Highly sensitive electrochemical detection of potential cytotoxicity of CdSe/ZnS quantum dots using neural cell chip

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A R T I C L E   I N F O

Article history:
Received 14 October 2011
Revised in revised form 14 December 2011
Accepted 20 December 2011
Available online 27 December 2011

Keywords:
CdSe/ZnS quantum dots
Cytotoxicity
Cyclic voltammetry
Differential pulse voltammetry
MTT assay
Neural cell chip

A B S T R A C T

Cell chip was recently developed as a simple and highly sensitive tool for the toxicity assessment of various kinds of chemicals or nano-materials. Here, we report newly discovered potential cytotoxic effects of CdSe/ZnS quantum dots (QDs) on intracellular redox environment of neural cancer cells at very low concentrations which can be only detected by cell chip technology. Green (2.1 nm in diameter) and red (6.3 nm in diameter) QDs capped with cysteamine (CA) or thioglycolic acid (TA) were found to be toxic at 100 μg/mL when assessed by trypan blue and differential pulse voltammetry (DPV). However, in case of concentration-dependent cytotoxicity, toxic effects of TA-capped QDs on human neural cells were only measured by DPV method when conventional MTT assay did not show toxicity of TA-capped QDs at low concentrations (1–10 μg/mL). Red-TA QDs and Green-TA QDs were found to decrease electrochemical signals from cells at 10 μg/mL and 5 μg/mL, respectively, while cell viability decreased at 100 μg/mL and 50 μg/mL when assessed by MTT assay, respectively. The relative decreases of cell viability determined by MTT assay were 15% and 11.9% when cells were treated with 5–50 μg/mL of Red-TA QDs and 5–30 μg/mL of Green-TA QDs, respectively. However, DPV signals decreased 37.5% and 39.2% at the same concentration range, respectively. This means that redox environment of cells is more sensitive than other components and can be easily affected by CdSe/ZnS QDs even at low concentrations. Thus, our proposed neural cell chip can be applied to detect potential cytotoxicity of various kinds of molecular imaging agents simply and accurately.

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1. Introduction

QDs are nanometer-sized semiconductor crystals whose excitons are restricted in three-dimensional space (Murray et al., 2000). Owing to their high fluorescence intensity with narrow emission band, QDs have gained considerable attention in the biotechnological field as efficient fluorophores for molecular imaging (Niemeyer, 2001; Trindade et al., 2001). Since QDs itself are normally capped by hydrophobic ligands for increased stability, ligand exchanging or attaching steps are essential for extending their application potential to the bio-imaging field (Yu et al., 2006). A variety of materials such as mercaptoacetic acid (Fujikawa et al., 2008), peptides (Zhou and Ghosh, 2007), lipids (Dubertret et al., 2002), streptavidin (Yum et al., 2009; Chen et al., 2007), and even dendrimers (Feng et al., 2008) have been utilized to fabricate water-soluble QDs with high stability, as well as to reduce their toxicity to both animal cells and animals itself. QDs consist of groups II–VI or III–V elements, but most of them consist of cadmium, selenium, or lead and are frequently fabricated as core–shell structures (Michalet et al., 2005). Unfortunately, these metals comprising QDs are well-known highly toxic materials that cause severe disease or adverse effects when exposed to humans or animals even at the low concentrations (Zhu et al., 1996; Elazzouzi et al., 1994). Consequently, various studies have reported the cytotoxicity of QDs capped with various kinds of ligands on different cell lines. Briefly, sheep serum albumin-capped CdSe/ZnS QDs were found to have acute toxic effects on EL-4 cells at a concentration of 0.1 mg/mL (Hoshino et al., 2004), and mercaptoundecanoic acid-capped CdSe/ZnS QDs were found to induce negative effects on Vero, HeLa, and human hepatocytes at concentrations of 0.2 mg/mL, 0.1 mg/mL, and 0.1 mg/mL, respectively (Shiohara et al., 2004). Single-core QDs such as CdSe or CdTe nanoparticles were found to have severe toxic effects on cells due to the absence of a zinc sulfide shell, which normally decreases the cytotoxicity of QDs (Lovric et al., 2005). Otherwise, QDs consisting of the same components but capped with different ligands sometimes show no toxicity in certain cell lines and even in vivo (Jaiswal et al., 2003). Hence, intensive assessment of the cytotoxicity of QDs in different kinds of cell lines should still be determined.

