living cells; CPPs dramatically increase the rate and efficiency of cellular uptake of QD probes. The optimal molecular ratio between arginine-rich CPPs and QD cargoes for cellular internalization is approximately 60:1. Upon entry into cells, the QDs are concentrated in the perinuclear region. There is no cytotoxicity following transport of QDs present at concentrations up to 200 nM. The mechanism for arginine-rich CPP/QD complexes to traverse cell membrane appears to involve a combination of internalization pathways. These results provide insight into the mechanism of arginine-rich CPP delivery of noncovalently attached cargoes, and may provide a powerful tool for imaging *in vivo*.

Keywords: Cell-Penetrating Peptide (CPP), Cellular Internalization, Polyarginine, Protein Transduction Domain (PTD), Quantum Dot (QD).

1. INTRODUCTION

Cell membranes prevent the entry of most polar molecules, including virtually all proteins, for which specific carriers do not exist. The cellular uptake of the transactivator of transcription (Tat) protein of the human immunodeficiency virus type 1 (HIV-1) was reported in 1988.[1,2] The membrane permeability of Tat requires a protein transduction domain (PTD) or called cell-penetrating peptide (CPP) that contains a large number of basic amino acids.[3] Subsequently, naturally-occuring CPPs were identified in a variety of other proteins, including antennapedia from *Drosophila*,[4] VP22 from herpes simplex virus.[5] These CPPs and a number of synthetic arginine-rich peptides[6] enhance the uptake of covalently or noncovalently attached cargo molecules, which may be proteins, nucleic acids or nanoparticles.[7,8] CPPs and their cargoes are delivered to the cytoplasm as well as the nucleus.[9] The exploitation of CPPs for the intracellular delivery of therapeutic and experimental molecules is receiving a great deal of attention.

In recent years, arginine-rich CPPs have been developed that are capable carry covalently[10,11] or noncovalently[12] attached cargoes through cell membranes. CPPs and their noncovalently-associated cargoes can enter various kinds of cells, including animal cells/tissues, plant cells/tissues, bacteria, archaea, and yeasts.[12–15] CPPs and their cargoes generally retain their biological activities after entry into cells. Bioavailability is a major challenge for the use of certain therapeutic molecules. CPPs represent a promising tool for improving cellular uptake of such molecules.[16] The advantages of CPPs as therapeutic carriers include low toxicity, rapid transduction rate, ease of use, and high efficiency of transduction.[17] Certain CPPs can be used at concentrations up to 100 μM without inducing signs of cytotoxicity.[17]

Fluorescent probes play an important role in the study of cellular structure, tracking of intracellular molecules, and monitoring dynamic processes in living cells.[18,19] However, fluorescent proteins have certain disadvantages,

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including broad emission spectra, limited brightness and low photostability, that limit their usefulness, especially for long-term observation.[18, 19] New inorganic fluorophores called quantum dots (QDs), or semiconductor nanocrystals, have been developed.[20, 21] In contrast with traditional fluorescent probes, QDs have a narrow range of emission wavelength, exceptional resistance to photo and chemical degradation, and high quantum yield.[22] These properties make QDs particularly useful for long-term observation of molecules in live cells, as well as tumor targeting and diagnostics *in vivo*. Other advantages of QDs in biomedical applications are their low cytotoxicity and inactivity with respect to the differentiation and proliferation of embryonic stem cells.[23–26] Improved solubility and efficiency of cellular uptake would increase their utility in biomedical applications. One solution to overcome these problems would be to bioconjugate QDs to CPPs.[27–39]

The mechanism underlying protein transduction across the membrane mediated by arginine-rich CPPs is uncertain, although some evidence indicates that CPPs enter cells by energy-, receptor-, and classical endocytosis-independent pathways.[12, 14, 15] One possible mechanism for cellular uptake of arginine-rich CPPs is macropinocytosis.[14, 15, 40–42] However, the variety of CPPs along with the different methods used to conjugate the CPP with their cargoes complicates the determination of the internalization mechanism. Moreover, there have been no reports of CPP-mediated transduction of noncovalently attached QDs, the focus of the present study.

In this study, the ability of an arginine-rich CPP (specifically synthetic nona-arginine; SR9) to deliver QDs into cells following a noncovalent association was studied using gel retardation assays, flow cytometric analysis and microscopic observations. Finally, we determined the cytotoxicity and subcellular localization of QDs, and assessed possible mechanisms for CPP-mediated cellular internalization of QDs.

2. MATERIALS AND METHODS

2.1. QDs and Peptides Preparation

Carboxyl-functionalized QDs with a CdSe/ZnS core–shell (T2-MP EviTags) were purchased from Evident Technologies Inc. (Troy, NY). The CPP used in the present study was synthetic nona-arginine (SR9).[14] Noncovalently associated SR9/QD complexes were prepared by gently mixing SR9 peptide with QDs at room temperature for 1.5 h.

2.2. Cell Culture

Human bronchoalveolar carcinoma A549 cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS) as previously described.[12, 13] Cells were seeded into either 24-well plates or 35-mm Petri dishes and allowed to attach for 48 h. Prior to experiments, the cells were washed three times with phosphate buffered saline (PBS). The culture medium was then switched to RPMI supplemented with 1% FBS for treatment with the SR9/QD mixtures.

2.3. Gel Retardation Assay

This experiment was conducted following previously described protocols.[43, 44] Various amounts of SR9 were mixed with stocks of QD in PBS at a QD concentration of 0.5 μM. These SR9/QD mixtures were incubated at 37 °C for 1.5 h. The different ratios of SR9/QD (0, 10, 20, 60, 100, 300, and SR9 only) were analyzed by electrophoresis on a 0.5% agarose gel (Multi ABgarsoe, ABgene, UK) in 0.5 × TAE (40 mM of Tris-acetate and 1 mM of EDTA, pH 8.0) buffer at 100 V for 30 min. Images were captured using a Fujifilm LAS-3000 luminescent image analyzer system (Fujifilm, Tokyo, Japan) with excitement at 488 nm. Data were collected and analyzed using the Multi-Gauge standard image analysis software (Fujifilm).

