Visualization of quantum dot-electron donor conjugate uptake and processing by living cells

Samuel J. Clarke, C. Annette Hollmann, and Jay L. Nadeau

Department of Biomedical Engineering, McGill University, 3775 Rue University, Montréal, QC H3A 2B4, samuel.clarke@mcgill.ca, caroline.hollmann@mcgill.ca, jay.nadeau@mcgill.ca

ABSTRACT

CdSe/ZnS quantum dot (QD) conjugates to specific electron donors, particularly dopamine, have particular spectral properties that allow visual distinction of various stages of their processing in living cells. Using these conjugates, we find that reduced QDs are more biologically accessible than those that are readily oxidized in solution. Little toxicity is seen when the particles are retained within lysosomes, although removal of the cap by lysosomal proteases can be observed. However, light, glutathione depletion, sucrose or chloroquine are all sufficient to release particles into other parts of the cell. Severe damage is seen when QDs associate with mitochondria, associated with oxidation of the QDs and blebbing and rupture of the mitochondrial membranes.

Keywords: quantum dot, cytotoxicity, singlet oxygen, endocytosis, bioavailability

1 INTRODUCTION

The interactions of semiconductor quantum dots (QDs) with living cells remain poorly understood. CdSe and CdTe QDs of different materials, sizes, colors, and surface coats demonstrate very different toxic effects to cells in culture. Much of the toxicity differences with a given type of nanoparticle are attributable to *bioavailability*: that is, whether the particles are able to enter the cell, escape from endosomes, and enter the nucleus or mitochondria. No satisfactory explanation exists for differing bioavailability among batches of particles, which appears to be loosely correlated with particle size, especially for nuclear entry[1]. However, because small size correlates with thiol cap stability [2], no firm conclusions can be drawn from this result.

A quantitative understanding of the fate of conjugated QDs in biological systems is hence critical if these particles are to be used in *in vitro* diagnostics or *in vivo* systems. The work here demonstrates that QD conjugates can be used not only as static fluorescent labels, but also as nanoscale sensors for intracellular processes such as endocytosis, lysosomal processing, and mitochondrial depolarization. The observed toxic effects also suggest that the electronic interaction of QDs and their conjugates determine toxicity as much as the size and composition of the nanocrystals themselves. A further quantification of these effects should enable the design of phototoxic drugs targeted to specific cells, and should aid environmental agencies in regulating nanoparticle disposal and release.

2 RESULTS

2.1 QD-dopamine fluorescence properties

Using QD-electron donor conjugates, specifically QD-dopamine, we found two effects that proved to be very useful for tracking the fate of QD conjugates inside and near cells: (a) the fluorescence of the conjugated QDs changes according to the number of conjugate molecules present on the surface, with quenching of the QD peak and enhancement of the dopamine peak proportional to the number of dopamines; (b) the fluorescence of the conjugated QDs is affected by changes in redox potential, with both the QD and dopamine peaks becoming brighter with oxidation (Fig. 1).

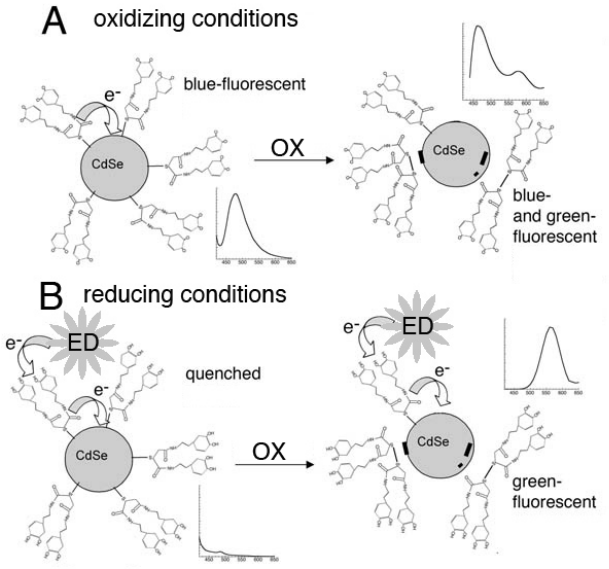

Figure 1. Mechanisms for changing spectral properties of QD-dopamine conjugates with changing environmental conditions. Typical spectra are in insets. (A) Under ambient conditions of water and oxygen (before light

exposure, i.e."oxidizing conditions"), QDs oxidize dopamine, leading to quenching of QD fluorescence. The only fluorescence is the blue peak of oxidized dopamine (excitation max 420 nm/ emission max. 470 nm). Under further oxidation from cellular processes or high-power UV exposure ("OX"), cap decay causes loss of dopamine and corresponding loss of particle solubility, simultaneous with enhanced fluorescence from the QDs (green). Thick black lines indicate formation of oxides, which passivate the surface and increase fluorescence but reduce solubility. (B) Under reducing conditions, additional electron donors such as glutathione are present ("ED"). Electron transfer between dopamine and QDs is not inhibited, but the generated oxidized dopamine may be re-reduced. Hence, dopamine remains soluble and attached to the QDs, but nonfluorescent. In this case, the conjugate is fully quenched. Under photooxidation or cellular processing ("OX"), the dopamine remains in its reduced state, but loss of this quencher from the surface allows the QD fluorescence to re-appear (green).

