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Quantum Dot Nanotoxicity Investigations Using Human Lung Cells and TOXOR Electrochemical Enzyme Assay Methodology

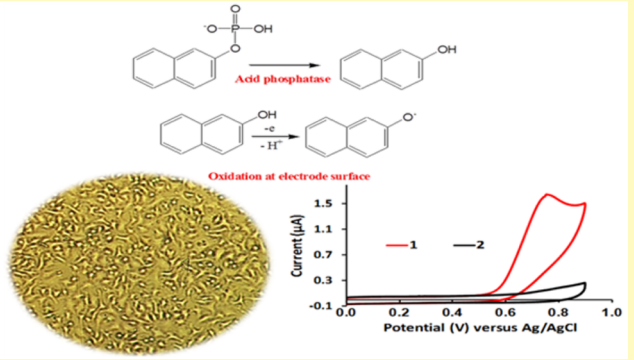
Tony O'Hara,[†] Brian Seddon,[‡] Andrew O'Connor,[§] Siobhán McClean,[§] Baljit Singh,[‡] Emmanuel Iwuoha,[∥] Xolile Fuku,[∥] and Eithne Dempsey*[,†,‡]

[†]Centre for Research in Electroanalytical Technology (CREATE) and [§]Centre for Microbial Host Interaction (CHMI), Department of Science, and [‡]MiCRA-Biodiagnostics Technology Gateway, CASH-Synergy Centre, Institute of Technology Tallaght, Dublin 24, Ireland

[∥]SensorLab, Department of Chemistry, University of the Western Cape, Private Bag X17, Bellville, 7535, South Africa

Supporting Information

ABSTRACT: Recent studies have suggested that certain nanomaterials can interfere with optically based cytotoxicity assays resulting in underestimations of nanomaterial toxicity. As a result there has been growing interest in the use of whole cell electrochemical biosensors for nanotoxicity applications. Herein we report application of an electrochemical cytotoxicity assay developed in house (TOXOR) in the evaluation of toxic effects of mercaptosuccinic acid capped cadmium telluride quantum dots (MSA capped CdTe QDs), toward mammalian cells. MSA capped CdTe QDs were synthesized, characterized, and their cytotoxicity toward A549 human lung epithelial cells investigated. The internalization of QDs within cells was scrutinized via confocal microscopy. The cytotoxicity assay is based on the measurement of changes in cellular enzyme acid phosphatase upon 24 h exposure to QDs. Acid phosphatase catalyzes dephosphorylation of 2-naphthyl phosphate to 2-naphthol (determined by chronocoulometry) and is indicative of metabolic activity in cells. The 24 h IC50 (concentration resulting in 50% reduction in acid phosphatase activity) value for MSA capped CdTe QDs was found to be 118 ± 49 μg/mL using the TOXOR assay and was in agreement with the MTT assay (157 ± 31 μg/mL). Potential uses of this electrochemical assay include the screening of nanomaterials, environmental toxins, in addition to applications in the pharmaceutical, food, and health sectors.



KEYWORDS: *cytotoxicity, electrochemical, biosensors, quantum dot, nanotoxicity*

QDs are inorganic nanoparticles whose electrons and holes are quantum confined in three spatial dimensions.[1] These unique semiconductors are emerging as alternative materials for displays, solar energy harvesting, and as complementary tools to organic fluorescent dyes for biological imaging.[2,3] QDs are composed of groups III−V (InP, InAs), groups II−VI (CdS, CdTe), or group IV (Si, Ge) elements.[4−6] The metal core is often covered or capped with an inorganic shell such as zinc sulfide and the particle coated with organic agents such as thioglycolic acid (TGA) in order to improve physiochemical properties and biocompatibility.[1,7−11] One of the advantages of QD nanomaterials over organic fluorescent dyes is their broad absorption characteristics and narrow emission profiles, a property which allows simultaneous, multiple color formation using a single light source.[12,13] Owing to their intended industrial uses, biological systems are most likely exposed to these small-scale nanomaterials.[14]