2.4. Cytotoxicity Measurement

After exposure to QDs, medium was removed and the cells were washed several times with PBS. Cell viability was determined using the sulforhodamine B (SRB) colorimetric assay.[45] Cells without any treatment represent negative controls, while cells treated with 100% DMSO serve as positive controls. After the exposure period of 24 h, the cells were fixed with 10% cold trichloroacetic acid (TCA; J. T. Baker, Phillipsburg NJ) for 1 h at 4 °C. The TCA solution was then discarded, and the cells washed three times with distilled water followed by complete drying. SRB (Sigma-Aldrich, St. Louis, MO), 0.2% in a 1% acetic acid, was added to each well for 30 min at room temperature. The staining solution was removed, and the cells were washed with 1% acetic acid to remove excess dye, and the cells were air-dried. The dye was dissolved in cold 10 mM of Tris buffer (pH 10.5) followed by repeated pipetting in each well to ensure complete dissolution of the protein-bound dye. The absorbance of the dye solution was measured at 540 nm using the FLUOstar microplate reader (BMG Labtech, Durham, NC).

2.5. Noncovalent Protein Transduction

A549 cells were treated with mixtures of 6 μM of SR9 and 100 nM of QD for 1 h unless otherwise noted.[15] For subcellular localization, the cells were treated with SR9/QD complexes for 20 min followed by Hoechst 33342 (Invitrogen, Carlsbad, CA) at a final concentration of 16.2 μM at 37 °C for 40 min, in accordance with the manufacturer's instructions. Some conditional samples were stained with 50 nM of LysoTracker DND-99 (Invitrogen) at 37 °C for 30 min, 50 nM of MitoTracker Deep Red FM (Invitrogen) at 37 °C for

30 min, or 5 μg/ml of N-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino)phenyl)-hexatrienyl)pyridinium dibromide (Membrane Tracker; FM 4-64, Invitrogen) at 4 °C for 1 min, according to the manufacturers' instructions.

The influence of a variety of pharmacological agents that disrupt different transport processes was examined by treating the cells with effective concentrations of the agent for 1 h at 37 °C prior to measuring SR9/QD uptake as described above. To interfere with endocytosis, cells were treated with 2 μM of nigericin (Fluka Chemie, Seelze, Germany), 2 μM of valinomycin (Sigma-Aldrich), 10 mM of sodium azide (Fluka Chemie), 1 mM of N-ethylmaleimide (NEM; Sigma-Aldrich) or 80 mM of sodium chlorate ($NaClO_3$; Sigma-Aldrich) at 37 °C.[10, 14] To interfere with lipid sequestration experiments, cells were treated with 5 μg/mL of filipin (Sigma-Aldrich), 2 mM of methyl-β-cyclodetritrin (MβCD; Sigma-Aldrich) or 5 μg/mL of nystatin (Sigma-Aldrich). Disruption of macropinocytosis and actin rearrangement was accomplished by treatment with 100 μM of 5-(N-ethyl-N-isopropyl)-amiloride (EIPA; Sigma-Aldrich) or 10 μM of cytochalasin D (CytD; Sigma-Aldrich). To induce microtubule depolymerization, cells were treated with 10 μM of nocodazole.

2.6. Flow Cytometric Analysis

Human A549 cells were seeded at a density of 2.5×10^5 cells/well. Control and SR9/QD mixture-treated cells were then harvested and analyzed using a flow cytometer. Adherent cells were washed twice with PBS after treatment with SR9/QD mixtures, and then digested with 0.25% trypsin-EDTA. The cells were then washed twice and resuspended in PBS. QD fluorescence was detected using a Cytomics FC500 flow cytometer (Beckman Coulter, Fullerton, CA) with FL1 filters (excitation at 488 nm and emission at 513 nm). Data were analyzed with the CXP software.[12, 15] Results are reported as the percentage of total cell population.

2.7. Fluorescent and Confocal Microscopy

Fluorescent and bright-field images were observed and recorded using an Olympus IX70 inverted fluorescent microscope (Olympus, Center Valley, PA) using a UPLFLN 60X NA 1.25 objective and WU, WB and WG filters (Brightline, Bridgeville, PA). Images were captured using a Hamamatsu ORCA285 CCD camera. Shutters, filters, and camera were controlled using SlideBook software (Intelligent Imaging Innovations, Denver, CO). Other fluorescent images were observed using the TCS SL confocal microscope system (Leica, Wetzlar, Germany), and the relative intensities of fluorescent images were quantified using the UN-SCAN-IT software (Silk Scientific, Orem, UT), as previously described.[15]

2.8. Statistical Analysis

Data are expressed as mean ± standard deviations (SDs). Statistical comparisons between the control and experimental groups were performed using the Student's t-test. Mean values and SDs were calculated for each sample in at least three independent experiments. The level of statistical significance was set at $P < 0.05$(*, ‡, †) or 0.01(**, ††).

