Peptide-mediated cellular delivery of semiconductor quantum dots

Kelly Gemmill[1], James Delehanty[1], Michael Stewart[2], Kimihiro Susumu[2], Eunkeu Oh[2], Jeff Deschamps[1], Juan Blanco-Canosa[3], Phil Dawson[3], Alan Huston[2], Igor Medintz[1]

Center for Bio/Molecular Science and Engineering[1] and Division of Optical Sciences[2]
US Naval Research Laboratory
Washington DC

Departments of Cell Biology & Chemistry[3]
The Scripps Research Institute
La Jolla, CA

1 ABSTRACT

Semiconductor CdSe/Zn quantum dots are ideal materials for biological imaging due to their superior optical properties over fluorescent proteins and organic dyes. We have previously demonstrated that a cell-penetrating peptide (CPP) derived from the HIV-Tat peptide, when conjugated to the QD surface via metal-affinity coordination between histidine residues and the ZnS shell, can facilitate cellular uptake of QDs. However, the QDs are taken up via the endocytosis pathway and remain trapped inside endosomal vesicles and are unavailable for cytosolic imaging or biosensing [1]. We describe here a palmitoylated peptide, Palm-1, that facilitates cellular uptake and endosomal escape of polyethylene glycol-modified dihydrolipoic acid (DHLA-PEG) coated quantum dots when conjugated to the QD surface in a variety of eukaryotic cell lines [2]. These findings, combined with the ability of QDs and other nanoparticles to bind a variety of cargo on their surface, will expand our capabilities for engineering multifunctional nanomaterials for biosensing, drug delivery, imaging and multifunctional theranostic applications.

Keywords: Nanoparticles, quantum dots, peptides, cells, imaging

2 INTRODUCTION

Quantum dots have long been pursued for biological imaging due to their high quantum yields, photostability, and ability to be conjugated to a variety of biomolecules. QDs can be delivered to cells in culture via passive delivery methods, facilitated delivery utilizing a delivery agent, or by active delivery such as microinjection [3]. Passive delivery is inefficient resulting in very little QD uptake into the cells. Microinjection, while effective, is cumbersome and not suitable for studies on large populations of cells. Facilitated delivery has shown the most promise, but the challenge has been to find delivery methods that are simple, versatile, biocompatible, non-cytotoxic, and deliver QDs to the cellular cytosol. Utilizing peptides as the delivery agent have been very effective in filling this role.

Cell penetrating peptides come from a variety of sources, both natural and synthetic, and can be easily conjugated by a variety of chemistries to a nanoparticle surface [4]. They are extremely small, allowing room for other modalities on the surface of multifunctional nanoparticles. They can also be easily engineered with a variety of functional domains for conjugation and can be engineered to be non-toxic.

The most commonly used cell penetrating peptide for use with quantum dots is derived from the HIV-Tat protein which contains a series of arginine residues that impart a positive charge and, when covering the surface of QDs, allows interaction of the QD with the cell surface and uptake via endocytosis [5] . While this peptide results in internalization of the QDs into the cell, the nanoparticles are trapped in endosomal vesicles once inside the cell and are unavailable for targeting or sensing throughout the cell [1].

Numerous methods have been tested to achieve cytosolic cellular delivery of quantum dots. Some have shown success, but they lack the versatility and benefits of peptide-mediated cellular delivery [6]. We have demonstrated that a palmitoylated peptide, Palm-1 allows for facilitated uptake of QDs and endosomal escape over the course of 48 hours. The peptide was non-toxic to the cells both alone and with the QD conjugate.

3 RESULTS

3.1 Cell- Penetrating Peptide Design

There are numerous characteristics that are needed in a QD-bound cell-penetrating peptide. We have previously shown that peptides can be conjugated to the ZnS shell of CdS/ZnS core/shell quantum dots via metal-affinity coordination of histidine residues . The binding is strong with Kd values in the nanomolar range and the number of peptides can be controlled by the molar ratios of the components in the simple self-assembly reaction [7]. Therefore the cell-penetrating peptides used here contain a hexahistidine motif.