To date there have been numerous reports suggesting that cadmium-based QDs are cytotoxic, hence limiting their applicability.[3,11,14−17] Toxicity appears to stem from the release of Cd^{2+} ions from the core due to imperfections in the surface which follow particle internalization by the cell.[18] Cd^{2+} is known to generate reactive oxygen species, the ion can affect mitochondrial function, damage DNA and induce apoptosis or cell death. Other properties of QDs believed significant to cytotoxicity include particle size, surface chemistry, and charge.[14]

For the most part, the cell toxicity of QDs and other nanomaterials has been assessed by colorimetric assays such as the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and lactate dehydrogenase (LDH) assays[2,11,14−16] with cellular internalization confirmed via confocal or transmission electron microscopy (TEM).[19,20] However, concerns have been raised that some nanomaterials can interfere with the colorimetric dyes used in cytotoxicity assays.[21−23]

In recent times attention has turned to electrochemical methods for cytotoxicity evaluation. Electric cell substrate

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impedance (ECIS) techniques have been used to evaluate the cytotoxicity of a variety of nanomaterials[24−32] with a number of advantages including suitability for high throughput screening and allowing for continuous online monitoring of cell viability throughout the exposure period (not possible with conventional end point cytotoxicity assays).

Other interesting whole-cell electrochemical biosensing technologies have also been developed to probe the toxicity of nanomaterials. Yoon et al. developed an electrochemical biosensor that measured concentration, size, and time-dependent cytotoxicity of graphene nanoflakes toward HeLa cells.[33] An ITO working electrode modified with a graphene/nafion nanocomposite film measured increased hydrogen peroxide release from cells upon exposure to graphene nanoflakes. Kim et al. reported an electrochemical cell chip to assess the cytotoxicity of CdSe/ZnS QDs. With the aid of differential pulse voltammetry, the device measured changes in the intracellular redox environment of human neuroblastoma cells and showed greater sensitivity when compared with the MTT assay.[34] One of the drawbacks of many such whole-cell electrochemical biosensing systems developed for cytotoxicity measurements is that they provide little direct information on the mode of action of toxins within the cell compared to the established MTT, LDH, and neutral red uptake assays. This disadvantage highlights the need for an electrochemical assay that provides greater insights into inhibiting effects of toxins toward cells.

Acid phosphatases are a family of enzymes belonging to the hydrolase class and catalyze the hydrolysis of orthophosphate monoesters under acidic conditions.[35] They are found widely in plants, animals, and microorganisms and play a role in the production, transport, and recycling of inorganic phosphate which is crucial to cellular metabolism and bioenergetics.[36] They are located in the cell membrane, cytosol, and lysosomes,[37] and a number of researchers have reported making optical measurements of changes in these enzymes' activity upon exposure of cells to cytotoxic drugs[38] and cigarette smoke condensate.[39]

In our previous work we reported the TOXOR electrochemical cytotoxicity assay, which makes electrochemical measurements of changes in intracellular acid phosphatase upon exposure to toxic chemicals.[40] Acid phosphatase converts substrate 2-naphthyl phosphate to 2-naphthol which is oxidized at a carbon electrode surface. Electrochemical detection of acid phosphatase offers a number of advantages such as high sensitivity, low cost, and potential for incorporation of the assay into a portable sensing device. In this paper we examine nanotoxicity screening applications of this electronic assay. MSA capped CdTe QDs were selected for initial screening in order to prove the capability of the electrochemical assay with respect to cytotoxicity measurements of a nanoparticle previously reported as being cytotoxic toward mammalian cells.[17,41] To the best of our knowledge this is the first such report of an electrochemical cytotoxicity assay for QDs based on this approach and validated against the MTT assay. This assay could be a useful tool for cytotoxicity screening of QDs as well as other nanomaterials.