3. RESULTS

Agarose-based gel retardation assays were conducted to determine whether SR9 forms stable complexes with QDs. SR9 peptide was mixed and incubated with carboxylated QDs in different ratios. QDs exhibited a reduced mobility when incubated with SR9, and the overall mobility decreased as the concentration of SR9 increased (Fig. 1(A)). The emission intensity of QDs decreased

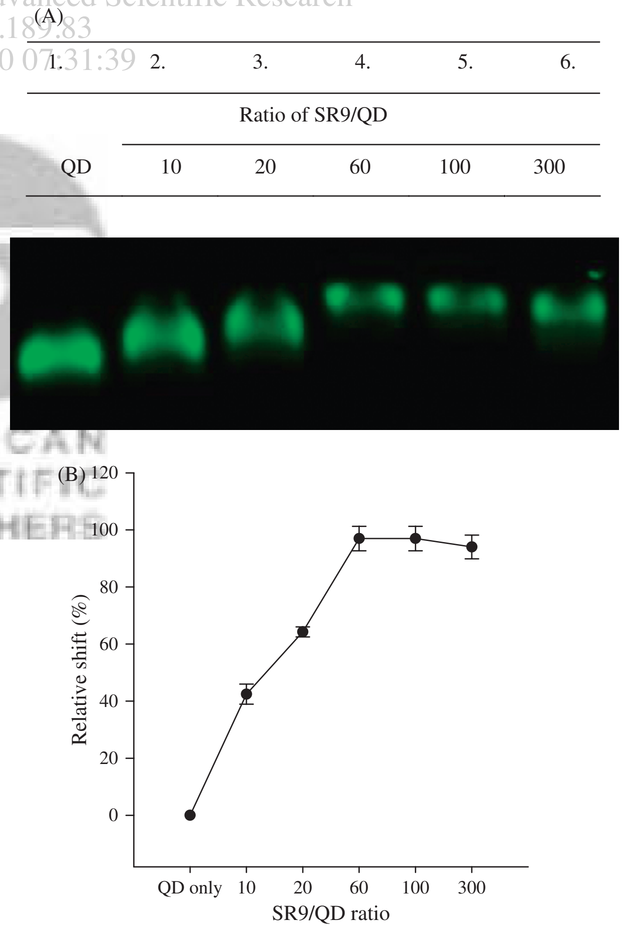

Fig. 1. Formation of noncovalent SR9/QD complexes. (A) Gel retardation assay revealing interactions between SR9 peptides and QDs. Different amounts of SR9 were mixed with QDs in molecular ratios of 0 (only QD), 10, 20, 60, 100 and 300. After the incubation with agitation for 1 h, the mixtures were analyzed by electrophoresis on a 0.5% agarose gel. QD fluorescence was visualized with excitation at 488 nm. (B) Relative mobility of SR9/QD complexes in an agarose gel.

slightly when the SR9/QD ratio exceeded 60, probably due to a shielding effect from the SR9. Moreover, a hyperbolic relationship was observed when the relative shift was plotted as a function of combination ratio (Fig. 1(B)). An optimal SR9/QD combination ratio of 60:1 was identified on the basis of maximal mobility reduction (Fig. 1(A), lane 4, and Fig. 1(B)). These data indicate that SR9 can interact with QDs to form stable, noncovalent SR9/QD complexes.

To confirm the protein transduction ability of SR9, we analyzed the kinetics of cellular internalization of SR9/QD complexes by flow cytometry. Cellular internalization in A549 cells was detected after a 60 min exposure to QD alone (Fig. 2(A)), although less than $1.0 \pm 0.3\%$ of cells showed internalization of QD at this time. This agrees with previous reports that QDs alone are rarely taken up by cells.[39, 46] However, fluorescence was observed in $2.1 \pm 1.1\%$ of cells 20 min after exposure of A549 cells to SR9/QD complexes at a combination ratio of 60:1. After a 60 min exposure, cells treated with SR9/QD complexes showed 14.9 times higher internalization than cells exposed to QD alone. Prolonged exposure to SR9/QD complexes beyond 60 min increased cellular internalization only slightly (data not shown); accordingly, we used a 60 min treatment period for subsequent uptake studies.

In order to understand relationship between SR9 and QD stoichiometry and cellular uptake in greater detail, we fixed the SR9 concentration and varied the QD concentration (Fig. 2(B)) or fixed the QD concentration and varied the SR9 concentration (Fig. 2(C)). Again, cellular uptake was monitored by flow cytometry. When A549 cells were treated with 30 μM of SR9 premixed with different