The QDs we utilize also contain a PEG layer for aqueous solubility. This layer adds considerable size to the QD core/shell structure [8]. Therefore, peptides must be engineered to penetrate through this layer, while remaining available to interact with the cell membrane. Lastly, the peptides must have a strong positive charge to facilitate interaction with the negatively charged cell membrane.

The cell penetrating peptide (CPP) utilized here is derived from HIV-Tat protein and contains a hexahistidine domain and a series of 8 arginines that impart a positive charge [1] . The Palm-1 peptide was originally designed for the delivery of palmitoyl-protein thioesterase 1 (PPT-1) inhibitors to neurons [9]. Palm-1, like CPP, contains a hexahistidine sequence and a lysine rich domain to impart a positive charge. It also contains a palmitoyl group attached to peptide backbone by a non-hydrolyzable amide linkage through diaminopropionic acid. The palmitoyl group is thought to help with cellular membrane insertion. The polyproline sequences forms a type II helix and is used to extend the peptide in a linear formation to help in extend through the PEG layer of DHLA-PEG QDs to the QD surface, which remaining available for interaction with the cell membrane [10].

Peptide	Sequence	Ref
CPP (JB434)	H_8WGLA(Aib)SGR_8	[1]
Palm-1 (JB577)	WG(Pal)VKIKKP9GGH_6	[10]

(Aib)-α-aminoisobutyric acid, (Pal)-palmitoyl groupl anchored by diaminopropionic acid (Dap)

3.2 CPP Cellular Delivery

CPP peptide was self-assembled to the QDs at various ratios. Twenty-five peptides per QD was found empirically to give optimal results [1]. The QD/peptide conjugate was added to both COS-1 and NIH3T3 cell monolayers for 1-2 hours at a 60nM QD concentration. The acute delivery is meant to optimize cellular uptake while minimizing cellular toxicity. Cells were washed with and returned to incubation in complete cell media for at least 24 hours. Images up to 72 hours later showed QD localization in punctate vesicles inside of the cell. These vesicles were on the same plane of as the DAPI staining showing internalization of the nanoparticles into the cell. The punctate staining strongly colocalized with labeled transferrin, a marker of endocytotic vesicles, indicating endocytosis delivery of quantum dots. QD labeled NIH3T3 cells are shown in Figure 1.

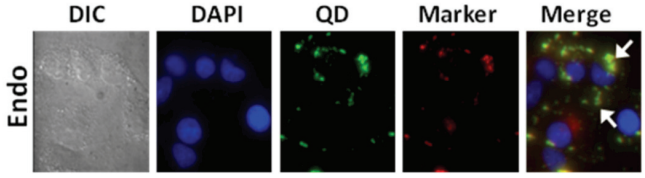

Figure 1: Cellular localization of 550nm DHLA-PEG QDs at a 60nM concentration with 25 CPP peptides per QD incubated for 1 hour with HEK 293T/17 cells. Alexa Fluor-647 labeled Transferrin was used as an endosomal marker. Arrows indicate colocalization of endosomal marker and QD signal in the merged image. Figure is adapted from [2].

3.2 Palm-1 Cellular Delivery

Palm-1 was conjugated to DHLA-PEG QDs and delivered to COS-1 cells as described above. The optimal peptide to QD ratio was determined to be 50-75 peptides per QD. While results up to 24 hours after cellular delivery were similar to those for CPP, the QD localization pattern changed after 48-72 hours. While some of the QD fluorescence remained endosomal, some fluorescence began to separate from the transferrin colocalization and become more cytosolic.

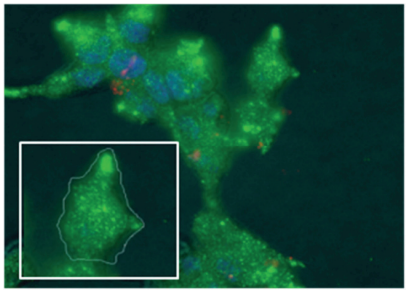

Figure 2: Palm-1 conjugated DHLA-PEG QDs in COS-1 cells. QD conjugates were delivered in growth media for two hours, and then cells were incubated in complete growth media for 48 hours. Reference adapated from [2]