EXPERIMENTAL SECTION

Materials. Cadmium chloride ($CdCl_2$, 99.99%), sodium borohydride (98%), tellurium powder (99.99%), MSA (≥99.99%), phosphate buffered saline (PBS) tablets, Roswell Park Memorial Institute (RPMI) medium containing 25 mM HEPES, trypsin 0.5%-EDTA 0.2%, L-glutamine, sodium acetate (>99%), and magnesium chloride hexahydrate were all purchased from Sigma-Aldrich. Fetal bovine serum (FBS) was supplied by Biochrom while penicillin/streptomycin 100× was from Applichem GmBH. All reagents used for cell culture were tissue culture grade. Other chemicals and suppliers included acetic acid (99% Riedel de Haen) and 0.22 μm cellulose acetate filters (GE-Whatman). Uvasol potassium bromide for IR spectroscopy and acetone (99.8%) were Merck brand. Deionized water was prepared using an ELGA PURELAB Option-S-7BP water purification system. Electrochemical sensors (30 mm × 8 mm) were fabricated using screen printing (DEK 248) on polyester strips. Sensors were designed with electrodes, including carbon and silver (Ag/AgCl) films (Gwent Ltd.) and a sensing area of 4 mm^2.

Synthesis of MSA Capped CdTe QDs. MSA capped CdTe QDs were prepared following a modified version of the procedure reported by Vaishnavi and Renganathan.[42] A typical synthesis involved dissolution of the metal precursor $CdCl_2$ (2 mmol), MSA (0.7 mmol) in 50 mL deionized water, and adjusting the pH to 12 with 1 M NaOH followed by mixing at room temperature for 30 min under nitrogen. Separately, a 50 mL solution of NaHTe was prepared in a 3-neck round-bottom flask via reduction of Te powder (0.5 mmol) with $NaBH_4$ (13 mmol) under nitrogen at 0 °C for 6 h. The purple/gray colored NaHTe was added slowly to the $CdCl_2$ precursor solution and then refluxed under nitrogen for 2 h at 100 °C resulting in the formation of a green colored suspension. Following reflux, the round-bottom flask was cooled on ice and 100 mL acetone added slowly while mixing. The precipitated QDs were collected by centrifugation (11 000 rpm, 5 min) and washed with acetone (3 times) followed by deionized water and then dried in an oven at 60 °C.

Characterization. UV−visible absorption spectra of QDs dispersed in DI water were recorded using a Hitachi U-2900 spectrophotometer. Fluorescence excitation and emission spectra were measured using a LS55 luminescence spectrophotometer, scan speed 500 nm/min, slit width 7.5 nm. Fourier transformed infrared (FTIR) spectra (400−4000 cm^{-1}) of QDs and MSA capping agent in KBr disks were obtained using an IR Prestige-21 Shimadzu spectrometer. Energy dispersive X-ray (EDX) analysis of QDs was carried out using a JEOL JSM-6390LV SEM instrument with Inca x-act (Oxford Instruments plc) EDX detector for elemental composition confirmation. SEM/EDX sample preparation involved dispersion of QDs in deionized water followed by sonication and drop casting onto an aluminum stub with samples dried overnight before analysis. Size distribution and selected area electron diffraction (SAED) pattern analysis were performed using a JEOL JEM-2100F transmission electron microscope (TEM) operating at 200 kV. Sample preparation for TEM involved drop casting a suspension of QDs (∼5 μL) onto carbon coated 200 mesh Cu grids followed by drying at room temperature. Powder X-ray diffraction (XRD) analysis of QDs was performed using a Rigaku D/MAX-PC 2500 X-ray diffractometer with a CuKa (θ = 1.54 Å) radiation source operating at 40 kV and 200 mA. MTT cytotoxicity assay absorbance measurements were made using a Biotek Synergy H1 Hybrid Reader. Electrochemical measurements of cellular acid phosphatase were made using a CH Instruments Inc. CHI630C potentiostat with screen-printed sensors. Agglomeration of QDs after 24 h suspension in cell culture medium was investigated by dynamic light scattering (DLS) using a Malvern instruments ZetaSizer Nanosizer.

