Cell Penetrating Peptide Mediated Quantum Dot Delivery and Release in Live Mammalian Cells

Jianquan Xu, Yiyi Yu, Hao-Chih Lee, Qirui Fan, Jessica Winter, Ge Yang

Abstract-**Quantum dots (QDs) are semiconductor nanocrystals whose unique fluorescence properties make them desirable biological imaging probes. However, reliable and efficient cellular delivery of QDs remains technically challenging. To address this problem, we developed a cell penetrating peptide (CPP) based approach that delivers QDs into mammalian cells with high reproducibility and efficiency and minimal cytotoxicity. To understand the delivery mechanism, we analyzed related cell uptake pathways. We followed internalization and endosomal release of CPP conjugated QDs (CPP-QDs) and found that although endocytosis (micropinocytosis) was the predominant pathway, some CPP-QDs were internalized through direct permeation of the plasma membrane. Internalized QDs could be released from endosomes to the cytoplasm if conjugated with an endosomolytic peptide (HA2), but most of released particles either were re-captured by lysosomes or aggregated in the cytoplasm. Together, our results provide insights into mechanisms of CPP mediated cellular delivery of quantum dots for intracellular imaging as well as therapeutic applications.**

I. Introduction

Recently, significant research efforts have been made on imaging cellular processes using QDs because of their unique fluorescence properties, such as high quantum yield, strong photostability and high multiplexity [1]. However, reliable and efficient cellular delivery of QDs into cells remains technically challenging. So far, methods for cellular delivery of QDs can generally be grouped into three categories: passive (e.g. QD surface functionalization), active (e.g. microinjection and electroporation) and facilitated (e.g. facilitation by proteins or peptides) [2]. Passive approaches rely on nonspecific functional modification of QD surfaces and therefore may increase cytotoxicity, and efficiency of passive approaches may vary among different cell types. Active delivery approaches are invasive and may cause cell death. In comparison, facilitated cellular delivery methods provide better specificity and flexibility.

Cell-penetrating peptides (CPP) are powerful tools for facilitated cellular delivery of macromolecule cargoes, such as DNAs, RNAs, proteins, polymers, liposomes, and nanoparticles [3-7]. Recent studies have also demonstrated CPP mediated cellular delivery of QDs [8-10]. However, the cellular uptake pathways of CPP conjugated cargoes are not well understood, and targeted release of internalized QDs remains a bottleneck.

To address these problems, we conjugated TAT and HA2 peptides to QDs (CPP-QDs) and analyzed their internalization and endosomal trapping and release in mammalian PC12 cells using high-resolution live cell imaging. We found that the CPP-QDs were mainly internalized through the endocytosis pathway. However, a small number of CPP-QDs were internalized through a much faster pathway, likely mediated by direct membrane permeation. Furthermore, we found that CPP-QDs could be released to the cytoplasm when conjugated to membrane disrupting HA2 peptide. Interestingly, endosome release could be enhanced by co-delivery of TAT and $HA2^T$ conjugated quantum dots. By analyzing internalization and endosome release of the CPP-QDs in live mammalian cells, our results provide insights into mechanisms of CPP mediated cellular delivery of quantum dots for cellular imaging as well as therapeutic applications.

II. CPP Mediated Cellular Delivery and Release

TAT peptide was derived from the transactivator of transcription of human immunodeficiency virus-1. HA2 peptide was derived from the hemagglutinin protein of Influenza, a virus that relies on endosomal acidification to deliver its nucleic acid content to host cells [11]. HA2 and its mutants has been studied extensively and shown to have membrane lysis abilities at low pH [12]. Biochemical analysis of HA2 was limited because of its poor water-solubility. Thus a TAT moiety was usually fused to the HA2 to enhance cellular delivery. TAT-fused HA2 peptide ($HA2^T$) was more widely used to enhance the cytosolic delivery of cargoes [13, 14]. $HA2^T$ has been used for delivery of quantum dots into cardiac myocytes, although the efficiency was very low [9].