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Most of the techniques for determining the cytotoxicity of QDs are colorimetric or fluorescence-based methods such as 3-(4,5-
dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) viability assay, fluorescence-activated cell sorting (FACS) assay, and fluorescence imaging (Cho et al., 2010; Mahto et al., 2010; Zhang and Monteiro-Riviere, 2009). These common methods are proven tools for validating changes in cell viability in response to treatment with specific chemicals, drugs, or inorganic materials. However, in case of QDs, their natural strong fluorescence may interfere with the signals required for the determination of cell viability and induce error signals, which are critical for the toxicity assessment of QDs, causing inaccuracy in the obtained results.

We previously introduced a cell chip that can detect cell viability simply and sensitively by the electrochemical method without using any additional fluorescence dyes or pre-treatment steps that are essential in conventional methods (Yea et al., 2007). The superior performance of the cell chip was proven by drug screening and toxicity assessment for various kinds of anticancer drugs (El-
Said et al., 2009a,b,c) or environmental toxins (Kafi et al., 2010), which showed reliable linearity between cell viability and the concentrations of chemicals applied to the different kinds of cell lines. Moreover, the electrochemical signals achieved from intracellular redox environment were found to be more sensitive than optical method that normally detect mitochondrial or cell membrane damages.

Hence, in this study, we report the effects of CdSe/ZnS quantum dots (QDs) on intracellular redox environment of human neuroblastoma cells using neural cell chip. The cellular uptake of different-sized QDs with a negative or positive surface charge was confirmed by fluorescence microscopy, and cytotoxicity was determined by DPV and trypan blue assay. Thereafter, the concentration-dependent toxicity of negatively charged QDs was assessed by DPV, trypan blue, and MTT viability assays to detect the damage to intracellular redox environment, cell membrane and mitochondrial activity, respectively. Finally, the results from MTT assay utilizing an optical source for the detection of mitochondrial activity was compared with that of the DPV method to prove the enhanced sensitivity of the cell chip for the determination of QDs-induced cytotoxicity.

2. Experimental details

2.1. Materials

CdSe/ZnS QDs 2.1 nm and 6.3 nm in diameter were obtained from Evident Technologies (New York, USA) and Sigma–Aldrich (Germany), respectively. Thioglycolic acid and cysteamine were purchased from Sigma–Aldrich (Germany). Synthesized peptides (RGD-MAP-C) were obtained from Peptron (Korea), and phosphate-buffered saline (PBS: pH 7.4, 10 mM) solution consisting of 136.7 mM NaCl, 2.7 mM KCl, 9.7 mM Na2HPO4, and 1.5 mM KH2PO4 was purchased from Sigma–Aldrich (St. Louis, MO, USA). Human neuroblastoma cells (SH-SY5Y) were obtained from Korean Cell Line Bank (Korea). Other chemicals used in this study were obtained commercially as reagent grade.

2.2. Fabrication of water-soluble QDs

QDs were made water-soluble by capping TA or CA as previously described (Strekal et al., 2008). Briefly, 100 μL of QDs in toluene were transferred to 100 μL of chloroform and added to 1 mL of TA. After vigorous shaking for 2 h, the QD mixture was washed three times with acetone to remove free TA ligand. CA-capped QDs were fabricated by a similar method as described above.

2.3. Fabrication of peptide-modified cell chip

The chip contained two (Au) working electrodes each with an area 5 mm2, separated by 2 mm, making the area for cell attachment approximately 2.6 mm2. Gold electrodes with a thickness of 45 nm were prepared by DC magnetron sputtering on the titanium (Ti) deposited glass substrate. Au electrode surface was then cleaned thoroughly with piranha solution and dried under nitrogen steam as previously reported (Lee et al., 2010).