Cell Culture. A549 human lung epithelial cells were cultured in T75 polystyrene tissue culture flasks containing RPMI medium plus 25 mM HEPES, 10% FBS, 1% L-glutamine, and 1% penicillin/streptomycin. The cells were grown in a 5% CO_2 atmosphere at 37 °C and were subcultured for a maximum of 20 passages. Prior to carrying out experiments, cells were examined under a light microscope to check they were in the logarithmic phase of growth and free from microbial contamination.

Electrochemical Cytotoxicity Assay. A 96 well plate was seeded with 4 × 10^4 A549 cells per microwell and incubated for 24 h (5% CO_2, 37 °C). Following incubation, the medium was removed and each microwell washed three times with PBS. Fresh medium (100 μL) was added to each microwell plus 100 μL of MSA capped CdTe QDs

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(0−1000 μg/mL). QDs were presterilized by exposure to UV light (254 nm) for 20 min before dispersion in sterile deionized H_2O. This approach was employed as previous attempts to sterilize suspensions of QDs using a 0.2 μm cellulose acetate filter resulted in loss of some material during filtration. Cells were exposed to QDs for 24 h after which time the particles were removed. The microwells were then gently washed three times with PBS followed by the addition of 10 mM 2-naphthyl phosphate in pH 4.5 acetate buffer, presterilized using a 0.22 μm filter. After 1 h, the supernatant was removed and pipetted into fresh microwells. Generation of 2-naphthol arising from cellular acid phosphatase activity was measured using chronocoulometry. Chronocoulometry involved a potential step of 0.2 to 0.8 V versus Ag/AgCl and a pulse width of 10 s. The charge at 10 s was used for cytotoxicity analysis. All cytotoxicity measurements were undertaken in triplicate and in three independent experiments. For each experiment, the IC50 value was determined by fitting the data with a four parameter nonlinear fit using GraphPad Prism 7 software. In order to test for potential interference of QDs with the signal from the cytotoxicity assay, the experiment was repeated following the same procedure with microwells filled with cell culture medium only (Supporting Information page S5).

MTT Cytotoxicity Assay. The cytotoxicity of QDs was also assessed using the colorimetric MTT cytotoxicity assay kit (Cayman Chemical Company). A549 cells were seeded on a 96 well plate and exposed to the nanoparticles as previously described. Following a period of 24 h incubation, 10 μL of 5 mg/mL MTT reagent was added to each sample. The plate was placed on an orbital shaker for 1 min and then incubated for 4 h in a tissue culture incubator (5% CO_2, 37 °C). After this time the toxin and medium were removed from the microwells and 100 μL of crystal dissolving solution was added to each well. The plate was then placed on an orbital shaker for 5 min and subsequently incubated at 37 °C for 10 min to ensure dissolution of formazan crystals. Formazan generation by cells was measured at 570 nm. Following each experiment, the IC50 value at 24 h was determined. Potential interference of nanoparticles with the cytotoxicity assay was also investigated by repeating the experiment in the absence of cells (Supporting Information page S5).

Cellular Uptake of MSA Capped CdTe QDs. Internalization of QDs by cells was investigated using an Olympus Flouview FV 1000 confocal microscope. Briefly, 100 μL volumes of cell suspension (4×10^5 cells per ml) were aliquoted into four wells of an eight well glass cover slide (Nunc Lab-Tech) and incubated for 24 h (37 °C, 5% CO_2). After 24 h, the medium was removed and the wells washed with PBS (three times, 100 μL each) followed by the addition of 100 μL of fresh RPMI medium. Aliquots, 100 μL of MSA capped CdTe QDs (600 μg/mL) dispersed in PBS, were added to two wells while 100 μL aliquots of PBS were added to two additional wells containing cells as a control. After 30 min, QDs and cell culture medium were removed from the wells which were subsequently washed with PBS (three times, 100 μL each) followed by fixation with 2.5% w/v paraformaldehyde dissolved in PBS. The cells were then stained with DAPI (VECTOR)H-1200) so as to allow visualization of cell nuclei. Confocal images of cells were obtained using three channels (CH1: excitation 405 nm, filter 425 to 491 nm (DAPI); CH2: excitation 405 nm, filter 535 to 580 nm (MSA capped CdTe QDs); and CH3: DIC imaging mode) using 40× oil immersion lens.

