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Intracellular oxidative stress and cadmium ions release induce cytotoxicity of unmodified cadmium sulfide quantum dots

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ABSTRACT

Objective: To fully understand the cytotoxicity of after-degradation QDs, we synthesized CdS QDs and investigated its toxicity mechanism.

Methods: Biomimetic method was proposed to synthesize cadmium sulfide (CdS) QDs. Thereafter MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay was conducted to evaluate their cytotoxicity. To investigate the toxicity mechanism, we subsequently conducted intracellular reactive oxygen species (ROS) measurement with DCFH-DA, glutathione (GSH) measurement with DTNB, and cellular cadmium assay using atomic absorption spectrometer. Microsized CdS were simultaneously tested as a comparison.

Results: MTT assay results indicated that CdS QDs are more toxic than microsized CdS especially at concentrations below 40 μg/ml. While microsized CdS did not trigger ROS elevation, CdS QDs increase ROS by 20–30% over control levels. However, they both deplete cellular GSH significantly at the medium concentration of 20 μg/ml. In the presence of NAC, cells are partially protected from CdS QDs, but not from microsized particles. Additionally, nearly 20% of cadmium was released from CdS nanoparticles within 24 h, which also accounts for QDs' toxicity.

Conclusion: Intracellular ROS production, GSH depletion, and cadmium ions (Cd^{2+}) release are possible mechanisms for CdS QDs' cytotoxicity. We also suggested that with QD concentration increasing, the principal toxicity mechanism changes from intracellular oxidative stress to Cd^{2+} release.

1. Introduction

Quantum dots (QD) are semiconductor nanocrystals (~2–100 nm) composed of groups II–VI or III–V elements (Hardman, 2006). Owing to the unique optical and electrical properties (Bruchez et al., 1998; Chan et al., 2002; Chan and Nie, 1998; Dabbousi et al., 1997; Han et al., 2001), QD shows great promise in biomedical imaging and electronics industries (Erogbogbo et al., 2008; Medintz et al., 2005; Michalet et al., 2005; Qi and Gao, 2008; Susumu et al., 2007; Weng et al., 2008). However, a number of significant barriers prevent widespread use of the technology (Jamieson et al., 2007). Amongst them, the toxicity of QDs is an urgent concern. Their use in human applications will be under question until enough data of their toxicity is available.

In past years, many studies are specifically designed for assessing the toxicity of QDs, but due to the lack of standardized QD synthesis protocols, coatings, solubilization ligands, dose, and cell systems, it is hard to obtain a straight answer (Maysinger et al., 2007). However, from this diversity, an inference can be safely made that the protection of QD surface play a key role in their toxicity (Maysinger et al., 2007). If the QD surface is well protected by polymers or proteins, it exerts little harm on cells or mammals (Derfus et al., 2004; Dubertret et al., 2002; Hanaki et al., 2003; Jaiswal et al., 2003; Jiang et al., 2009; Larson et al., 2003; Voura et al., 2004). Otherwise, it manifests noticeable toxicity. Basically, at least four possible mechanisms were proposed to explain the toxicity of QDs: (i) release of toxic metals from the core of nanoparticles (Cho et al., 2007; Derfus et al., 2004; Kirchner et al., 2005a,b; Mancini et al., 2008), (ii) generation of reactive oxygen species (ROS) (Cho et al., 2007; Clarke et al., 2006; Green and Howman, 2005; Ipe et al., 2005; Lovric et al., 2005; Lu et al., 2008), (iii) nanoparticle aggregation on the cell surface (Kirchner et al., 2005a), and/or (iv) cytotoxicity of surface-covering molecules of QDs (Hoshino et al., 2004a).

It is noteworthy that even though the QD surface is well protected by multiple inorganic/organic coatings, the subcellular localization of QD, low pH environment, penetration of UV light through skin and/or oxidation induced by inflammatory responses could degrade the coatings *in vivo* and yield "naked" inorganic

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cores (Derfus et al., 2004; Lovric et al., 2005; Maysinger et al., 2007). Therefore, while the endeavor on modification of QD surface is indispensable to reduce its toxicity for wide use, it is also necessary to assess the toxicity of inorganic QD core, which indicates the potential hazard of QD under certain conditions or after long term use.

