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Quantum dots induce heat shock-related cytotoxicity at intracellular environment

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Abstract Quantum dots (QDs) are semiconductor nanocrystals with unique optical properties. Different proteins or polymers are commonly bound to their surfaces to improve biocompatibility. However, such surface modifications may not provide sufficient protection from cytotoxicity due to photodegradation and oxidative degradation. In this study, the cytotoxic effects of QDs, CdTe, and CdSe/ZnS were investigated using cadmium-resistant cells. CdTe QDs significantly reduced cell viability, whereas, CdSe/ZnS treatment did not markedly decrease the cell number. CdTe QDs were cytotoxic in cadmium-resistant cells suggesting that internalized QDs degraded and cadmium ions contributed to the cytotoxic effects. CdTe QDs were consistently more cytotoxic than CdSe/ZnS QDs, but both QDs as well as cadmium ions activated heat shock protein 70B′ promoter. QDs themselves are likely to contribute to HSP70B′ promoter activation in cadmium-resistant cells, because CdSe/ZnS QDs do not release sufficient cadmium to activate this promoter.

Keywords Quantum dots (QDs) · Cytotoxicity · Cadmium · Heat shock protein (HSP) · Nanomaterial

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Introduction

Quantum dots (QDs) are semiconductor nanocrystals a few nanometers in diameter that are used extensively for in vitro observations of cellular mechanisms and for in vivo studies aimed at understanding the biodistribution of nanoparticles upon systemic injection (Bruchez et al. 1998; Chan et al. 2002; Alivisatos 2004). With the emergence of QDs detectable with light in the near IR window, clinical applications can be foreseen under certain situations (Pansare et al. 2012; Peng et al. 2012; Qian et al. 2012). The advantages of QDs over organic fluorescent dyes lie primarily in their high quantum yield, exceptional photostability, coupled with multicolor capability and size-tunable emission color. However, the most useful QDs contain toxic elements, mostly cadmium. Although QDs injection in animals does not trigger nefarious events over a limited timeline (up to a few mo) (Rak-Raszewska et al. 2012), concerns have risen about the long-term cytotoxicity of QDs as a consequence of slow degradation of the shell built around the CdSe core upon exposure to the biological milieu (Derfus et al. 2004; Michalet et al. 2005; Cho et al. 2007; Winnik and Maysinger 2013).

From the numerous studies of QD cytotoxicity, it has emerged that one of the possible mechanism responsible for the QDs toxicity involves the formation of reactive oxygen species (ROS) through cadmium ions released within cells by degradation of internalized QDs core. Photogeneration of free

radicals upon irradiation of QDs has also been invoked as a potential origin of QD-induced oxidative stress. (Lovric et al. 2005a, 2005b). Thus, Cho et al. found that QDs' cytotoxicity may not be attributed solely to the toxic effect of released cadmium ion, and concluded oxidative stress is generated both by free cadmium and by photooxidation processes to QDs in a polar aerobic environment (Cho et al. 2007). Moreover, Hoshino et al. gave strong evidence that QD toxicity is related to the chemical structure of the ligand bound to the QDs surface molecules (Hoshino et al. 2004).

Heat shock protein (HSP)70 is a suitable gene for the construction of sensor cells. Kato et al. described that HSP expression is useful for the investigation of biological responses to biomaterials (Kato et al. 1997, 1998, 2001). We established a stress-response element in the heat shock protein 70B′ (HSP70B′) gene and, using this functional element with reporter genes, detected cadmium chloride and a wide range of cytotoxic stimuli (Wada et al. 2005, 2007; Okuda-Shimazaki et al. 2007).

In this study, we used cadmium-resistant cells to investigate (1) if QDs in the absence of measurable amounts of free cadmium ions reduce cell viability and (2) to reveal the mechanism involved in QD-induced cell death. The cells had down-regulated Zrt/Irt-related protein (ZIP)8 transporter, which is associated with cadmium ion uptake (Yanagiya et al. 1999; Fujishiro et al. 2009). In these cells, the transport of cadmium ion through the cell membrane is prevented. Hence, a cytotoxic response of cadmium-resistant cells, if detected, must be induced by cadmium ions released by QDs after cellular uptake. The viability of cadmium-resistant cells treated with CdTe or CdSe/ZnS QDs was assessed by water-soluble tetrazolium salts-1 (WST-1) assay for cell counting. The activation of heat shock protein 70B′ promoter by the two types of QDs was achieved by employing the luciferase reporter gene assay. Our results strongly support the hypothesis that, even in the absence of internalized cadmium ions, QDs lead to cell death in naive and in cadmium-resistant cells by activation of the HSP70B′ promoter, a well-established marker for the heat shock-induced cellular impairments.