Not all QD preparations behaved identically under dopamine conjugation, and the differences could be used to predict their behavior in cells. QDs that showed the most complete quenching and the greatest degree of unquenching under UV exposure were the most readily taken up by cells. QDs showing poor initial quenching formed poorly soluble clusters in solution that were not taken up by cells. These clusters were highly fluorescent in the QD peak but not the dopamine peak, suggesting that the dopamine had been lost. This was most often seen with larger nanocrystals (yellow- and red-emitting), whereas the green- and blue-emitting particles showed more reliable bioavailability.

2.2 Endocytosis

QD-dopamine is readily endocytosed by cells bearing dopamine receptors. Little or no binding is seen to cells without dopamine receptors, and the QD conjugates are readily washed away. The QDs taken up into the endosomes initially remain quenched. Fusion of lysosomes leads to QD peak fluorescence inside these organelles, with no associated dopamine fluorescence. Thus, it can be seen that these QDs have lost their surface cap due to the acidic and proteolytic environment of the lysosome (Fig. 2).

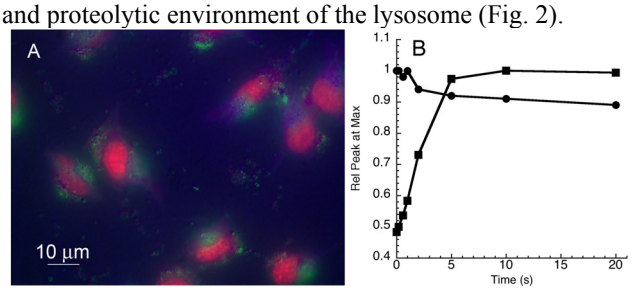

Figure 2. Subcellular differences in QD-dopamine fluorescence. (A) Image under DAPI filter of cells labeled with QD-dopamine (and with SYTO red to show nuclei). The green regions show QD fluorescence but no dopamine fluorescence. The violet areas show dopamine fluorescence and no initial QD fluorescence; QD fluorescence appears after UV illumination. Double-labeling experiments identified the green areas as lysosomes (not shown). (B) Time course of QD peak fluorescence under UV illumination for a violet region (squares) and a green region (circles).

The cytoplasm of a normal cell is reducing due to the presence of the glutathione redox couple, with more oxidizing environments found in subcellular compartments such as lysosomes and mitochondria. Cells can be made even more reducing by addition of cell-permeant glutathione, or mitochondria may be depolarized by electron-transport blocking drugs (e.g. sodium azide) or by fixation of the cells. Under all of these circumstances, the only QD fluorescence seen in the cells is lysosomal (Fig. 3).

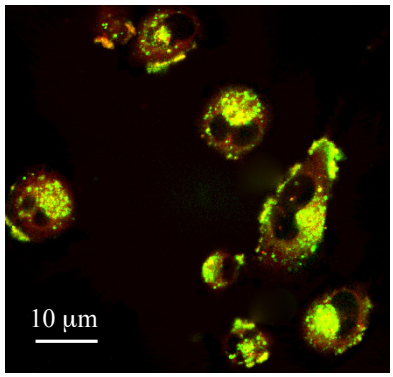

Figure 3. Lysosomally-labeled cells after incubation with QD-dopamine and cell-permeant glutathione. The appearance of the clusters is typical of lysosomal labeling; the yellow color indicates co-labeling with LysoTracker Red.

2.3 Escape from endosomes

Cells under normal conditions show QD escape from endosomes to at least some extent. It is likely that this occurs before fusion with lysosomes, as these QDs are quenched: that is, they do not appear upon initial fluorescence imaging. However, photobrightening leads to the appearance of these QDs in < 1 s of UV exposure. The distribution is primarily perinuclear, with significant overlap with mitochondrial-targeting dyes (Fig. 4 A).

When the cytoplasm is made more oxidizing by suppression of glutathione, there are no apparent lysosomes with either QD-dopamine or LysoTracker staining. QD-dopamine fluorescence appears throughout the cell (Fig. 4 B).

Addition of chloroquine or sucrose leads to swelling of endosomes. Upon initial light exposure, QD fluorescence is contained within these swellings; however, it quickly (< 10 s) escapes and spreads throughout the cell.