To investigate the cytotoxicity of QD core, we synthesized a kind of "naked" CdS QDs with biomimetic method. As for the cellular model, we selected Chinese Hamster Lung (CHL) Cells because it has been reported that the lung and spleen of mice had the most accumulation of CdSe/ZnS-SSA QDs after up to 7 days' treatment (Hoshino et al., 2004b). Moreover, QDs, as nanoparticles, may be inhaled by human and reach the deep lung, where it could interact with the alveolar epithelium (Oberdorster et al., 2005). Taking these factors into account, CHL cell can act as a good model to investigate the cytotoxicity of QDs.

Our observations in this study led us to conclude that ROS production, GSH depletion, and free Cd^{2+} release could induce cytotoxicity of CdS QDs in a CHL culture model. The rising QD concentration results in the elevation of Cd^{2+} concentration, which might replace the oxidative stress-induced toxicity and become the primary cytotoxicity pathway.

2. Materials and methods

2.1. Synthesis of unmodified cadmium sulfide QDS

All reagents were purchased from Sigma–Aldrich (Shanghai, China). Span 80 (5 ml) and tributyl phosphate (5 ml) were dissolved in kerosene (40 mL), followed by adding sodium sulfide (50 mL, 0.2 M aqueous solution). The mixture was stirred at room temperature for 10 min (3000 rpm) to obtain an emulsion liquid membrane (ELM) system. Then ELM (50 ml) was added to cadmium chloride (100 ml, 0.1 M aqueous solution) and the reaction mixture was stirred for 10 min (300 rpm). QDs were collected by centrifugation and washed 3 times with dimethylbenzene and absolute ethanol to remove organic impurities. The precipitation was kept in absolute ethanol.

2.2. Particle characterization

X-ray diffraction (XRD) was carried out on a Philips Pw1700 X-ray diffractometer. Transmission electron microscopy (TEM) images were taken on a Philips EM400ST transmission electron microscopy, with an accelerating voltage of 100 kV.

2.3. MTT assay

With RPMI 1640 medium (Gibco, America) containing 10% fetal bovine serum (Gibco, America), CHL cells (CTCCCAS, Shanghai, China) were seeded (37 °C, 5% CO_2) in 96-well plates (Cosmo Biosciences Inc., Hong Kong) at a density of 5×10^4 cells/ml. Cells were treated with CdS QD or microsized CdS solution (dissolved with dimethyl sulfoxide) and their final concentrations ranged from 2.5 μg/ml to 80 μg/ml. After the 24 h of incubation, the medium was removed and replaced with drug-free, serum-free medium (500 μL/well). MTT (Sigma–Aldrich, Shanghai, China) stock solution (5 mg/mL) was added to each well, and cells were then incubated for 4 h at 37 °C. Media were removed and cells were lysed with dimethyl sulfoxide (Sigma–Aldrich, Shanghai, China). Absorbance at 570 nm was measured by using a microplate reader (Multiskan MK3, Thermo Labsystems, Finland). All measurements were done in triplicate.

To investigate the effect of N-acetylcysteine (NAC), NAC was dissolved in PBS (400 mM), and was added to the culture medium 2 h ahead of CdS treatment (Lovric et al., 2005). All measurements were done in triplicate.

Before MTT solution was added to the 24 h exposure cells, their morphological changes were observed with Inverted Microscope (TE2000, Nikon, Japan).

2.4. Intracellular ROS measurement

ROS levels were determined by measuring the oxidative conversion of 2′,7′-dichlorofluorescin diacetate (DCFH-DA) to fluorescent compound dichlorofluorescin (DCF) (Jia et al., 2006; Lin et al., 2006). Briefly, cells in 96-well plates were incubated with control media or CdS QD/microsized CdS solution for 24 h. Thereafter, the medium was removed and replaced with serum-free medium. DCFH-DA (Beyotime Institute of Biotechnology, Jiangsu, China) stock solution (10 mM) was diluted 1000-fold in serum-free medium, and was added to each well of 96-well plates (final concentration 10 μM). Cells were incubated for 25 min and then DCF fluorescence was determined at 485 nm excitation and 520 nm emission using a fluorescence microplate reader (Safire2, Tecan, Switzerland). All measurements were done in triplicate.