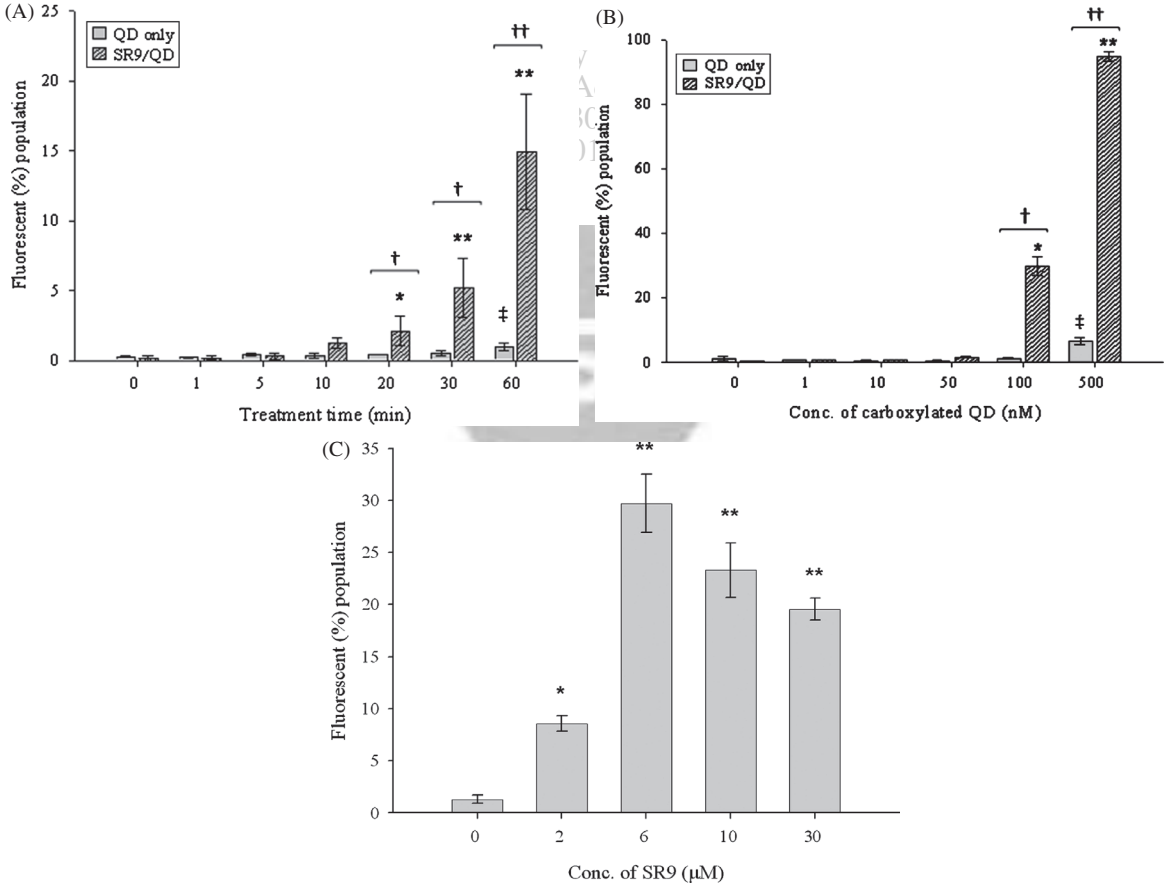

Fig. 2. Influence of time and SR9/QD ratios on complex uptake by A549 cells. (A) Intracellular fluorescent intensity of QD at different times following exposure to A549 cells. Cells were treated with either QD only or SR9/QD mixtures (60/1) for 0, 1, 5, 10, 20, 30 and 60 min. Cells were counted and their fluorescence detected using a flow cytometer. The fraction of cells with QD fluorescence is indicated as the mean $\pm$ SD. Fluorescent cell populations at each time point of SR9/QD group were compared to the population displaying fluorescence at 0 min. Differences are noted at $P < 0.05$(*) and $P < 0.01$(**) levels of significance. Fluorescent population in each time point of QD only group was compared to that at 0 min of QD only. Significance was shown for $P < 0.05$(‡). Each population of SR9/QD group was compared to that of QD only at the same time of treatment. Significant differences were shown for $P < 0.05$(†) and $P < 0.01$(††). (B) Fluorescent population of cells in various concentrations of QDs. Cells were treated with 0, 1, 10, 50, 100 and 500 nM of QDs mixed with or without 30 μM of SR9 for 1 h. Each SR9/QD bar was compared to the SR9/QD in 0 nM, and significances were shown for $P < 0.05$(*) and $P < 0.01$(**). Each QD only bar was compared to the QD only in 0 nM, and significance was shown for $P < 0.05$(‡). Each SR9/QD bar was also compared to QD only in the same dosages of QD. Significances were shown for $P < 0.05$(†) and $P < 0.01$(††). (C) Fluorescent population in different dosages of SR9. Cells were treated with different amounts of SR9 (0, 2, 6, 10, and 30 μM) premixed with 100 nM of QDs for 1 h. Significances were shown for $P < 0.05$(*) and $P < 0.01$(**).

concentrations of QD for 1 h, cellular uptake was increased only when 30 μM of SR9 was mixed with QD at concentrations above 100 nM (Fig. 2(B)). On the other hand, significant uptake was notedx when 100 nM QD was mixed with SR9 at concentrations as low as 2–30 μM (Fig. 2(C)). The highest fluorescent signal obtained in these experiments was when 6 μM of SR9 was mixed with 100 nM of QD. These results are consistent with the data presented in Figure 1, and suggest that the optimal SR9/QD ratio for cellular internalization is approximately 60:1.

Our previous reports indicated that SR9 is not toxic in living cells, as revealed in trypan blue or 3-(4,5-dimethylthiazol-2-yl)-2,5-diohenyltetrazolium bromide (MTT) based assays.[10, 12–14, 43, 44] To test the effect of QDs on cell viability, cells were treated for 24 h with different amounts of QDs with or without SR9, and cell density was determined using the SRB assay. QDs at concentrations either from 12.5 to 200 nM with SR9 at a ratio of 1:60 (Fig. 3(A)) or from 0 to 500 nM with 30 μM of SR9 (Fig. 3(B)) did not decrease cell viability. In addition, SR9 at concentrations from 0 to 30 μM with 100 nM of QD (Fig. 3(C)) caused no significant damage on cell viability.

Fluorescent and confocal microscopies were used to determine the intracellular distribution of SR9/QD complexes and their effects on cellular morphology. Untreated cells were dark when observed under the confocal microscope (Fig. 4(A), up-left); only background fluorescence was observed when cells were treated with SR9 alone (Fig. 4(A), down-left). A few cells ($2.7 \pm 1.6\%$) emitted green fluorescence when they were treated with QD only, indicating a limited cellular uptake (Fig. 4(A)). In contrast, abundant and evenly distributed fluorescence was observed in cells ($67.6 \pm 6.9\%$) treated for 1 h with SR9/QD complexes at a combination ratio of 60:1 (Fig. 4(A), middle). Higher magnification images are presented in the right panel of Figure 4(A). Fluorescent and bright-field views revealed the cellular distribution of QD (Fig. 4(B)). QDs were observed mainly in the perinuclear and membrane region; they did not appear to enter the nucleus within the first 60 min. QDs tended to be membrane-associated immediately after protein transduction (data not shown), and moved to the perinuclear region with time. To confirm this localization, cells exposed to 60:1 SR9/QD complexes were stained with Hoechst, LysoTracker, MitoTracker or Membrane Tracker for visualization of nuclear, lysosomal, mitochondria or membrane targets, respectively (Fig. 4(C)). QDs were only detectable in green fluorescent channel, but not in red fluorescent channel (Fig. 4(C), the first row). The merged image from treatment of SR9/QD complexes and nuclear stain (Hoechst) demonstrated that QDs do not enter nuclei (Fig. 4(C), first row, 4th column). Merged images of SR9/QD complexes with stains for the organelles indicated that QDs overlapped with neither lysosomes nor mitochondria (Fig. 4(C), the second