The chamber with dimensions of 2 cm × 2 cm × 0.5 cm (length × breadth × height) was attached onto the Au/Ti/Glass substrate by polydimethylsiloxane (PDMS). Peptide layer was fabricated on the gold surface by adding RGD-MAP-C peptide solution (0.05 mg/mL) for 12 h at 4 ºC. Finally, the peptide-modified gold surfaces were washed three times with PBS.

2.4. Cell culture and cell counting

SH-SY5Y cells (passage 20–30) were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) (Invitrogen, Carlsbad, USA) containing 10% heat-inactivated fetal bovine serum and 1% antibiotics (streptomycin + penicillin). The cells were grown at 37 ºC in a humidified atmosphere of 5% CO2. Approximately 2 × 104 cells were then seeded on the chip surface as determined by trypan blue assay.

2.5. Electrochemical detection of cell chip

Cyclic voltammetry (CV) and differential pulse voltammetry (DPV) were performed using a potentiostat (CHI-660, CHInstru-
maments, Austin, TX, USA). The three-electrode system composed of a cell-attached working electrode, a platinum (Pt) auxiliary electrode, and an Ag/AgCl reference electrode were used as previously described (Kafi et al., 2010).

For electrochemical measurement, cells were washed with PBS (0.01 M, pH 7.4), after which redox characteristics at the cell–electrode interface were determined properly. The scan rate for all of the voltammetric measurements was 50 mV/s.

2.6. MTT cell viability assay

Approximately 4 × 104 cells were seeded in a 96-well microtiter plate to determine the mitochondrial activity of the cells treated with different concentrations of TA-capped QDs by MTT (3-(4,5-
dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma) assay. After 24 h of treatment, medium was discarded and replaced with QD-free media. Then, 20 μL of stock MTT (5 mg/mL) was added to each well, followed by incubation for 3 h at 37 ºC, 5% CO2. Media were removed, cells were lysed, and formazan was dissolved with DMSO. Absorbance was measured at 540 nm using a Benchmark microplate reader (Bio-Rad, Mississauga, ON, Canada). All measurements were carried out in triplicate in three or more independent experiments.

2.7. Fluorescence microscopy

Cells were grown in 4-well chamber slides (Lab-Teck, Nalge Nunc International, Rochester, NY, USA). QDs were diluted with serum-free media (100 μg/mL) and then added to each well. After 6 h of treatment, cells were washed five times with PBS and then fixed with 4% paraformaldehyde solution in PBS. Thereafter, cells were washed three times with PBS, and DAPI (0.1 mg/mL) solution diluted with PBS at 1:100 was added to stain the nuclei. Fluorescence and DIC images were achieved using a fluorescence microscope (Eclipse Ti-U, Nikon instruments, Japan) controlled by NIS-elements basic research.
2.8. Data analysis

The intensity of the cathodic peaks (I_<sub>pc</sub>) in CV and DPV were used for quantitative analysis. Data were analyzed using the computerized statistical program “Origin 8”. Data are expressed as mean ± SE (N = 3).

3. Results and discussion

3.1. Strategy for the detection of QDs-induced cytotoxicity

Fig. 1 shows the strategy for detecting the cytotoxicity of QDs with different sizes and ligands. Since QDs are normally dissolved...
in organic solvent to maintain their stability and to prevent aggregation, attachment of polar ligands on the surface to make them water-soluble is essential for cellular research. CA and TA, which are positively and negatively charged, respectively, were used for the fabrication of water-soluble QDs, and ligand- or concentration-dependent cytotoxicity was determined by both trypan blue assay and the DPV method. MTT viability assay was further conducted to compare the changes in mitochondrial activity corresponding to different concentrations of QDs with those of DPV and trypan blue assay. Owing to the strong fluorescence of QDs, QDs that exist in the cell cytosol or remain on the bottom of the cell culture plate can influence the optical signals and induce signal interference, which may affect the absorbance values during the colorimetric assay. Hence, we hypothesized that the detection of signal changes of intracellular redox environment is more proper for the sensitive detection of the QD-induced cytotoxicity compared to colorimetric or fluorescence assay.