2.5. Glutathione (GSH) measurement

The GSH Quantification Kit was purchased from Jiancheng Bioengineering Institute (Nanjing, China). The concentration of intracellular GSH was determined according to the manufacturer's instructions.

2.6. Cellular cadmium assay

Cells cultured (37 °C, 5% CO_2) in the cell culture flask (Cosmo Biosciences Inc., Hong Kong) were treated in the same manner as for MTT assay with CdS QD/microsized CdS solution. After 24 h of incubation, the cell supernatant was collected in 1.5 ml Eppendorf tube (Cosmo Biosciences Inc., Hong Kong) and centrifuged for 15 min (800 rpm). As for the determination of extracellular Cd^{2+} concentration, an aliquot of the supernatant (1 ml) was collected in a 1.5 ml Eppendorf tube. After dilution, the Cd^{2+} in the supernatant was measured using Atomic Absorption Spectrometers (AAS) (WFX-210, Rayleigh, Beijing). All measurements were done in triplicate.

As for the determination of intracellular Cd^{2+}, 1 ml of dimethyl sulfoxide was added to the cell culture flask and the cell suspension was transferred to the corresponding Eppendorf tube mentioned above. The precipitation at the bottom of the tube was lysed with dimethyl sulfoxide. After vortex and centrifugation, the Cd^{2+} in the solution was measured using AAS. All measurements were done in triplicate.

2.7. Statistics

Data were analyzed by using SPSS 13.0 for Windows XP. One-way ANOVA was used to determine the difference between groups. Differences were considered significant when $P < 0.005$.

3. Results

TEM image of the CdS QDs which are prepared in this study is shown in Fig. 1A. The measured particle size is about 5–7 nm. Their XRD pattern is shown in Fig. 1B. All the peaks in the pattern can be identified as the known cubic structure of CdS.

The absorbance of MTT-reduced compound formazan can be a measure of cell metabolic activity. A higher value indicates an averagely active and well cell state. Our findings indicate that cell

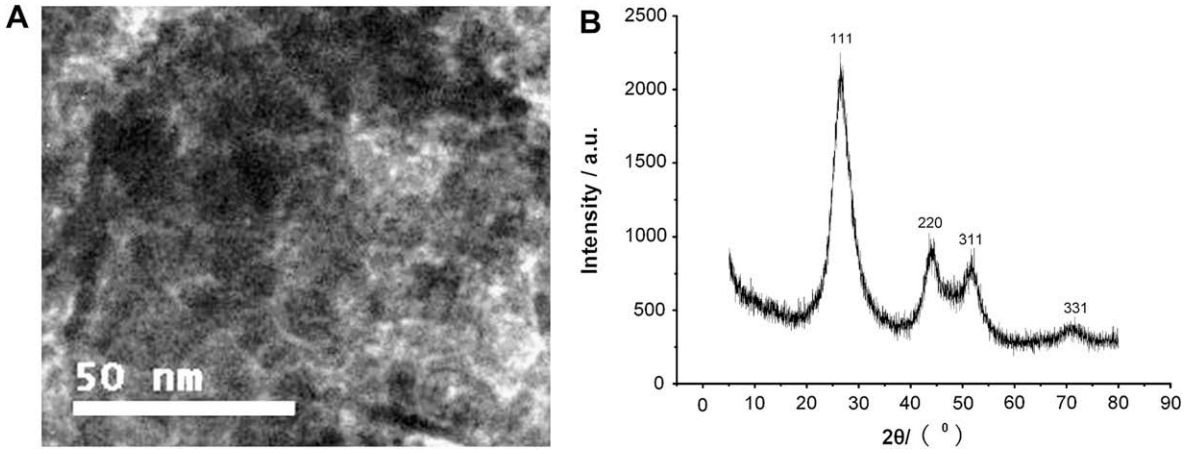

Fig. 1. Characterization of CdS QDs: (A) TEM image (B) XRD.

metabolic activity was slightly affected (84% of control cells) by CdS QDs at low QD concentration (2.5 μg/ml). When the QD concentration was up to 20 μg/ml, cell activity was severely damaged (19% of control cells). The damage seems to be stable regardless that the QD concentration continues to increase from 20 μg/ml. For the microsized CdS, its cytotoxicity manifested in a similar manner with CdS QDs except that its cytotoxic effect began to stabilize at a higher concentration (40 μg/ml). See Fig. 2A.