Statistical Analysis. The results of all cytotoxicity experiments were analyzed by one-way ANOVA plus Dunnett's comparison using Minitab 16 statistical software package with $p < 0.05$ considered statistically significant.

RESULTS AND DISCUSSION

Characterization of QDs. TEM analysis (Figure 1a) was performed in order to determine the size distribution of synthesized CdTe nanoparticles. High resolution (HR)-TEM images of QDs (Figure 1b) showed the presence of lattice fringes revealing their crystalline nature. However, in some images the QDs appeared agglomerated. This could possibly be reduced by employing different ligands to cap the nanoparticles, e.g., mercaptopropionic acid (MPA). Size distribution analysis (Figure 1d) was performed by measuring 200 particles over randomly selected areas during HR-TEM imaging. Particle size measurements were made with the aid of ImageJ software (http://rsb.info.nih.gov/ij). From this analysis, the average size of the QDs was estimated to be 3.3 ± 0.7 nm. The QDs were shown to be distributed within a narrow size range with 85% of the particle population within a 2.1−4.1 nm size distribution. The interplanar spacing (d) of QDs was investigated using SAED analysis (Figure 1c) and found to be 0.39, 0.33, and 0.24 nm. EDX analysis was performed to confirm the elemental composition of MSA capped CdTe QDs with peaks for Cd, Te, O, and S detected. This is in agreement with the synthetic composition and supports the formation MSA capped CdTe QDs. After initial washing with acetone (during the synthesis stage), Na and Cl peaks were detected in the EDX spectrum. This was due to the presence of unreacted starting materials, suggesting the need for additional washing of the material. Further washing with deionized water removed the unreacted precursors as confirmed by the comparison of EDX analysis before and after washing stages. The final EDX profile is shown in Figure 1e. EDX provided useful insights regarding removal of precursor residues and purity of QDs—crucial when performing cytotoxicity evaluation of any nanomaterial. The presence of such unreacted starting materials may influence and introduce additive or synergistic cytotoxicity toward cells resulting in false observations.

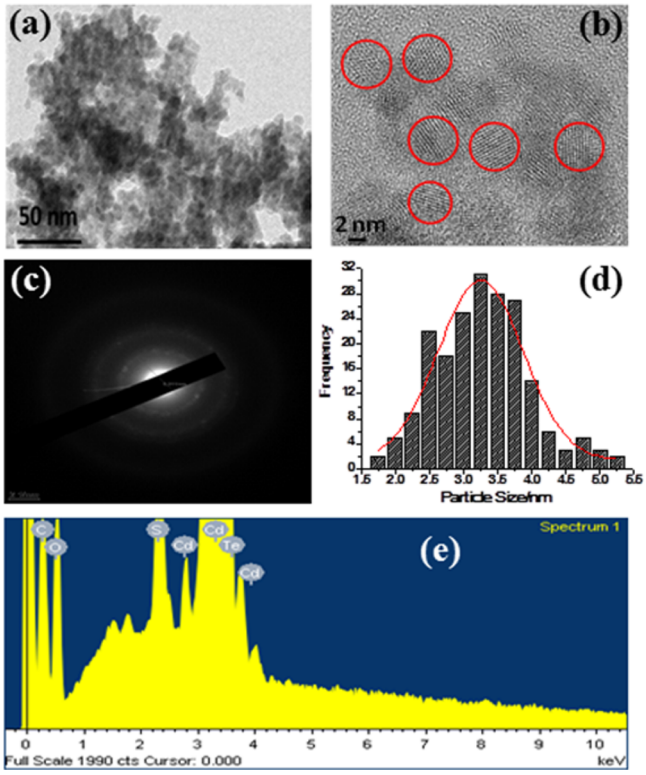

Figure 1. Results from the electron microscopy studies: (a,b) TEM images, (c) SAED pattern, (d) size distribution histogram, (e) EDX spectra of MSA capped CdTe QDs.