Materials and Methods

Materials. Cysteamine-coated CdTe and CdSe/ZnS QDs were synthesized, purified, and characterized as described previously (Al-Hajaj et al. 2011). Their physicochemical properties are presented in Table 1. The emission wavelength and the particle diameter were 564 and 3.17 nm for CdTe and 614 and 4.72 nm for CdSe/ZnS. Both QD samples had ζ-potential of ~+40 mV.

Cell culture. Metallothionein-null cadmium-resistant cells were established by a previous paper (Yanagiya et al. 1999).

Table 1 Physicochemical property of QDs.

Quantum dots	λ_{em} (nm)	Diameter (nm)	ζ-potential (mV)
CdTe-Cys	564	3.17	+41.9
CdSe/ZnS-Cys	614	4.72	+41.9

Cadmium-resistant cells, which were from metallothionein KO-mouse cells, were selected by $CdCl_2$ resistant. Cadmium-resistant cells were down-regulated ZIP8 transporter. NIH/3T3 cells, cadmium-resistant cells, and parental cells were cultured in Dulbecco's modified Eagle's medium containing 4,500 mg/L glucose (Sigma-Aldrich, St. Louis, MO) and supplemented with 10% fetal bovine serum, penicillin, and streptomycin. Cultured cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2, and the media were changed every 2–3 d.

Cell viability test. Cells were seeded at 1×10^4 cells per well into the 96-well plate. After incubation, cells were treated with either a solution of $CdCl_2$ or QDs solution for 24 h. Cell-Counting Kit solution (Doujin Chemical Co., Kumamoto, Japan) was added to each well, and then were incubated for 2 h at 37°C. The principle of this kit is measurement of water-soluble formazan generated from reduction of WST by mitochondrial activity. The absorbance at 450 nm was measured in xMark microplate reader (Bio-Rad Laboratories, Hercules, CA).

Luciferase reporter assay. Luciferase reporter plasmid, pHSP-Luc which contained HSP70B′ promoter −287 to + 118-bp region (Wada et al. 2007; Migita et al. 2010), was used to analyze the ability of either $CdCl_2$ or QDs sample to induce the cytotoxic response within cells. Cadmium-resistant cells and parental cells were co-transfected with 250 ng pHSP-Luc, 25 ng of pRL-CMV (renilla luciferase control plasmid; Promega Corp., Madison, WI) using Lipofectamine LTX (Invitrogen, Carlsbad, CA). At 12 h after transfection, cells were exposed to $CdCl_2$ or QDs solution, and were incubated for 12 h. Then, cells were lysed in 1×lysis buffer (Promega) and luciferase, and renilla light units were measured in Lumat LB9507 (Berthold Technologies, Bad Wildbad, Germany) luminometer according to the manufacturer's protocol for the Dual Luciferase assay (Promega).

Confocal microscopic observation. Confocal laser scanning microscopy was carried out using a Zeiss LSM510 microscope (Carl Zeiss, Inc., Oberkochen, Germany). One day before treatment of QDs, cadmium-resistant cells and parental cells were cultured on the cover slips (13-mm diameter; Matsunami Glass Ind., Ltd., Osaka, Japan). The concentration of 10 μg/mL CdTe QDs was added to the cells, and they incubated for 6 h. After incubation, the cells were

washed with PBS, and fixed with 4% paraformaldehyde for 5 min, and then they were stained lysosome, mitochondria, and nuclei by 50-nM LysoTracker Red DND-99 (Invitrogen), 500-nM MitoTracker Deep Red 633 (Invitrogen), and 1 μg/mL Hoechst33342 (Doujin Chemical) for 30 min at CO_2 incubator, respectively. Figures were created using NIH Image J software.