3.2. Electrochemical characterization of SH-SY5Y cells and QDs

For the electrochemical characterization of SH-SY5Y cells (passage < 20), Au surface was modified with RGD-MAP-C peptide by self-assembly technique to enhance cell binding affinity to the working electrode surface. It was previously shown that small living organisms including bacteria, animal cells, and even fungi have their own electrochemical characteristics, which can be detected by electrochemical tools (Li and Ci, 2000; Subrahmanyam et al., 2000; Feng et al., 1997). The exact mechanism of the electrochemical signals from cells is still unknown; however, intracellular redox system and the mitochondrial energy system have been considered as the origin of cellular redox peaks (El-Said et al., 2009a,b,c; Li and Ci, 2000), which can be used as an indicator of cell viability (El-Said et al., 2009a,b,c).

The electrochemical characteristics of SH-SY5Y cells used in this study were first confirmed by CV following the immobilization of cells on the RGD-MAP-C-modified Au working electrode. As shown in Fig. 2a, a quasi-reversible voltammogram was obtained from SH-SY5Y cells with a cathodic peak (IpC) at 250 mV and an anodic peak (IpA) at 365 mV. We also verified the effects of RGD-MAP-C peptide film on the Au surface by CV technique and obtained very weak redox peaks compared to the cell-immobilized electrode. Since QDs used in this study have electronic properties intermediate between those of bulk semiconductors and those of discrete molecules (Murray et al., 2000), the effects of QDs on the voltammetric response also need to be confirmed before the determination of cytotoxicity. As shown in Fig. 2b, QDs decreased the redox peaks from bare Au in 0.01 M PBS solution (pH 7.4) as well as increased the current intensity in the potential range from 400 mV to 600 mV. However, these effects were negligible since all of the IpC values obtained from QDs containing electrolyte were below 0.5 μA, whereas the IpC value from SH-SY5Y cells on the Au/RGD-MAP-C working electrode surface was 3.6 μA, which is seven times higher than that from QDs (Fig. 2c).

Finally, fluorospectrophotometry was performed to determine the exact emission peaks of TA and CA-capped green (2.1 nm in diameter, λex = 500 nm) and red (6.3 nm in diameter, λex = 625 nm) QDs. The strong emission peaks of CA-capped green (Green-CA), TA-capped green (Green-TA), CA-capped red (Red-CA), and TA-capped red (Red-TA) QDs were detected at 530 nm, 536 nm, 625 nm, and 625 nm, respectively. Hence, determination of cell viability (mitochondrial damage) via the optical/fluorescence method can be influenced by the strong fluorescence from QDs, which may overlap with the optical/fluorescence signal that represents cell viability.

![Fig. 3. Effects of different kinds of QDs on SH-SY5Y cells: (a) differential pulse voltammetry of SH-SY5Y cells treated with different kinds of QDs, (b) changes in intensity of DPV peak current from SH-SY5Y cells, and cell viability corresponding to the different kinds of QDs. Pulse amplitude and pulse width used for DPV were 50 mV and 50 ms, respectively. Error bars are the mean ± standard deviation of three different experiments.](image)