The optical properties of the QDs were examined by UV−vis (Supporting Information Figure S-1) and fluorescence spectroscopy (Figure 2). MSA capped CdTe QDs showed an absorbance at 465 nm, with excitation at this wavelength resulting in a fluorescence emission at 550 nm. The

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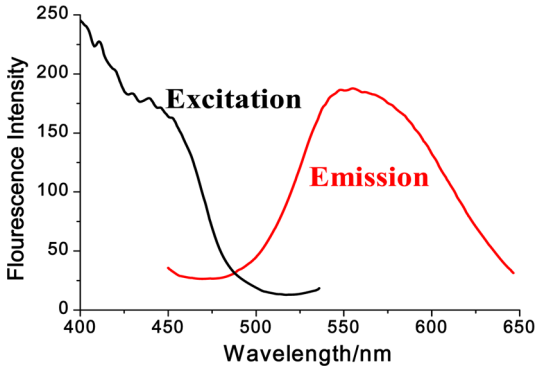

Figure 2. Fluorescence excitation and emission spectra of MSA capped CdTe QDs.

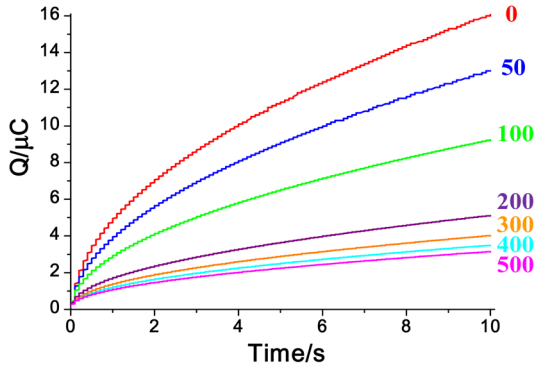

Figure 3. Chronocoulometry data (0−500 μg/mL) following 24 h exposure to QDs.

fluorescence properties of QDs is known to depend on particle size with a red shift in emission wavelength occurring with increased particle size due to a reduction in the bandgap energy.[16,43] Additionally, FTIR analysis confirmed successful capping of QDs with MSA (Supporting Information Figure S-2). Powder XRD analysis of QDs (Supporting Information Figure S-3) revealed a diffraction pattern that could be indexed to CdTe crystal phase in agreement with the databases references (CdTe: 04−004−8429). The peaks at 2θ° positions 23.080°, 27.620°, 38.340°, 46.010°, and 62.890° correspond with the (111), (200), (220), (311), and (420) CdTe crystal planes. Four additional peaks were also evident in the diffraction pattern at 2θ positions 40.570°, 43.440°, 49.800°, and 57.010° which corresponded with the $Cd_2Te_3O_9$ oxide phase.

The presence of this oxide phase was most likely due to surface oxidation of QDs which like other nanoscale particles are very active and prone to surface oxidation. During preparation, the vigorous washings (removal of precursor impurities) with acetone and deionized water could significantly contribute to the oxide phase. Washing was necessary to remove precursor impurities as investigated and confirmed during SEM/EDX studies. It has been previously reported that oxidation of both CdSe and CdTe QDs can lead to greater release of Cd^{2+}ions from the core of QDs giving rise to enhanced cytotoxicity.[44,45] It is therefore likely that this factor contributed to the cytotoxicity of QDs investigated during this study. The lattice constant (a) for the cubic CdTe phase was found to be 0.66 nm which was in agreement with literature.[46] In addition, the interplanar spacings (d) observed from XRD analysis of the (111), (200), and (220) planes were in agreement with those calculated from the SAED-TEM analysis.

Cytotoxicity Assessment. The cytotoxicity of MSA capped CdTe toward human cells was investigated using the electrochemical cytotoxicity assay and the data was correlated with a standard MTT assay. A concentration dependent reduction in acid phosphatase activity was detected in cells following 24 h exposure to ≥50 μg/mL CdTe QDs. The chronocoulometry traces (labeled for different concentrations of particles (μg/mL)) following 24 h exposure are shown (Figure 3).