Mechanisms of cellular uptake of CPP conjugated cargoes may depend on a variety of factors, such as chemical properties of the peptides as well as cargoes, concentrations of peptides, and types of cells. All these factors can significantly affect the efficiency of cell internalization [15, 16]. Although different mechanisms have been suggested for CPPs-mediated cellular uptake, endocytosis (micropinocytosis) was proposed as the predominant pathway for CPP-mediated cellular uptake of large molecules and nanoparticles [17]. Overall, however, the mechanism of cargo internalization and the fate of internalized cargoes are still not well understood. In this study,

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we addressed these questions by following the uptake and release of CPP-QDs in mammalian PC12 cells using high-resolution live cell imaging.

Cell delivery of QDs also provides a model for therapeutic delivery applications. For these applications, internalized cargoes usually must be released into the cytoplasm before they can reach their intended sites of biological functions. However, a major technical problem is that endosomal release is often not efficient. Instead, internalized cargoes often remain trapped inside endocytic organelles, such as vesicles, endosomes, and lysosomes [18, 19]. Several strategies have been developed to enhance endosomal release of CPP-cargos [20], such as using multivalent CPPs, CPP-mediated photochemical photosensitizers, and pH-dependent membrane active peptides. For example, quantum dots were coated with PEG and a disrupting polymer polyethyleneimine (PEI), by utilizing the PEI's "proton-sponge effect" (an endosmolytic effect mediated by the large number of amines present on PEI) for enhanced release to the cytoplasm [21]. Overall, to use pH-dependent membrane active peptide is the most adopted strategy to enhance the endosomal release of CPP-cargoes. It is based on the principle that pH in the endosome changes from neutral to acidic during endosome maturation, and these peptides are able to disrupt endosomal membranes and cause lysis without damaging the plasma membrane or membranes of other organelles [22, 23]. As a representative of these pH-dependent peptides, HA2 peptide and its analogues have been used to enhance endosomal release [13, 14]. In this study, we developed a novel strategy for enhancing endosomal release by co-delivery of TAT conjugated QDs with HA2 conjugated QDs.

III. Materials and Methods

Materials: Biotinylated TAT (47-57) and TAT-HA2 fusion ($HA2^T$) peptides were purchased from AnaSpec (Fremont, CA). Streptavidin-coated QDs (emission wavelengths at 525 nm and 655 nm, respectively), DiO dye and Hoechst 33342 were purchased from Invitrogen (Carlsbad, CA). PC12 cells were a gift from Dr. Manojkumar Puthenveedu (Carnegie Mellon University). Glass-bottom cell culture dish were purchased from MatTek (Ashland, MA).

Cellular Uptake and Imaging: Peptide conjugated QDs were prepared by incubating streptavidin-coated QDs with biotinylated peptides at a ratio of 1 to 100 for 1 hour at room temperature. Cells were plated onto a MatTek dish at an initial confluence of ~20%. The peptide-QDs were added to the culture and incubated for time ranging between 5 minutes to 16 hours, depending on different experiment designs. Cells were counterstained with Hoechst 33342 (blue) to label the nucleus. After incubation, cells were washed using fresh culture medium three times and then imaged using a Nikon Eclipse Ti-E inverted microscope with a Photometric CoolSnap HQ2 camera (Roper Scientific) and a 60×/1.41NA oil objective lens.

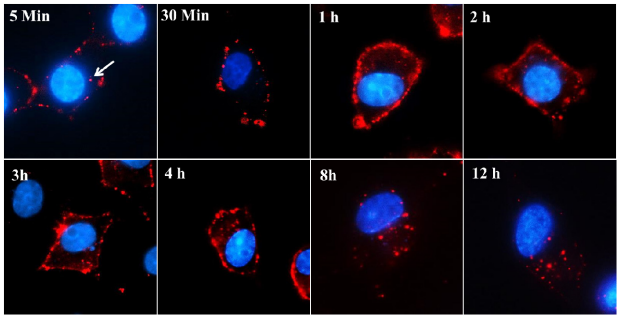

Figure 1. Cellular uptake of TAT conjugated quantum dots in PC12 cells over a 12-hour period, as observed by time-lapse live cell imaging. Red: QDs. Blue: cell nuclei.