From images taken by inverted microscope (Fig. 2B), the conclusion derived from MTT assay can be approved. Cells treated with CdS QDs manifested shrinkage and deformation at a relatively low concentration (10 μg/ml). However, cells treated with the microsized CdS only showed abnormal shapes at high concentration (40 μg/ml).

The DCF fluorescence intensity is a good indicator of ROS level in cells. ROS generation in CHL cells following 24 h of exposure to QDs and microsized CdS at 10, 20, 40, 60, 80 μg/ml is shown in Fig. 3A. While microsized CdS treatment of cells did not show the rise of ROS level, CdS QD treatment resulted in an increase (0.2–0.3 fold) of ROS generation over control levels.

GSH is the most abundant thiol compound which plays many roles including protection against ROS and maintenance of protein thiol groups in animal tissues. As GSH is an important component of cellular antioxidant defense system (Halliwell and Cross, 1994; Hernandez et al., 1995), its depletion signals the intracellular oxidative stress. After 24 h treatment with QDs or microsized CdS (20 μg/ml), CHL cells manifested a significant decrease (50%) of intracellular GSH level (Fig. 3B). Interestingly, QDs and microsized CdS showed no difference in depleting GSH.

As a thiol compound which can act as a direct antioxidant and cysteine source for the repletion of intracellular glutathione, NAC is an effective scavenger of ROS and reduce oxidative stress in cells. With a concentration of 4 mM, NAC can mitigate QD-induced decrease of cell metabolic activity, especially when QD concentra-