A similar concentration dependent decrease in metabolic activity in cells was observed with the MTT assay. For comparison the results of both assays were normalized against untreated control cells (Figure 4a,b). The IC50 values, i.e., the concentration of MSA CdTe QDs resulting in 50% acid phosphatase or 50% mitochondrial dehydrogenase inhibition at

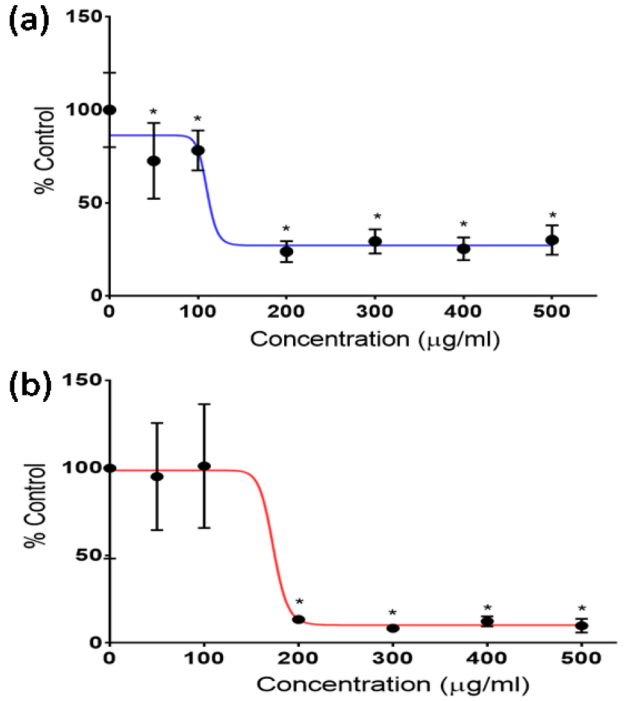

Figure 4. Normalized TOXOR electrochemical (a) and (b) MTT cytotoxicity assay data following 24 h exposure of A549 cells to QDs (0−500 μg/mL). Results are the mean of three independent experiments. Error bars represent SD (n = 9). Data points marked with an asterisk are statistically significant from controls $P < 0.05$.

24 h were determined from the average of three experiments (118 ± 49 μg/mL TOXOR assay and 157 ± 31 μg/mL MTT cytotoxicity assay). Dynamic light scattering data revealed that after 24 h suspension in cell culture medium, QDs (500 μg/mL) had agglomerated, achieving a particle size of 355.3 nm (Supporting Information Figure S-4) with zeta potential measurements (−19.7 ± 1.9 mV) suggesting that the suspended QDs were unstable (below ±30 mV).[14] Therefore, agglomeration is likely to have contributed to cytotoxicity toward cells.

With the aid of the MTT assay a number of groups have investigated the toxic properties of CdTe QDs toward mammalian cells with primary cell lines showing the greatest sensitivity.[47,48] Yan et al. reported a concentration dependent drop in the metabolic activity of primary human umbilical vascular endothelial cells (HUVECs) upon 24 h exposure to 0.1−100 μg/mL QDs with an IC50 value of 10.06 μg/mL,[41]

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while Nguyen et al. examined concentration- and time-dependent cytotoxicity of QDs toward human hepatocellular carcinoma cells (HepG2) reporting 75% reduction in cell viability after 24 h exposure to 100 μg/mL.[11]

There was no evidence that QDs had interfered with the electrochemical assay signal (Supporting Information Figure S-5a). In the case of the MTT assay, increased absorbance (most likely due to adherence of agglomerated QDs to the surface of the microwell during incubation) was measured at 570 nm in the absence of cells when microwells were filled with ≥300 μg/mL of QDs dispersed in cell culture medium (Supporting Information S-5b). However, it is unlikely that these small increases in absorbance (≤0.13 AU) had a significant impact on the results of the MTT assays because at ≥300 μg/mL concentrations, ≥88% of the metabolic activity in cells was inhibited.