3.3. Ligand-dependent toxicity of QDs based on DPV and trypan blue assay

The toxic effects of QDs on SH-SY5Y cells were studied by the DPV method using PBS (0.01 M, pH 7.4) as an electrolyte. The potential range was −0.2 to 0.6 VAg/AgCl at a scan rate of 50 mV s−1 with a pulse amplitude and pulse width of 50 mV and 50 ms, respectively (Fig. 3a). A well-defined peak current was detected at 280 mVAg/AgCl. Approximately 100 μg/mL of Green-CA, Green-TA, Red-CA, and Red-TA QDs were added to the cells on the chip surface and incubated in common incubator maintained at 37 °C, 5% CO2. After 24 h of incubation, cells were washed with PBS (0.01 M, pH 7.4), and the electrochemical signals were achieved by the DPV method using the same parameters as the control group. It was found that all of the QDs decreased SH-SY5Y cell viability at a concentration of 100 μg/mL, and the current decrease was dependent on the type of ligand attached to the QD surface. Both TA-capped red and green QDs decreased current intensity of DPV by about 58% compared to QD-untreated cells. Two kinds of CA-capped QDs also decreased the DPV signal; however, the percentage of decrease was less compared to that of TA-capped QDs. Since the intensities of the DPV peaks obtained from cells are a measure of cell viability, a decrease in the DPV signal upon QD treatment suggests that ligand-capped CdSe/ZnS core–shell nanoparticles are cytotoxic (El-Said et al., 2009a,b,c). Various kinds of factors may influence the cytotoxicity of nanoparticles, including the size of nanoparticles, particle composition, culture conditions, and cell type (Hardman, 2006).
However, there was no external factor that influenced the cytotoxicity of QDs, since we used the same cell line (SH-SY5Y) under the same culture conditions. As shown in Fig. 3b, no decrease in current intensity was evident with respect to the size of the nanoparticles, which was confirmed by trypan blue assay. CA and TA attached on the QD surface to increase water solubility were only the factors that had toxic effects on SH-SY5Y cells. Cells generally prefer positively charged particles due to their membrane characteristics (Liang et al., 2011); however, in the case of QDs, their small size may eliminate the importance of surface charge in cellular uptake, making solubility a more significant factor for the penetration of nanoparticles into the cytosol. To confirm the cellular uptake of different kinds of QDs, QDs in serum-free medium (100 μg/mL) were added to cells and incubated for 6 h, followed by DAPI staining. Fig. 4 shows the cellular uptake of different kinds of QDs and shows that TA-capped QDs more easily penetrated the cell membrane than CA-capped QDs, which induced ligand-dependent toxicity.

3.4. Concentration-dependent toxicity of QDs as determined by DPV, MTT, and trypan blue viability assays

We have previously shown that cell chip could be excellent tool for the assessment of potential cytotoxicity of toxins at very low concentrations due to its high sensitivity (Kafi et al., 2010). Since zinc sulfide encapsulated QDs have been considered as relatively low toxic nanoparticles compared to bare CdSe or CdTe QDs,
CdSe/ZnS QDs with different diameter that emit green and red fluorescence each were treated to SH-SYSY cells immobilized on chip surface. Fig. 5a shows the electrochemical responses of SH-SYSY cells treated with different concentrations of Red-TA QDs. Peak current intensity at 280 mV achieved by the DPV method was found to decrease with increasing concentration of Red-TA QDs (1, 5, 10, 30, 50, and 100 µg/mL). This negative relationship was confirmed by quantifying the voltammetric signals at each concentration of Red-TA QDs. MTT viability assay that detect mitochondrial activity in cell cytosol also showed negative effects of Red-TA QDs on SH-SYSY cells; however, cell viability achieved from MTT assay was decreased only 15% while DPV signals decreased 37.5% at the same range of concentrations of Red-TA QDs (5–50 µg/mL). The threshold concentration that significantly decreases cell viability was also different from each methods; the decrease of cell viability was evident at 100 µg/mL of Red-TA QDs when assessed by MTT assay, while DPV signals start to decrease at 10 µg/mL of Red-TA QDs (Fig. 5b). Trypan blue assay which detect cell membrane damage showed similar results with that of DPV signals, indicating that very small amount of Red-TA QDs can give damage to neural cell membrane and intracellular redox environment which can be sensitively detected by electrochemical tools. The concentration-dependent toxicity of other TA-capped QDs with different diameter, Green-TA QDs, was also determined by the DPV method, which showed a decrease in peak current at 280 mV (Fig. 6a). A negative linearity between the concentration of QD and cell viability was obtained, similar to that of Red-TA QDs (Fig. 6b). Cytotoxicity of Green-TA QDs confirmed by MTT (mitochondrial damage) and trypan blue (cell membrane damage) were evident at 50 µg/mL and 10 µg/mL of Green-TA QDs, respectively. However, DPV signals linearly decreased through whole concentrations, from 5 µg/mL to 100 µg/mL. The signal differences between the DPV and MTT techniques were evident at a concentration of 30 µg/mL of Green-TA QDs, as MTT assay indicated a slight increase in cell viability while the DPV signal decreased from 0.44 µA to 0.3 µA. This signal variance of MTT assay may be attributed to the strong fluorescence of Green-TA QDs, as the emission band overlapped with the absorbance value at 540 nm used for the determination of cell viability (Fig. 2d). The relative decrease of cell viability was 11.9% while DPV signals decreased 39.2% at the concentration range of 5–30 µg/mL. This indicates that the DPV method is an effective tool for the toxicity assessment of QDs, especially at low concentrations. QDs are semiconductor nanoparticles which can absorb the optical energy from extracellular environment and also can transfer its energy to the various kinds of oxygen-containing molecules in cell cytosol. These characteristics of QDs lead to the generation of reactive oxygen species (ROS) including superoxide, hydroxyl radical and singlet oxygen and affect the redox environment of cells and/or mitochondrial energy system. (Romoser
et al., 2011) Since the developed cell chip may detect the electron generated from intracellular redox system and/or the mitochondrial energy system, the negative effects of QDs on cells can be more sensitively detected by the DPV tool than other methods that utilize optical source for the determination of cell viability. Inductively coupled plasma atomic emission spectroscopy (ICP-AES) was further conducted to discover the possible correlations between the concentrations of Cd, Se and Zn leached out from CdSe/ZnS QDs and the cell viability. In case of Red-TA QD, Cd and Se were not increased with increasing the concentrations of QDs until 50 µg/mL. However, concentrations of Cd and Se were dramatically increased at 100 µg/mL which could be a possible reason for the sudden decrease of cell viability when measured by MTT viability assay (Fig. S1a). Cd and Se measured from Green-TA QDs almost linearly increased with increasing the concentrations which are consistent with the decrease of cell viability shown in Fig. 6 (Fig. S1b). Hence, Cd and Se leached out from TA-capped CdSe/ZnS QDs have clear negative effects on SH-SYSY cells and decreased cell viability especially at the high concentrations of QDs. Since DPV signals from cells can be easily obtained using our fabricated cell chip, this technology can be used as an effective prediction and analysis tool for the toxicity assessment of various kinds of fluorescence-emitting materials with high reliability and reproducibility.