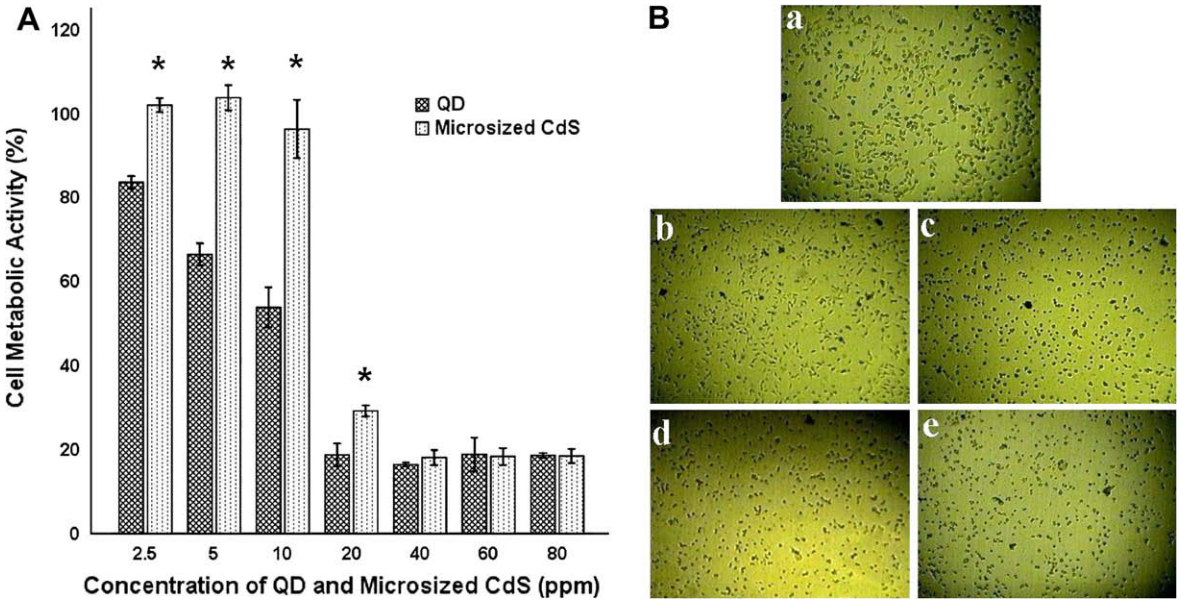

Fig. 2. (A) Effect of CdS QDs and microsized CdS particles on CHL cell metabolic activity. Cells were treated with different concentrations of CdS QDs or microsized CdS for 24 h. At the end of the incubation period, cell metabolic activity was determined by MTT assay. Control cells cultured in particle-free media were run in parallel to treatment groups. Their metabolic activity was set as 100% and then that of treatment groups was calculated by OD values comparison. * $p < 0.005$ as determined with one-way ANOVA. (B) Morphological changes of CHL cells. Cells were treated with different concentrations of CdS QDs and microsized CdS, and were incubated for 24 h at 37 °C in a 5% CO_2 atmosphere. At the end of 24 h exposure, cells were visualized under inverted microscope (Original magnification, ×200). (a) Control; (b) 10 μg/ml of microsized CdS; (c) 10 μg/ml of QDs; (d) 40 μg/ml of microsized CdS; (e) 40 μg/ml of QDs.

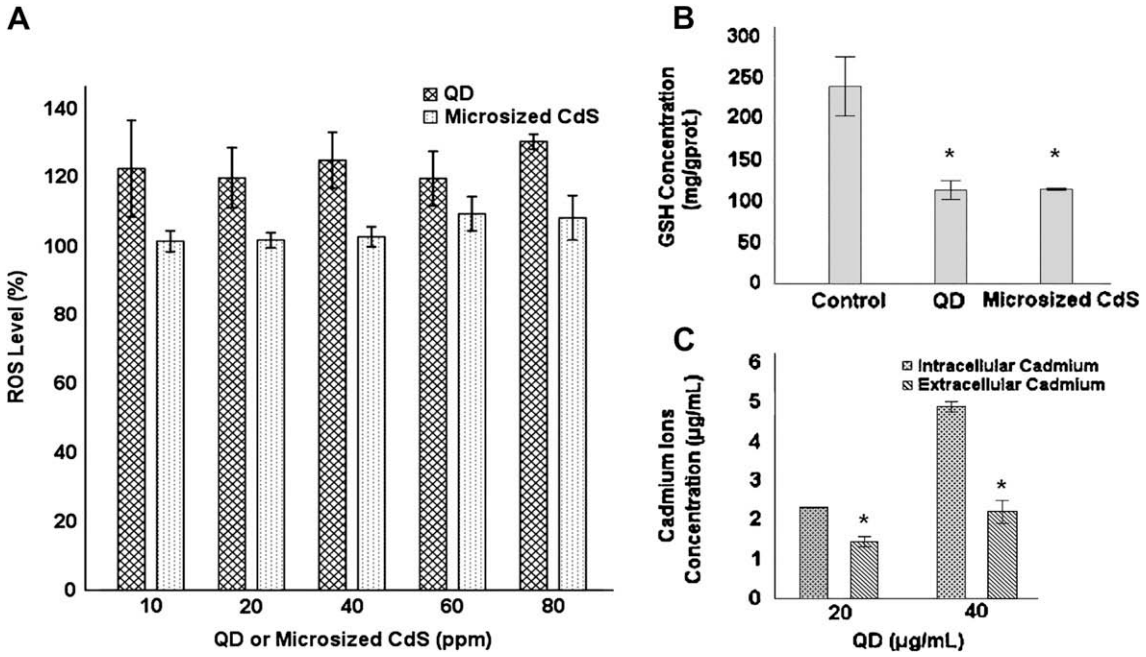

Fig. 3. (A) QDs induce generation of intracellular ROS. CHL cells were treated with different concentrations of QDs or microsized CdS for 24 h. Subsequently, intracellular ROS level was determined by DCFH-DA method. ROS level of control cells were set as 100%. (B) Effect of QDs and microsized CdS on GSH levels in CHL cells. Cells were treated with 20 μg/ml of QDs or microsized CdS for 24 h. At the end of the exposure, GSH levels were measured. Control cells cultured in particle-free media were run in parallel to treatment groups. $^{*}p < 0.005$ as determined with one-way ANOVA followed by a post-hoc Dunnett's test. (C) Intracellular and extracellular Cd^{2+} concentrations. CHL cells were treated with 20 μg/ml and 40 μg/ml of QDs for 24 h. Thereafter the concentration of liberated Cd^{2+} within cells and in the media was determined by AAS technique as described in the MATERIALS AND METHODS section. $^{*}p < 0.005$ as determined with one-way ANOVA.