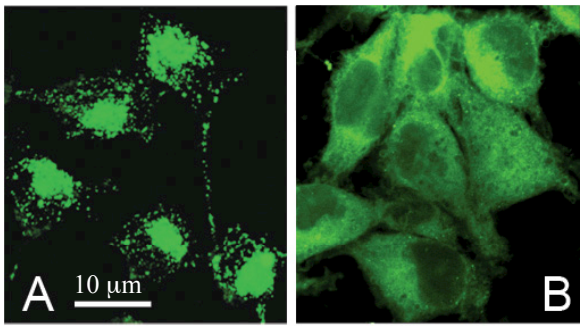

Figure 4. Progressive oxidation and QD-dopamine labeling. (A) Normal, actively-dividing cells labeled with QD-dopamine. The clusters correspond to lysosomes, and the filamentous structures to mitochondria. (B) Cells labeled with QD-dopamine made more oxidizing by suppression of glutathione. Note the labeling of the entire cell.

2.4 Interaction with mitochondria and DNA

Nuclear uptake is seen rarely with QD-dopamine. On the few occasions in which particles entered the nucleus, they were the smallest size of QD tested (emission peak 545 nm, core size ~ 2.5 nm) (Fig. 5 A).

In contrast, labeling in the mitochondrial region was the strongest labeling seen apart from that in lysosomes. Co-labeling with mitochondrial-targeting dyes suggested that at least some of the QDs were entering the mitochondrion (Fig. 5 B, C).

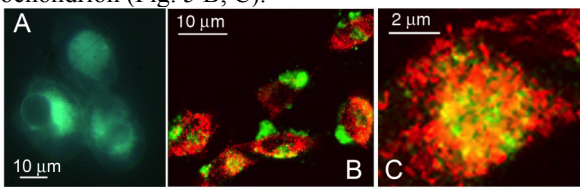

Figure 5. Penetration of QD-dopamine conjugates into subcellular compartments. (A) In this cluster of three cells, only the uppermost shows nuclear labeling, with the other two cells demonstrating a typical perinuclear concentration of QDs. (B) Co-labeling of mitochondria with RedoxSensor Red dye (RSR) and green QD-dopamine. RSR is fluorescent only inside mitochondria, thus overlap (yellow) should occur only with QD penetration into mitochondria. (C) Cells in B showing both overlap and non-internalized QDs (green).

Exposure to light led to rapid death of mitochondrial-labeled cells, with blebbing of the mitochondria and eventually the entire cell membrane. The time course required for cell death depended upon the redox state of the cell and the intensity of light used (2-5 minutes for unprotected UV illumination in cells untreated with glutathione agents; 20-30 minutes for intermittent confocal laser exposure). This is almost certainly due to release of reactive oxygen species, which is greater with QD-dopamine than with unconjugated QDs (not shown).

2.5 No unquenching without membrane potential

QD-dopamine fluorescence in cells changes greatly upon UV illumination. Lysosomal fluorescence appears first, followed by mitochondrial fluorescence and eventually fluorescence throughout the rest of the cell. These changes occur in live cells over 0.5 – 2 s, slowly enough as to be observed as a "migration" of QDs from the lysosomes/endosomes to the rest of the cell (Fig. 6 A). This phenomenon could be due to either (or both) of two causes: the rupture of endosomes/lysosomes by photooxidation, leading to QD release into the rest of the cell; and to QD unquenching by the light itself, leading to previously dark QDs becoming visible.

The question can be addressed by looking at the time course of fluorescence in fixed cells or cells with depolarized mitochondria. In these cases, the apparent "migration" does not occur after 10 s of high-intensity UV illumination (Fig. 6 B). This suggests that QDs are not being unquenched by light exposure alone. Thus, endosomal rupture is probably allowing access to the rest of the cell by the QDs, but fluorescence is only visible in oxidizing regions.

If fixed cells are illuminated for longer periods (60 s or more), some fluorescence often appears throughout the cell (not shown). Thus, the illumination light can eventually unquench the QD-dopamine, but is less effective than the mitochondria.

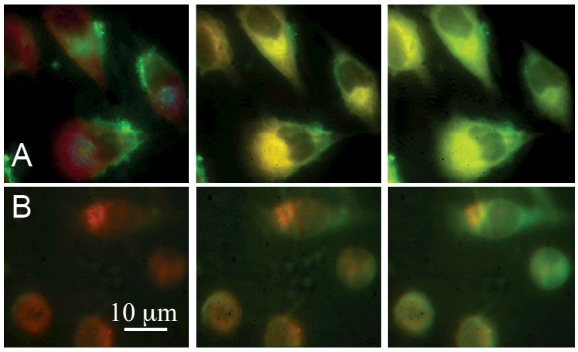

Figure 6. (A) Live cells co-labeled with QD-dopamine and a mitocondrial stain (MitoTracker Red) and a lysosomal marker (LysoTracker Blue) and oxidized by light exposure (left to right, high-intensity UV illumination for 0.6 s, 2 s, and 10 s). Note how QDs originate in lysosomes, and nearly complete overlap of QD labeling and Mitotracker as the cell becomes more oxidizing. (B) Fixed cells labeled with green QD-dopamine and LysoTracker Red and exposed to high-intensity UV illumination for 0.6 s, 2 s, and 10 s. Note how QD fluorescence brightens, but distribution does not change significantly with increasing oxidation.