IV. Results

To determine the uptake pathways of CPP-QDs, we followed cellular internalization of TAT conjugated QDs (TAT-QDs) into PC12 cells over a 12-hour period using time-lapse live cell imaging (Fig. 1). Within 5 minutes of incubation, TAT-QDs could be observed inside cells (see e.g QDs marked by the arrow in Fig. 1). Since an interval of 5 to 15 min is required for endosome formations in endocytosis, our data indicated that at least some TAT-QDs were internalized through a separate and faster pathway [24]. The observed fast uptake was likely through direct membrane permeation mediated by electrostatic interactions between the negatively charged cell surface and the positively charged sequences of amino acids [10, 25]. Nevertheless, most of the TAT-QDs were internalized through the endocytosis pathway. At 30 min, cell boundaries were clearly delineated by QD fluorescence, suggesting that substantially more QDs had attached to the cell surface, although very few were found inside the cell. This further supported the observation that only a small number of TAT-QDs were internalized by direct plasma membrane permeation. Within 1 hour to 4 hours of incubation, many more QDs were found inside the cells and accumulated in an area near the cell nucleus, likely the microtubule organizing center (MTOC) [8]. After 8 hours, few QDs were found on the cell surface. Instead, larger and brighter clusters of QDs were observed inside the cells, presumably caused by aggregation of QDs or formation of multi-organelle structures.

To further analyze the co-localization of quantum dots with intracellular organelles, we used the DiO dye, which labels intracellular membrane (Fig. 2A-C). After two hours of incubation, most of the QDs distributed around the MTOC formed aggregates, consistent with results shown in Fig. 1. The punctate distribution and the co-localization of QDs with organelles indicated that most QDs were internalized through endocytosis and trapped in intracellular organelles. Nucleus staining confirmed that internalized QDs did not enter the cell nucleus (Fig. 2D), presumably because QDs were trapped in large intracellular organelles and/or unable to interact with nuclear import factors (e.g. importin β) for nucleus transport [26].

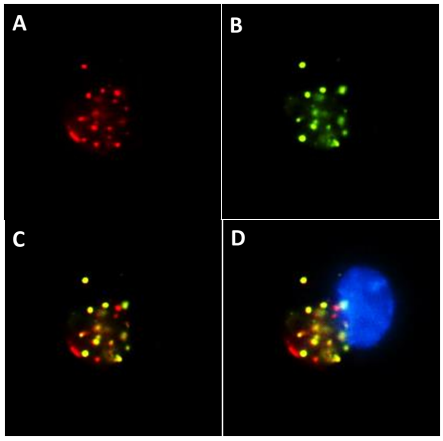

Figure 2. (A) QDs (red). (B) Organelles labeled with DiO (green). (C) An overlay image showing colocalization of QDs with organelles. (D) Distributions of QDs and organelles relative to the cell nucleus (blue).

To investigate endosomal release of CPP-QDs, we conjugated HA2^T to quantum dots (HA2^T-QDs). PC12 cells incubated with HA2^T-QDs for 4 hours showed intense punctuate fluorescence, indicating endocytosis mediated internalization (Fig. 3A). Lack of diffusive fluorescence signal indicated that endosomal release efficiency was very low. However, strong diffusive fluorescence signal was detected throughout cells co-incubated with TAT-QDs and HA2^T-QDs (Fig. 3B). This demonstrated that co-delivery of TAT-QDs and HA2^T-QDs enhanced endosomal release of QDs. Furthermore, all internalized QDs showed strong aggregation after 16 hours of incubation, including even those QDs already released from the endosomes (Fig. 3C-D). This could be due to formation of multi-organelles, or aggregation or recapture of unanchored QDs after endosomal release.

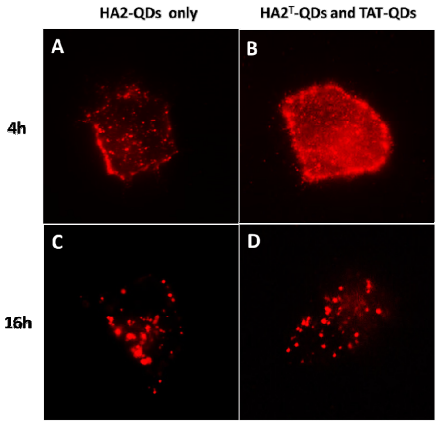

Figure 3. (A, C) PC12 cells treated with HA2^T-QDs only at 4 h and 16 h, respectively. (B, D) PC 12 cells co-treated with HA2^T-QDs and TAT-QDs at 4 h and 16 h, respectively.