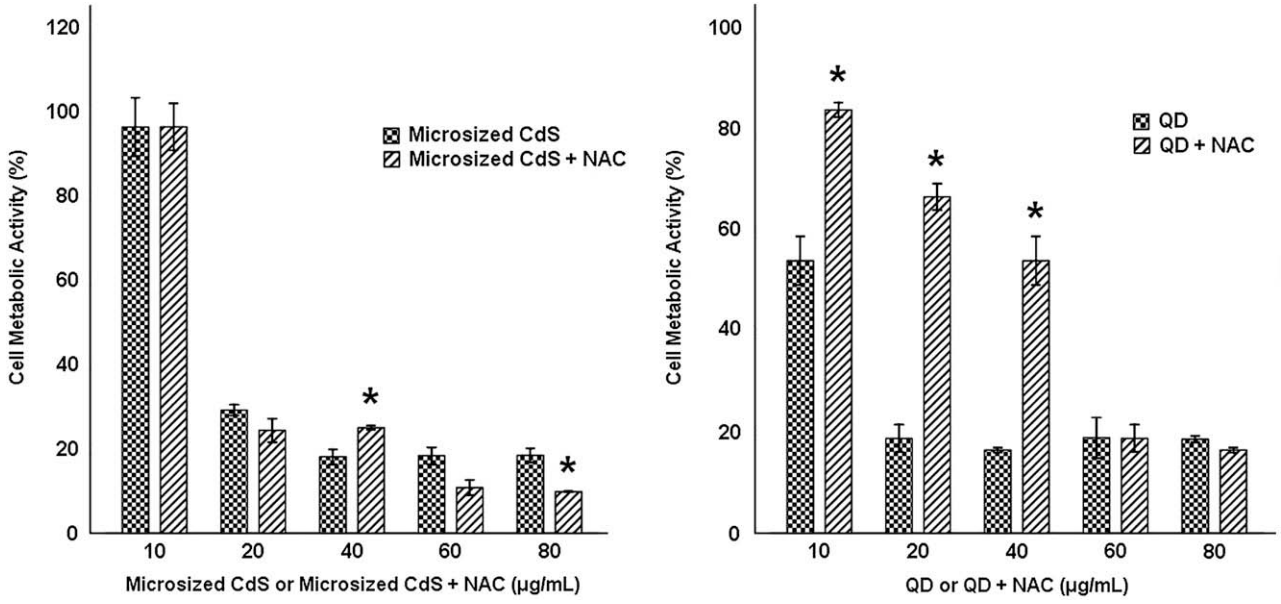

Fig. 4. Metabolic activity of CHL cells treated with different concentrations of QDs or microsized CdS in the presence and absence of NAC (4 mM). Control cells cultured in particle-free media were run in parallel and their metabolic activity was set as 100%. $^{*}p < 0.005$ as determined with one-way ANOVA.

tions were low (<40 μg/ml). But for the cells treated with microsized CdS, NAC did not inhibit their damage (Fig. 4). Same concentration of NAC, reported by Lovric et al. (2005), prevented CdTe QD-induced cellular damage.

By using AAS, the concentration of intracellular and extracellular Cd^{2+} can be measured and the results are presented in Fig. 3C. Intracellular Cd^{2+} of QDs at 20 and 40 μg/ml were 2.3 μg/ml and 4.9 μg/ml respectively, which were higher than the extracellular concentration (1.6 fold and 2.2 fold, respectively). By dividing the sum amount of intra- and extra-cellular Cd by initial Cd content in CdS QDs, we estimated that nearly 20% of cadmium was released from CdS QDs.

4. Discussion

Results in our study suggested that: (i) CdS QD core is toxic to CHL cells and this cytotoxicity becomes significant when its concentration rises, (ii) ROS production, GSH depletion, and Cd^{2+} re-

lease could jointly induce the cytotoxicity of CdS QDs, (iii) while at low concentrations ROS chiefly mediates the QD cytotoxicity, free Cd^{2+} plays a primary role in QD toxicity at high concentrations, and (iv) NAC can partially protect CHL cells from QD damage.