Cellular Uptake of QDs. Confocal microscopy was employed to investigate internalization of MSA capped CdTe QDs within cells. Optical sectioning (Figure 5c) and *XZ* plane imaging (Figure 5d) shows the entry of QDs within the plane of the cells after as little as 30 min (presented as green in confocal images), indicating internalization. This was confirmed using phase contrast microscopy (not shown). These findings are supported by the work of a number of researchers who have employed confocal microscopy to investigate internalization of QDs by mammalian cells.[3,16] Wang et al. studied the time dependent internalization of MSA capped CdTe particles within HeLa cells, reporting the entry of green colored entities into the cytoplasm following 10 min incubation with the accumulation of bright clusters of particles within cells over time.[49] Su et al. investigated the internalization of CdTe QDs in human embryonic kidney cells (HEK cells) and reported shrinkage and deformation of nuclei after 24 h exposure to particle concentrations on the order of 150 nM.[50] Nanoparticles such as QDs are believed to enter cells by both diffusion and endocytosis mechanisms[51] with factors such as particle size and capping agent influencing cellular uptake.

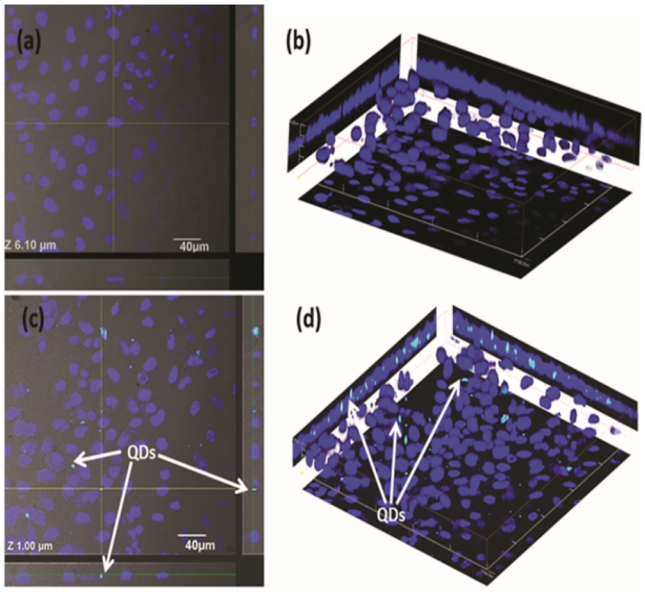

Figure 5. Confocal images of A549 cells following 30 min exposure to either 50/50 RMPI medium and PBS (Control) (a and b) or MSA capped CdTe QDs (300 μg/mL) (c and d). Cell nuclei (blue) were stained using DAPI. Representative optical section at (1 μm) (c) and 3D reconstructions (d) both show MSA capped CdTe QDs (green) were evident in cells following 30 min exposure.

CONCLUSION

The cytotoxicity of MSA capped CdTe QDs toward mammalian cells was examined for the first time using an in house TOXOR electrochemical cytotoxicity assay. The 24 h IC50 value (118 ± 49 μg/mL) determined by the electrochemical assay was in good agreement with that found using the MTT cytotoxicity assay (157 ± 31 μg/mL). Importantly there was no evidence that the nanoparticles had interfered with the electrochemical assay signal. Future work will therefore focus on the use of this electrochemical cytotoxicity assay to screen other types of nanomaterials, in particular, those reported to interfere with colorimetric cytotoxicity assay measurements, e.g. CNT and TiO_2 nanoparticles. Finally, work is currently underway to develop this assay in more robust fish cell lines with the view to developing a portable biochip for environmental toxicant and nanomaterial screening applications.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssensors.6b00673.

Additional characterization of QDs including powder X-ray diffraction pattern, UV−vis, and FTIR spectra. Results of investigations into potential interference of QDs with electrochemical and MTT cytotoxicity assay signals are also included. (PDF)

AUTHOR INFORMATION

Corresponding Author

*E-mail Eithne.Dempsey@ittdublin.ie. Phone +353 1 404 2862.

ORCID

Eithne Dempsey: 0000-0003-3607-5015

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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