To determine what roles TAT and HA2 play respectively during the endosome release, we conjugated the two types of peptides to QDs of different colors. TAT was conjugated to the green (525 nm emission wavelength) QDs and the HA2^T was conjugated to the red (655 nm emission wavelength) QDs. The two types of QDs showed good co-localization when incubated with PC12 cells for 2 hour (Fig. 4), indicating that TAT and HA2^T conjugated QDs accumulated together in endocytic organelles during endocytic uptake. After 4 hours, diffusive fluorescence was found in the green channel (Fig. 4B), indicated the endosomal release of the green QDs which were conjugated to the TAT peptide. However, the distribution pattern of the red QDs was found to be more punctate, suggesting the HA2^T conjugated red QDs were not released as effectively as TAT conjugated green QDs.

It has been well characterized that the HA2 lyses the endosomal membrane in a pH dependent manner because these peptides insert themselves into the lipid bilayers and form membrane pores that mediate the endosome escape [14, 27]. It was proposed that a HA2 fusion tag caused the retention of its fused cargo inside endosomes even after lysis took place [28]. Cells after membrane lysis remained permeable, and the HA2 remained tightly bound to the membranes after lysis [14]. This could explain why the TAT-QDs have higher escape

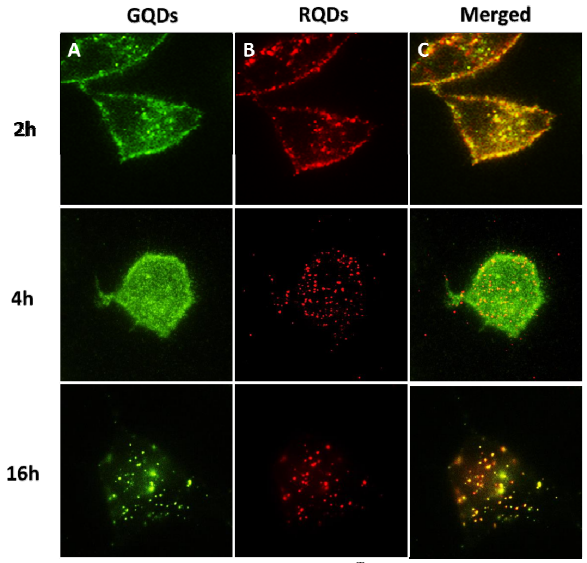

Figure 4. PC12 Cells co-treated with HA2^T-655nm QDs (RQDs) and TAT-525nm QDs (GQDs) for 2, 4, and 16h. A: green channel, B: red channel, and C: merged.

efficiency than the HA2^T-QDs even when membrane lysis was caused by the HA2^T peptide. After the endosome lysis, TAT-QDs inside endosomes were released from the permeable pores to the cytoplasm, while many HA2^T-QDs remained trapped inside endosomes because of the strong affinity of HA2^T for membrane lipid bilayer. Additionally, by incubating with both TAT-QDs and HA2^T-QDs, the total cellular uptake efficiency was enhanced by the outstanding delivery ability of TAT, which increased the concentration of peptide conjugated QDs inside endosomes. A certain concentration of HA2 was required to trigger endosome lysis. This may be another reason for the higher release activity when co-incubating with two peptides. Most of the peptide conjugated QDs, whether released from the endosome or not, either aggregated or were re-captured after 16 hours (Fig 4C).

V. Conclusion

In this study we analyzed internalization and endosomal

release of CPPs conjugated QDs in living mammalian cells. We showed that endocytosis (micropinocytosis) is the dominant but not exclusive pathway for CPP-QDs uptake. We showed that co-delivery with $HA2^{T}$-QDs enhanced cytosolic release of TAT-QDs and resulted in a diffuse cytosolic distribution. The fusogenic HA2 tag facilitated release of endocytosed TAT-QDs from endosomes into the cytoplasm but caused retention of its conjugated cargoes. This co-delivery approach provides a simple and efficient way to enhance cytoplasm release of target cargoes. Our results provide not only insights into the uptake mechanism of CPPs conjugated QDs in living cells but also a method for enhancing endosomal release, which is essential to therapeutic applications.

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