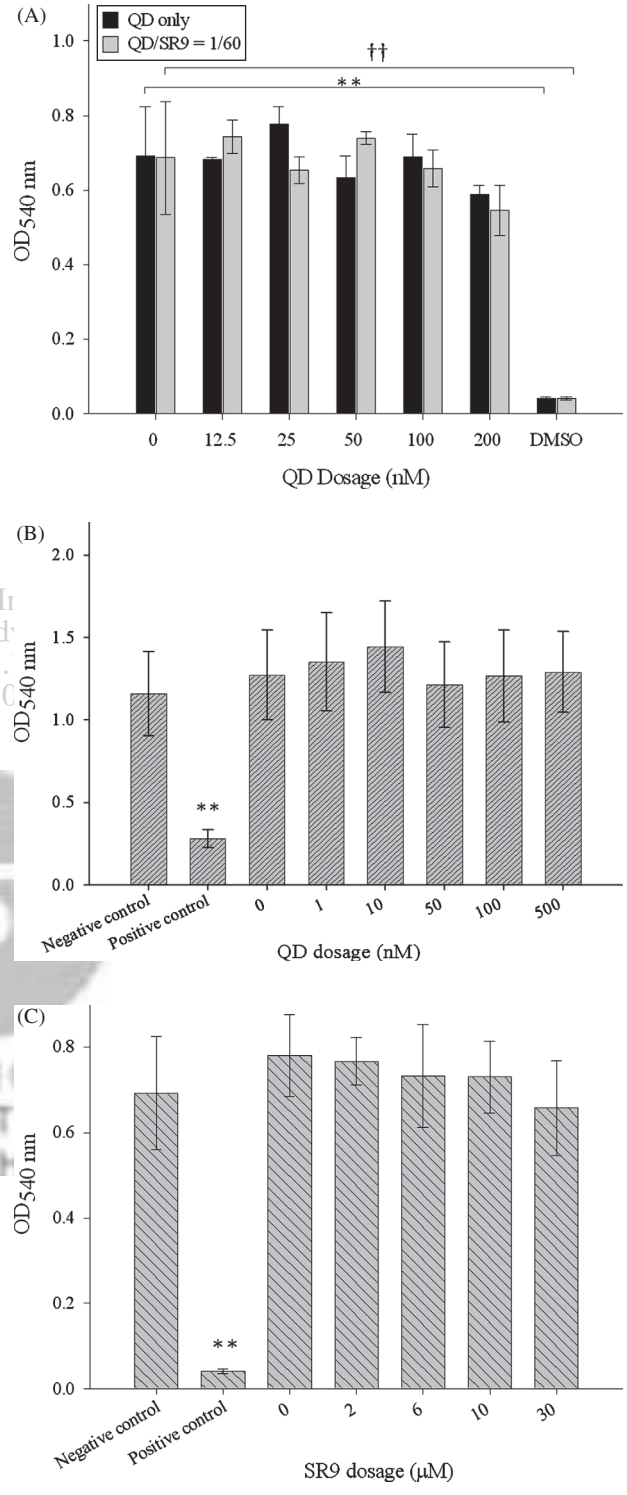

Fig. 3. Cytotoxicity of QDs in human cells. (A) Cells were treated with various amounts of QD (12.5, 25, 50, 100 and 200 nM) only or SR9/QD complexes at a fixed molecular ratio of 60:1 for 24 h. (B) Cells were treated with various amounts of QD (1, 10, 50, 100 and 500 nM) in the presence of 30 μM of SR9. (C) Cells were treated with various amounts of SR9 (2, 6, 10 and 30 μM) in the presence of 100 nM of QD. Cell viability was detected with the SRB assay. Cells without any treatment or treated with 100% DMSO serve as negative and positive controls, respectively. Values were represented by the mean $\pm$ SD. Significant differences were shown for $P < 0.01$(** or ††).