In this paper, we have proposed the biomimetic method to synthesize "naked" CdS QD core. XRD and TEM techniques were used to analyze the particle shape and size (spherical, ~6 nm). Without any protection on its surface, this QD can act as a model of degraded surface-modified QDs. By studying its toxicity and behavior in cells, we could figure out the hazard of long term use of QDs. Admittedly, well protection of QD core with coatings and/or ligands can increase the stability of QDs and slow their oxidative process in cells (Derfus et al., 2004), which reduces their toxicity; however, when subcellular localization of QDs or their local environment changes, or after they are retained in cells or body for a long term, the degradation of coatings can occur and expose their inorganic core (Akerman et al., 2002; Derfus et al., 2004; Gao et al., 2004; Lovric et al., 2005). Therefore, our study can represent the potential hazard of QDs under certain conditions or in the long run use.

Mitochondria is a significant organelle in QD-induced toxicity (Cho et al., 2007; Lovric et al., 2005). Chan et al. (2006) demonstrated that CdSe-core QD treatment induced mitochondrial-dependent apoptotic process *in vitro*. Mitochondrial function is severely impaired by CdS QDs beginning at a low concentration (20 μg/ml) and this toxicity does not show a dose-dependant increase when the concentration is above 20 μg/ml. Compared with microsized CdS, CdS QDs showed higher toxicity when they were at low concentrations (<20 μg/ml) but similar toxicity when their concentrations were high (>40 μg/ml). That indicates the CdS QDs should have a lower EC_{50} value and thus is more toxic than microsized CdS. Because they have the same chemical compositions, their toxicity difference should come from the size effect. The greater surface area per mass of nanosized particles compared with larger particles of the same chemistry could increase particle surface-dependent reaction including generation of ROS (Choi et al., 2007; Oberdorster et al., 2005; Yacobi et al., 2007).

CdS QDs may raise the ROS level through a couple of pathways: (i) upon excitation, QDs can form electron-hole pairs to transfer electron to oxygen (Cho et al., 2007), (ii) intracellular antioxidant system may be directly damaged via interaction with QDs, and/or (iii) Cd^{2+} released from QDs causes intracellular ROS elevation (Dailianis et al., 2005; Yang et al., 2007). The overloaded ROS in cells can damage membrane lipids, proteins, and DNA (Finkel and Holbrook, 2000). It has been reported that QDs produced ROS in the absence or presence of light (Green and Howman, 2005; Lovric et al., 2005). With a fluorescence microplate reader, we measured the ROS production in cells which were individually treated with QDs or microsized CdS. While the microsized CdS did not increase the ROS level, the CdS QDs promoted the intracellular ROS level by 20–30% over control cells. This difference implies that ROS production of QDs should be at least partially attributed to pathway (i) and (ii) mentioned above.

Besides ROS stimulation, intracellular GSH depletion has been reported important to induce cellular oxidative stress, which is considered to be one main mechanism of cytotoxicity (Osseni et al., 1999). GSH is widely distributed in many animal cells and is involved in ROS scavenge, protecting against toxins, protein and DNA synthesis, maintenance of membrane integrity and regulation of enzyme activities (Shekar et al., 2006). In the GSH measurement results, we have demonstrated that GSH depletion in CHL cells signaled the impairment of cellular antioxidant system by CdS QDs. Besides, GSH level could be reduced by cadmium and this depletion may be mainly due to its chelation of cadmium (Ohta et al., 1997; Shekar et al., 2006; Singhal et al., 1987; Vido et al., 2001). This is probably an important mechanism considering that microsized CdS depleted GSH to a similar extent as QDs did.