3.3 Cytotoxicity

We have previously shown that CPP is non-toxic both alone and when conjugated to QDs for cellular delivery [1]. We tested the cytotoxicity of Palm-1 peptide in a similar manner by measuring cellular proliferation rates. Proliferation was measured 72 hours after a one hour incubation with DHLA-PEG QDs, Palm-1 peptide, or Palm-1/QD conjugates. Cytotoxicity of Palm-1/QD conjugates was found to be comparable to DHLA-PEG QDs or Palm-1 peptide alone in COS-1 cells. Results were similar for NIH3T3 cells. This indicates that Palm-1 is a relatively non-toxic peptide and acceptable for cellular applications within the indicated ranges.

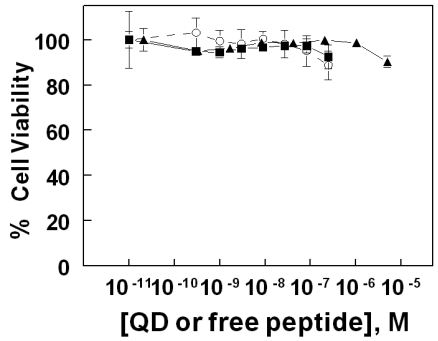

Figure 3: Cytotoxicity of DHLA-PEG QDs (open circles), PALM-1 peptide (closed triangles) and QD-Palm-1 conjugates (closed squares) incubated for 3 hours with COS-1 cells. Viability assays were performed 72 hours after delivery with Promega's CellTiter 96 Cell Proliferation Assay. Figure adapted from [2]

4 MATERIALS AND METHODS

4.1 QD synthesis

CdSe–ZnS core–shell QDs with 550nm emission maxima were synthesized and made hydrophilic by exchanging the native trioctylphosphine/trioctylphosphine oxide (TOP/TOPO) capping shell with dihydrolipoic acid (DHLA) or polyethylene glycol appended dihydrolipoic acid (DHLA-PEG) ligands as described previously [8, 11].

4.2 Peptide synthesis

All peptides were synthesized using Boc-solid phase peptide synthesis, purified by HPLC, and characterized by electrospray ionization mass spectrometry [1, 12]. All peptide sequences are written in the conventional amino-to carboxy terminus orientation.

4.3 Cell Culture.

Human embryonic kidney (HEK 293T/17) and African green monkey kidney (COS-1) cell lines (ATCC, Manassas, VA) were cultured in complete growth medium (Dulbecco's Modified Eagle's Medium (DMEM; purchased from ATCC)) supplemented with 1% (v/v) antibiotic/antimycotic and 10% (v/v) heat inactivated fetal bovine serum (ATCC). Cells were cultured in T-25 flasks and incubated at 37 °C under 5% CO_2 atmosphere and a subculture was performed every 3–4 days.

4.4 Self assembly and cell delivery

QD–CPP and QD-Palm-1 bioconjugates were formed by diluting a stock solution of preformed peptide-QD complexes (50-100nM QD assembled with 25 CPP or 75 Palm-1 peptides per QD) into DMEM contain 25mM HEPES (DMEM-HEPES). These peptide: QD ratios were determined experimentally for each peptide to be the ratio that yielded the optimal degree of cell uptake. The self-assembled bioconjugates were then incubated with cells as described in the text. For monitoring the intracellular fate of QD-peptide assemblies, AlexaFluor 647-transferrin was added at the manufacturer's recommended concentrations. Prior to imaging, the cells were washed with phosphate buffered saline, fixed with 3.7% paraformaldehyde in PBS (20 min) and nuclei were stained with 1 µg/ml DAPI (Sigma) for 10 minutes. Imaging and analysis was performed as described below.

4.5 Imaging

The intracellular distribution of QDs was analyzed by differential interference contrast (DIC) and epifluorescence microscopy using an Olympus IX-71 total internal reflection fluorescence microscope equipped with a 60x oil immersion lens. Samples were excited using a Xe lamp and images were collected using standard filter sets for DAPI, FITC (for QDs), and Cy5 (for AF647-transferrin). Merged images were generated using Adobe PhotoShop.