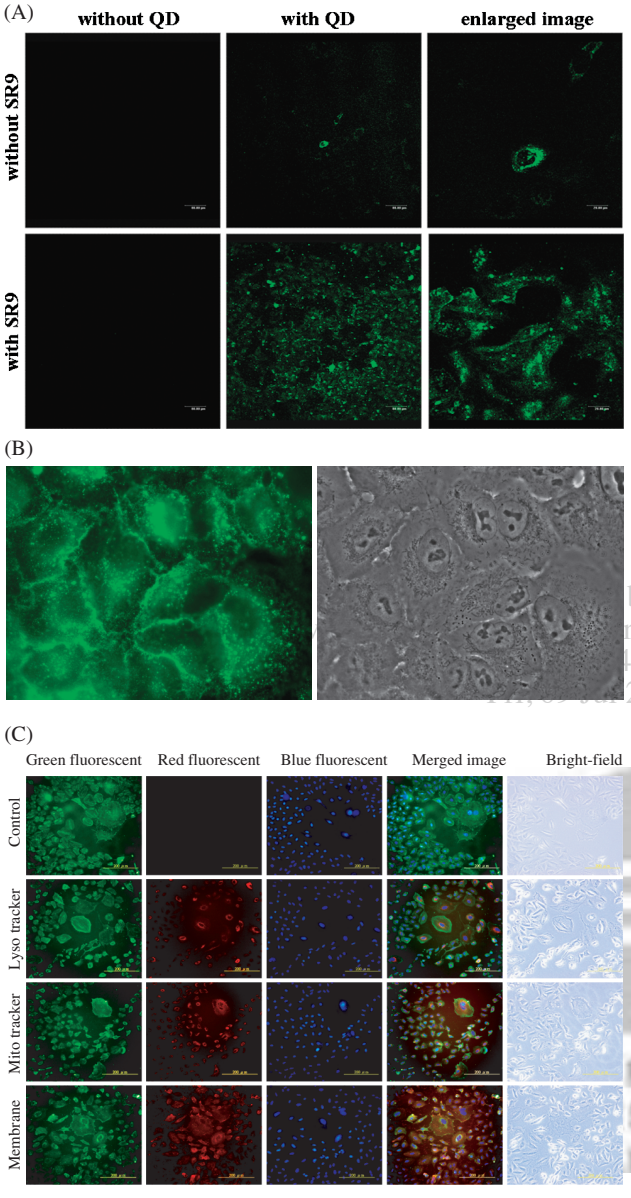

Fig. 4. Images of cellular uptake of SR9/QD complexes. (A) Confocal images of cells treated in various combinations of 100 nM of QDs and 6 μM of SR9. Cells were mock-treated as a negative control (up-left). Scale bar is 80 μm. Cells were treated with 6 μM of SR9 only (down-left), 100 nM of QDs (up-middle), or 6 μM of SR9 and 100 nM of QD mixtures (down-middle). Images shown in the most right column were enlarged images of cells treated with QD only (up-right) or SR9/QD mixtures (down-right). Scale bar is 20 μm. (B) Fluorescent (left) and bright-field (right) images of cells treated with SR9/QD mixtures in a ratio of 60. (C) The distribution of SR9/QD complexes in living cells. Cells were treated with SR9/QD complexes in a ratio of 60:1 and stained with Hoechst 33342 and either LysoTracker, MitoTracker or Membrane Tracker. Green fluorescent channel indicated the distribution of SR9/QD in cells (the most left column). Red fluorescent channel indicated the location of different organelles or cell membrane (the second left column). Blue fluorescent channel indicated the location of nucleus (the middle column). There was no emission light leaking in red fluorescent channel (the first row, second left position). The merged image (the second right column) indicated the location of QD in green, organelles in red, overlapping in yellow, and nucleus in blue. Images of bright-field view were shown (the most right column). Scale bar is 200 μm.

and third rows). However, the distribution of QD did overlap with cell membrane (Fig. 4(C), the fourth row). These results indicate that QDs is associated with cell membranes 1 h after treatment uptake.

Disruptors of cellular processes were used to identify the mechanism of cellular uptake of SR9/QD complexes. A549 cells were incubated with reported concentrations of modulators of uptake processes for 1 h at room temperature. The pharmacological actions of these agents and their effects on SR9/CQ uptake (as reflected in the fraction of cells taking up the SR9/CQ complex) are summarized in Table I and Figure 5. Inhibitors of energy-dependent transport process, namely nigericin, valinomycin, sodium azide, sodium chlorate, nocodazole and NEM, did not disrupt uptake (Fig. 5(A)). Similarly, inhibitors of clathrin- and caveolae-mediated uptake, including nocodazole, NEM, MβCD, CytD, filipin and nystatin, did not inhibit SR9/QD uptake (Figs. 5(B) and (C)). Note that nocodazole also promotes microtubule depolymerization, and NEM also disrupts uptake by clatharin- and caveolae-dependent pathways. However, the macropinocytosis inhibitor EIPA reduced SR9/QD uptake by $60 \pm 26\%$ (Figs. 5(D) and (F)), while the ionophore nystatin reduced SR9/QD uptake by $58 \pm 17\%$ (Figs. 5(C–F)). In contrast, MβCD enhanced the fraction of cells taking up SR9/QD complexes $57 \pm 27\%$ (Figs. 5(B–F)). These results suggest that SR9/QD complexes enter cells by a combination of internalization pathways.

4. DISCUSSION

It has been previously shown that covalent linkage with different CPP carriers increases the efficiency of cellular entry of QDs. However, there have been few reports of enhanced cellular uptake of QDs following noncovalent association with CPPs. In this study, we demonstrated that a nontoxic nona-arginine peptide (SR9) forms a relatively stable noncovalent complex with QDs that increases the rate and efficiency of QD uptake by A549 cells. Arginine-rich CPPs (including the SR9 used in the present study) are also recognized as nuclear localization signal (NLS) peptides which were expected to target the cargo to nuclei. In previous studies we demonstrated that arginine-rich peptides are able to deliver cargoes, including proteins,[12–15] DNA[43] and RNA,[44] into cells following noncovalent association. In many cases, arginine-rich peptide/cargo complexes were predominantly localized in the nucleus.[12, 14, 43] In the present study we used QDs as the cargo. Following a 1 h uptake, SR9/QD complexes were observed in cell membranes and in the perinuclear region (Fig. 4(C)), and not in the nucleus. However, QDs taken up alone (i.e., without benefit of SR9) were localized in nuclei in the few cells that took them up (<1%; data not shown). Chen and Gerion reported a nuclear localization of NLS-QD complexes.[23] The reason for this difference in cellular

Table I. Summary of results of modulator assays.