3 DISCUSSION

The overall goal of this work is to develop a general fluorescent tool for tracking QD uptake and breakdown in

cells. This will aid in the development of models of QD accessibility to subcellular regions and of predictive models for QD toxicity.

4 METHODS

4.1 QD synthesis

CdSe/ZnS core-shell nanocrystals were synthesized as previously described [3, 4]. In brief, CdSe/ZnS QDs were synthesized as follows: 0.024g CdO was added to a reaction flask containing 0.44g stearic acid and heated to 180°C under inert conditions, forming a colorless solution. The solution was allowed to cool, and afterwards 5g TOPO and 2g octadecylamine was added to the flask. The flask was then evacuated and filled with inert gas several times, and the solution was heated to 200°C-300°C (exact temperature depends on the desired size). 0.2g Se was then dissolved in 2-4mL TOPO under inert conditions, and added to the reaction flask. Finally, 0.4mL of $Zn(Me)_2$ was added to 0.07mL $(TMSi)_2$ under an inert atmosphere, and added to the reaction flask. Finally the solution was allowed to cool, dissolved in $CHCl_3$ and precipitated with MeOH. The precipitate was collected by centrifugation and washed several times with MeOH. These TOPO passivated nanocrystals were then dispersed in the desired solvent, including toluene, $CHCl_3$ and hexane. QDs were solubilized using mercaptosuccinic acid (MSA). Aqueous QD solutions were diluted in H_2O to an optical density (OD) of 0.1 at the exciton peak. This corresponds to an approximate concentration of 1μM [3]. All QDs were stored in the dark until ready for use.

4.2 Conjugation to dopamine

1mg 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) was added to 0.2 mL of QDs in aqueous solution and 0.3 mL phosphate-buffered saline (PBS) solution. The tubes were covered in foil and put on a shaker for one hour. Afterwards dopamine was added to a final concentration of 2 mM, and PBS was added to a final volume of 1 mL. The tube was again covered in foil, and agitated on a shaker for 2 h. Solutions were dialyzed against PBS for 1 h in order to remove excess dopamine. All handling of dopamine solutions and QD-dopamine was performed in a glove bag under nitrogen to avoid oxidation of dopamine and further stored under an inert atmosphere until ready for use. The reducing agent β-mercaptoethanol (βME) was added to a concentration of 5.7 mM when used.

4.3 Incubation of QDs with cells

Experiments with cell lines were performed using mouse epithelial A9 cells stably transfected with human D2 dopamine receptors (ATCC CRL-10255). QD-dopamine conjugates were applied directly into serum-free medium at a concentration of ~50 nM particles. All cells were washed several times with sterile PBS after 60 min of incubation and imaged in PBS. For co-labeling with SYTO Red, MitoTracker Red, Redox Sensor Red or Lysotracker Blue (Molecular Probes), dyes were added to cells at a concentration of 1μM and co-incubated for the last 15 minutes of the QD incubation. The intracellular environment was rendered more reducing by addition of glutathione monoethyl ester (GSH-MEE) and more oxidizing by exposure to L-buthionine-sulfoximine (BSO) [5]. Cells were treated with 0.1, 1, or 10 mM GSH-MEE or BSO in DMEM for 4 hr. Following this treatment, the cells were washed, and QDs and dyes were added as above.

2.6 Spectroscopy and Microscopy of Cells

Absorbance and emission spectra were recorded on SpectraMax Plus and SpectraMax Gemini readers (Molecular Devices). Cells were examined and imaged with an Olympus IX-71 microscope and a Nuance multispectral imaging system. The objective lens was a Nikon PlanFluor 100× (N.A. = 1.30). UV illumination was through a 'quantum dot' filter cube set (excitation = 380-460 nm, dichroic = 475 nm, emission = 500 LP) a 'DAPI' filter cube set (excitation = 350/50 nm, dichroic=400 nm, emission = 420 LP) or a 'TRITC' filter set (excitation = 540 nm, dichroic = 565 nm, emission 605 LP).

Confocal imaging was performed on a Zeiss 510 LSM with a PlanApo 100× oil objective. QDs were excited with an Ar ion laser (458 nm line); LysoTracker Red and RedoxSensor Red were excited with a HeNe laser (543 nm line). Cells labeled with > 1 probe were examined for channel bleed-through before imaging.

5 REFERENCES

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