4.6 Cytotoxicity

Cellular toxicity was assessed using the CellTiter 96 Cell Proliferation Assay (Promega, Madison WI) according to the manufacturer's instructions. This assay is based upon the conversion of a tetrazolium substrate to a formazan product by viable cells at the assay end point. 5000 cells per well were cultured in 96-well microtiter plates in complete growth medium in the presence of increasing concentrations of QDs, free peptide, or QDs in complex with peptide. In each case, the materials were incubated with the cells for the time required for efficient QD uptake. The materials were subsequently replaced with complete growth medium and the cells were cultured for 72 hours before reading in a Tecan Safire Dual Monochromator Multifunction Microtiter Plate Reader (Tecan, Research Triangle Park, NC) according to manufacturer's instructions.

5 DISCUSSION

While various methods exist to deliver quantum dots into live cells, peptide-mediated cellular delivery has become the favored delivery method in recent years. However, the field has been hindered by the endosomal sequestration of the nanoparticles after cellular delivery. Palm-1 is a novel peptide shown to facilitate cytosolic delivery of quantum dots. This adds to our existing toolbox of QD cellular delivery methods. As other biomolecules can be co-loaded onto Palm-1 decorated QDs, cytosolic delivery will advance the use of QDs in biological imaging, sensing, and even drug drug delivery. Further testing of this peptide will focus on its

method of action, its utility with other types of nanoparticles, and other applications.

6 ACKNOWLEDGEMENTS

The authors would like to acknowledge DTRA, DARPA, NRL Nanosciences Institute and ONR for their support.

7 REFERENCES

1. Delehanty, J.B., et al., *Self-assembled quantum dot-peptide bioconjugates for selective intracellular delivery.* Bioconjug Chem, 2006. 17(4): p. 920-7.
2. Delehanty, J.B., et al., *Self-Assembled Quantum Dot-Peptide Bioconjugates for Selective Intracellular Delivery.* Bioconjugate Chemistry, 2006. 17(4): p. 920-927.
3. Algar, W.R., et al., *Semiconductor Quantum Dots in Bioanalysis: Crossing the Valley of Death.* Analytical Chemistry. 83(23): p. 8826-8837.
4. Liu, B.R., et al., *Cell-penetrating peptide-functionalized quantum dots for intracellular delivery.* J Nanosci Nanotechnol. 10(12): p. 7897-905.
5. Schmidt, N., et al., *Arginine-rich cell-penetrating peptides.* FEBS Letters. 584(9): p. 1806-1813.
6. Delehanty, J., H. Mattoussi, and I. Medintz, *Delivering quantum dots into cells: strategies, progress and remaining issues.* Analytical and Bioanalytical Chemistry, 2009. 393(4): p. 1091-1105.
7. Sapsford, K.E., et al., *Kinetics of metal-affinity driven self-assembly between proteins or peptides and CdSe-ZnS quantum dots.* Journal of Physical Chemistry C, 2007. 111(31): p. 11528-11538.
8. Mei, B.C., et al., *Modular poly(ethylene glycol) ligands for biocompatible semiconductor and gold nanocrystals with extended pH and ionic stability.* Journal of Materials Chemistry, 2008. 18(41): p. 4949-4958.
9. Dawson, G., C. Schroeder, and P.E. Dawson, *Palmitoyl:protein thioesterase (PPT1) inhibitors can act as pharmacological chaperones in infantile Batten disease.* Biochem Biophys Res Commun. 395(1): p. 66-9.
10. Delehanty, J.B., et al., *Delivering quantum dot-peptide bioconjugates to the cellular cytosol: escaping from the endolysosomal system.* Integrative Biology. 2(5-6): p. 265-277.
11. Mattoussi, H., et al., *Self-assembly of CdSe-ZnS quantum dot bioconjugates using an engineered recombinant protein.* Journal of the American Chemical Society, 2000. 122(49): p. 12142-12150.
12. Schnolzer, M., et al., *In situ neutralization in Boc-chemistry solid phase peptide synthesis. Rapid, high yield assembly of difficult sequences.* Int J Pept Protein Res, 1992. 40(3-4): p. 180-93.