	Pharmacological actions					
Agent	Energy-dependent endocytosis[a]	Clathrin-dependent endocytosis[a]	Caveolae-dependent endocytosis[a]	Macro-pinocytosis[a]	References	Effect on SR9/QD uptake[b]
Nigericin, 2 μM	↓				[47, 50, 51]	n.e.
Valinomycin, 2 μM	↓				[47, 51]	n.e.
Sodium azide, 10 mM	↓				[57, 58]	n.e.
Sodium chlorate, 80 mM	↓				[49, 56, 58, 59]	n.e.
Nocodazole, 10 μM	↓	↓			[57, 71]	n.e.
NEM, 1 mM	↓	↓	↓		[52–55]	n.e.
Filipin, 5 μg/mL			↓		[58, 59]	n.e.
Nystatin, 5 μg/mL			↓	↓	[57, 58, 60, 61]	↓
MβCD, 2 mM		↓	↓	↓	[60, 62–66]	↑
CytD, 10 μM		↓	↓	↓	[57, 61, 64, 66–69]	n.e.
EIPA, 100 μM				↓	[48, 60, 69, 70]	↓

Note: Concentrations of agents used reflected the reported values for inhibition of transport processes. ↓—reduced; ↑—enhanced; n.e., no effect. [a]results from previously published papers, [b]cytometric results of SR9/QD internalization in this study.

distribution of SR9/QD complexes may reflect the use of different cells, CPPs, QDs, conjugation linkages or mechanisms of cellular entrance.

We attempted to investigate the mechanism of arginine-rich peptides-mediated QD internalization using twelve various physical treatments or pharmacological modulators. Endocytic modulators, including nigericin, valinomycin, NEM, sodium chlorate and sodium azide did not affect cellular uptake of SR9/QD complexes (Table I). Nigericin and valinomycin are ionophores that cause leakage of potassium to the cytosol, thereby dissipating membrane potential.[47] The metabolic inhibitors sodium chlorate and sodium azide inhibit energy-dependent molecular movement.[48, 49] These agents did not affect SR9/QD uptake suggesting that internalization of SR9/QD complexes does not involve an energy-dependent endocytic pathway (Table I). This is consistent with a recent conclusion from Futaki's group.[41] NEM inhibits not only energy-dependent pathways, but also caveolae-dependent endocytosis and clathrin-dependent endocytosis of transferrin receptor;[54, 62] NEM did not affect SR9/QD uptake. Filipin is a cholesterol-binding agent that interrupts transport via the caveolae-dependent pathway;[54] filipin did not affect SR9/QD uptake. Nystatin and MβCD sequester cholesterols.[49, 62] Nystatin is a caveolae and macropinocytosis inhibitor with no effect on the clathrin-mediated pathway; MβCD inhibits all 3 pathways (Table I). These results are not consistent with either a clathrin- nor caveolae-dependent pathway being the major route by which SR9/QD complexes pass through cell membranes (Table I). The ability of EIPA, an inhibitor of Na^+/H^+ exchange proteins, to inhibit SR9/QD uptake suggests a role for macropinocytosis in the uptake.[40–42, 48] More work is required to resolve these issues, but it appears that SR9/QD complexes enter cells by a combination of internalization pathways.[41]

There have been a number of studies on the mechanism of action of CPPs in recent years. It has been suggested that endocytic pathway involvement should not be considered as exclusive insofar as more than one entry mechanism may be operational for a given CPP in a particular cell type.[72] Mechanisms for cellular internalization of CPPs can be classified into three categories: clathrin-dependent endocytosis, caveolae-dependent endocytosis, and macropinocytosis.[72] Therefore, our results from the treatment of inhibitors were cross-referred to previous studies (Table I). Cellular internalization of SR9/QD complexes seems is not affected by cytoskeleton modulators, such as nocodazole and CytD[14, 15, 73] but is disrupted by agents that affect membrane-lipids, such as filipin, nystatin and MβCD.[54, 62] Our results are consistent with the involvement of multiple pathways in SR9/QD uptake with the following order of importance: macropinocytosis > caveolae-dependent endocytosis > clathrin-dependent endocytosis. Inhibition of macropinocytosis and caveolae-dependent endocytosis caused the greatest decrease in uptake. When both macropinocytosis and caveolae-dependent endocytosis are blocked, cells might resort to apply clathrin-dependent endocytosis for the uptake of SR9/QD complexes. However, this picture is complicated in the effects of MβCD and CytD (Table I). CytD inhibits F-actin polymerization is thought to block macropinocytosis.[14, 34, 74] However, CytD also inhibits clathrin-dependent endocytosis[61] and caveolae-dependent endocytosis.[66] Similar to CytD, MβCD reduces entry by all three classes of transport through the depletion of membrane cholesterol.[48, 60, 61, 66] Our results indicate that cell uptake is not affected by CytD treatment and is actually enhanced by MβCD treatment (Fig. 5). To resolve conflict, we suggest when the macropinocytic pathway is inhibited, cellular uptake is maintained by alternative mechanisms,[75] such as the direct membrane translocation pathway.[76] However, when macropinocytosis,