4. Conclusion

A cell chip was fabricated to detect the toxic effects of CdSe/ZnS QDs on intracellular redox environment of neural cells based on electrochemical method. The cathodic peak (Ipc) and anodic peak (Ipa) from SH-SYSY cells immobilized on RGD-MAP-C peptide-modified electrode were detected at 250 mV and 365 mV, respectively. 100 µg/mL of TA or CA-capped green (2.1 nm in diameter) and red (6.3 nm in diameter) QDs were found to be toxic to SH-SYSY cells, as determined by DPV and trypan blue assay. After confirming the cytotoxicity of ligand-capped CdSe/ZnS QDs, the concentration-dependent toxicity of TA-capped red and green QDs was assessed by DPV, trypan blue, and MTT viability assays. Acute toxicity of Red-TA QDs and Green-TA QDs were observed at 100 µg/mL and 50-100 µg/mL based on results of MTT assay, respectively, while DPV signals that detect intracellular redox state were decreased at 10 µg/mL and 5 µg/mL, respectively. Trypan blue assay that measures cell viability through cell membrane damages indicated the toxic effects of Red-TA and Green-TA QDs at 10 µg/mL which were consistent with DPV results. The decreases of cell viability determined by MTT assay were 15% and 11% when cells were treated with 5–50 µg/mL of Red-TA QDs and 5–30 µg/mL of Green-TA QDs, respectively, while DPV signal decreased 37.5% and 39.2% at the same concentration range, respectively. From these results, it can be concluded that intracellular redox environment is more sensitive than other components (mitochondria, cell membrane) and can be useful indicator for the assessment of cytotoxicity of nanoparticles which have potential toxicity at low concentrations. Hence, our fabricated cell chip can be applied to detect the effects of various kinds of fluorophores with high accuracy, reliability, and reproducibility.

Acknowledgments

This work was supported by a National Research Foundation of Korea (NRF) grant funded by the Korea government (MEST) (2011-000384), by the Nano/Bio Science & Technology Program (M10536050001-05N3609-00110) of the Ministry of Education, Science, and Technology (MEST), and by the Ministry of Knowledge Economy (MKE) and Korea Institute for Advancement in Technology (KIAT) through the Workforce Development Program in Strategic Technology.

Appendix A. Supplementary data


References