Cadmium can perturb lipid composition and enhance lipid peroxidation (Gill et al., 1989). Some antioxidant enzymes, specifically glutathione peroxidase and superoxide dismutase may be depleted by cadmium effect (Jamall and Smith, 1985). The intracellular Ca^{2+} concentration could also be promoted by cadmium (Yamagami et al., 1998). It has been reported that QDs can release free cadmium which results in cell damage (Cho et al., 2007; Derfus et al., 2004). Derfus et al. (2004) theorized that O_2 molecules oxidize chalcogenide atoms on QD surface to form oxides and the oxides desorb leaving dangling Cd atoms behind, which can easily desorb from the QD core. In our study, nearly 20% Cd atoms were released from CdS QD core. This value was significantly higher than that released from surface-modified CdSe or CdTe QDs especially the CdSe/ZnS (Cho et al., 2007; Derfus et al., 2004). It seems that ligands on QD surface are effective in preventing surface corrosion and cadmium release, and the ZnS shell is especially effective. The intracellular Cd^{2+} concentration (63 μmol/L and 34 μmol/L, respectively) is almost two-fold of extracellular Cd^{2+} concentration and is within the range of Cd^{2+} concentration which leads to significant cell death (>30 μmol/L) (Takagi et al., 2002).

As we have demonstrated with MTT assay, CdS QDs and microsized CdS showed similar toxicity when their concentrations were high (>40 μg/ml), which suggests that the chemical composition plays a leading role in their cytotoxicity at high concentration. We propose that the primary toxicity pathway of QD changes from oxidative stress to free Cd^{2+} release as its concentration ascends. This could be approved by the result of MTT assay with NAC in our study, NAC was a well-known effective antioxidant and precursor of GSH (Gillissen and Nowak, 1998; Henry et al., 1997). It could protect cell metabolic activity from QD toxicity in several aspects: directly scavenge the ROS, promote the synthesis of GSH, improve QD surface passivation, and/or possibly inhibit the Cd^{2+} damage (Lovric et al., 2005). Our study showed that NAC could not protect cells treated with microsized CdS. However, for cells treated with CdS QDs, NAC could improve their metabolic activity when the QD concentration was not high. Since NAC-induced protection against Cd damage may be mainly due to the lowered uptake of Cd^{2+} into cells (Wispriyono et al., 1998), and the QD cytotoxicity most likely arises from the intracellular QD level (Chang et al., 2006), we speculated that the release of Cd^{2+} from cell-uptaken QD resulted in the ineffectiveness of NAC to inhibit cytotoxicity of high-concentration-QD. The protection of NAC to low-concentration-QD treated cells reflected that NAC reduced oxidative stress in cells. Its ability to inhibit low-concentration-QD toxicity and its uselessness to the high-concentration-QD toxicity are evidence of our suggestion that as QD concentration rises, its primary toxicity pathway changes from oxidative stress to free Cd^{2+} release.

In summary, we have shown that surface-unmodified CdS QD are indeed cytotoxic and its cytotoxicity is higher than microsized CdS. The ROS production, GSH depletion, and free cadmium release are probably its toxicity mechanisms. From low concentration to high concentration, the leading pathway which mediated the cytotoxicity of QDs seems to be different.

When we review previous studies of QD cytotoxicity, we may feel somewhat confused with the diversity of its toxicity mechanism interpretation. For example, while Derfus et al. (2004) demonstrated that the cytotoxicity of QDs correlated well with the release of cadmium ions, Lovric et al. (2005) reported that ROS played the leading role in QD-induced cellular damage. Although diverse QD, coatings, cell systems all influenced the cytotoxicity results, the different QD concentration that the two studies used might explain the different toxicity mechanism that they observed. While in the former study, the QD concentration was very high (62.5 μg/ml, 250 μg/ml, and 1000 μg/ml), the concentration was significantly lower in the latter study (1 μg/ml, 5 μg/ml, and

10 μg/ml). Their individual interpretation of QD toxicity mechanism accords well with our proposal in this study.

In order to reduce QD-induced toxicity and eliminate this barrier for its extensive use in biological labeling or imaging, researchers have attempted to modify QD surface with ligands or shells. Indeed, surface modification is an effective way to inhibit deleterious effects of QDs. However, even those modified QDs may degrade over time *in vivo*. The inorganic QD core, like that we have investigated in this study, can be harmful. Much effort is still required to avoid the decay of QD *in vivo*. Notably, we may develop the non-toxic QDs based on the dose injected in the body. At a relatively high dose or long term use of QD, the cadmium release should be the central concern and researchers need to put forward preventive measures of Cd^{2+} release.

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