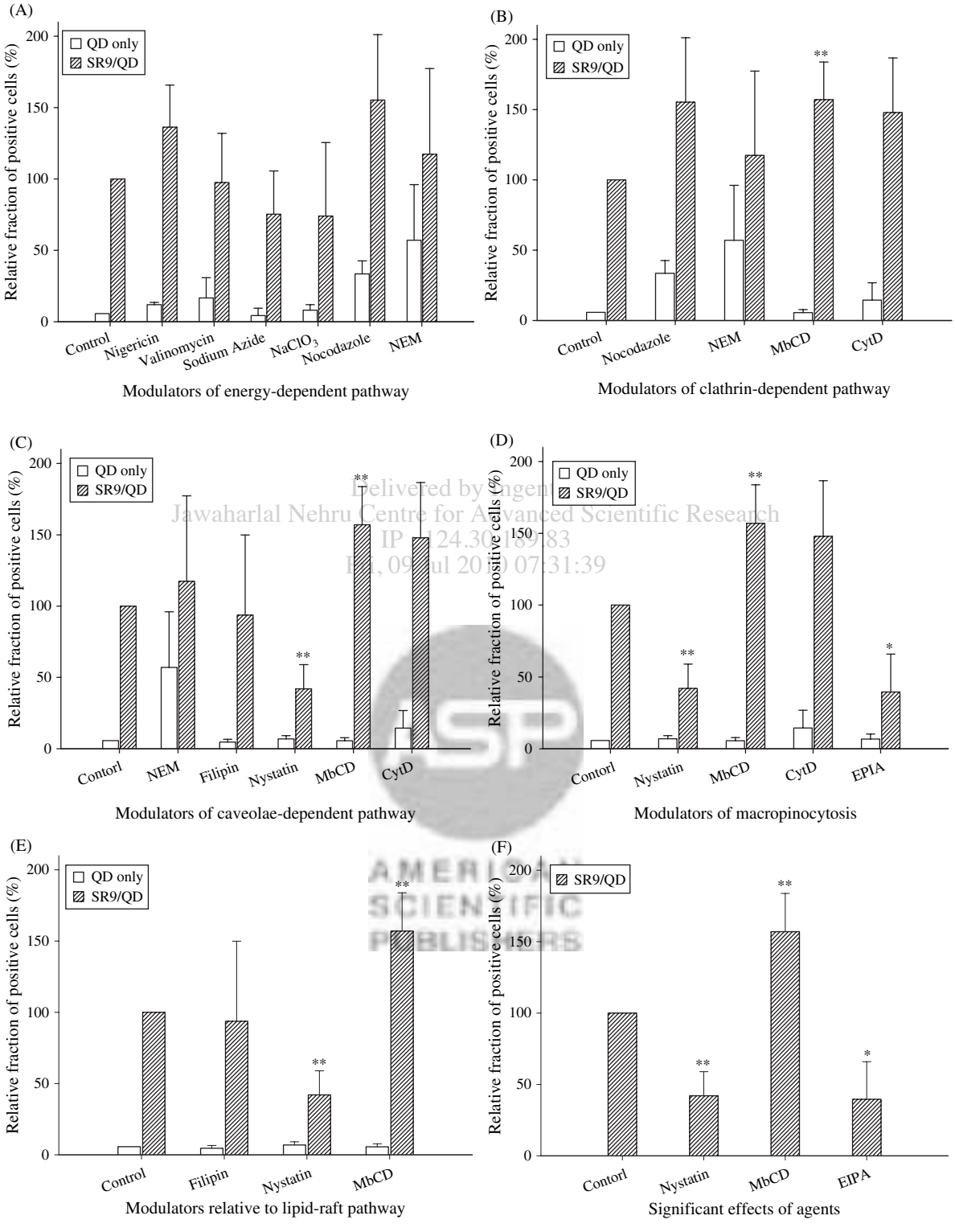

Fig. 5. Influence of pharmacological agents that disrupt transport processes on the uptake of SR9/QD complexes. (A) Cellular uptake of QD or SR9/QD complexes in the presence of modulators of energy-dependent process. Before incubation with QD or SR9/QD complexes, cells were treated with various agents for 1 h. The relative population of cells with fluorescence was represented by the mean ± SD. Each population was compared to the control, and significances of $P < 0.01$ are indicated (**). (B) Cellular uptake of QD or SR9/QD complexes in the presence of modulators of clathrin-mediated process. (C) Cellular uptake of QD or SR9/QD complexes in the presence of modulators of caveolae-dependent process. (D) Cellular uptake of QD or SR9/QD complexes in the presence of modulators of macropinocytosis. (E) Cellular uptake of QD or SR9/QD complexes in the presence of modulators of lipid-raft dependent pathway. Among agents which did not affect SR9/QD uptake were nigericin, valinomycin, sodium azide, sodium chlorate, nocodazole, NEM, filipin and CytD (for the pharmacological actions of these agents, see Table I). (F) Summary of cellular uptake of SR9/QD complexes affected by agents tested.

caveolae-dependent endocytosis and clathrin-dependent endocytosis were all blocked, we propose that a fourth, cryptic pathway associated with membrane lipid rafts could be adopted. Conner and Schmid have outlined four basic mechanisms contributing to pinocytosis: clathrin-dependent endocytosis, caveolae-dependent endocytosis, macropinocytosis, and clathrin- and caveolae-independent endocytosis.[77] Currently, the mechanism of clathrin- and caveolae-independent endocytosis is not understood; it is defined only in negative terms.[77] Our investigations into cellular internalization of SR9/QD complexes may shed some light on this cryptic mechanism.

5. CONCLUSIONS

An arginine-rich cell-penetrating peptide (SR9) facilitates the delivery of noncovalently associated QDs into A549 cells. Internalization of SR9/QD is nontoxic and highly efficient. QDs were associated with cell membranes and accumulated in the perinuclear region; QDs did not accumulate in lysosomes, mitochondria, or the nucleus. Pharmacological analysis was inconclusive with respect to the mechanism of uptake, but evidence was obtained that was consistent with the involvement of a combination macropinocytosis, and caveolae- and clatharin-mediated endocytosis. This study provides insights into the mechanisms of CPP-mediated delivery as well as the utility of simply prepared QD/CPP complexes for studying cell function and for introducing cargo proteins (potentially including diagnostic and therapeutic agents) into cells.

ABBREVIATIONS

CPP	cell-penetrating peptide
CytD	cytochalasin D
EIPA	5-(*N*-ethyl-*N*-isopropyl)-amiloride
MβCD	methyl-β-cyclodetritrin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diohenyltetrazolium bromide
NEM	*N*-ethylmaleimide
NLS	nuclear localization signal
PBS	phosphate buffered saline
PTD	protein transduction domain
QD	quantum dot
SRB	sulforhodamine B
SR9	synthetic nona-arginine
Tat	transactivator of transcription.

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