Statistical analysis. Three samples ($n=3$) were used for cell viability test and luciferase reporter assay. One-way ANOVA was used to examine the differences among different groups.

Results

First, we evaluated the cytotoxicity of the CdTe and the CdSe/ZnS conjugated with cysteamine by the WST-1 assay. In preliminary experimentation, the peak value of cell damage was 18 to 24 h after QDs exposure. The time course of cell damages by QDs was similar to cadmium chloride. Therefore, we checked the cell viability in 24 h after QDs exposure. Treatment of NIH/3T3 cells with either CdTe or CdSe/ZnS QDs led to a concentration-dependent reduction in cell viability after 24 h of exposure (Fig. 1). The viability of cells treated with CdSe/ZnS exceeded 80% at concentrations below 10 μg/mL, but the viability of cells treated with CdTe QDs drastically decreased upon treatment with QDs of concentration as low as 0.5 μg/mL.

Figure 2 shows the viability of cadmium-resistant cells and parental cells treated either with QDs or a $CdCl_2$ solution. Treatment of cells with cadmium chloride (0.5 and 1 μM) for 24 h, significantly reduced the viability of parental cells, but it had no cytotoxic effect on cadmium-resistant cells. Treatment of parental or cadmium-resistant cells with CdSe/ZnS QDs (10 μg/mL, 24 h) hardly affected their viability. In contrast, the viability of cadmium-resistant and parental cells treated with CdTe QDs (10 μg/mL, 24 h) decreased remarkably and to a similar extent.

To unravel the mechanism underlying the toxicity of CdTe QDs towards cadmium-resistant cells, it was necessary to determine the cellular localization of QDs in cadmium-resistant and parental cells. The cells exposed CdTe QDs was morphological change in 12 h. Then we observed cellular localization of QDs at 6 h. Imaging of cells by confocal laser microscopy was performed on cells subjected to a 6-h exposure to QDs. QDs were predominantly localized in lysosomes for both cadmium-resistant and parental cells (Fig. 3*a* and *d*). Furthermore, small amounts of QDs could be detected in mitochondria and in the nucleus (Fig. 3).

To estimate the effect on cellular stress, we determined HSP70B′ gene promoter activation in cells exposed to QDs using a reporter assay system. In preliminary experimentation,

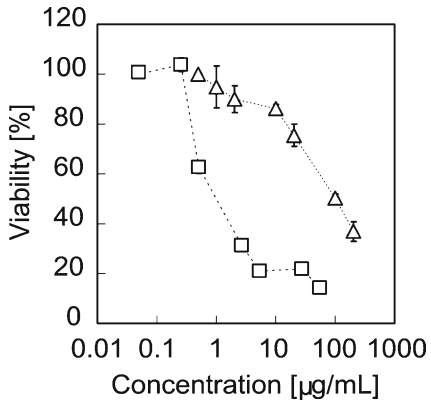

Figure 1 Viability of NIH/3T3 cells treated with cysteamine conjugated CdTe (*squares*) and CdSe/ZnS (*circles*). The cells were exposed to anionic QDs samples for 24 h in serum-free media. Values represent mean ± SD ($n=3$).

the peak value of HSP70B′ gene promoter activation was 12 h after QDs exposure. Therefore, we checked the HSP70B′ gene promoter activation in 12 h after QDs exposure. Figure 4 shows the activity of engineered HSP70B′ gene promoter of cadmium-resistant cells treated either with QDs or with cadmium chloride. There was no effect on the HSP70B′ gene expression when the cells were treated with cadmium chloride. Treatment of cells with 10 μg/mL CdTe for 12-h induced luciferase activity (expressed as relative luminescent units) in cadmium-resistant cells; however, although CdSe/ZnS QDs did not release cadmium ions, they slightly enhanced HSP promoter activity.

Discussion

The viability of cadmium-resistant cells treated with CdTe or CdSe/ZnS QDs was assessed by a WST-1 assay for cell counting. The activation of heat shock protein 70B′ promoter by the two types of QDs was achieved by employing the luciferase reporter gene assay. Our results strongly support the hypothesis that, even in the absence of internalized

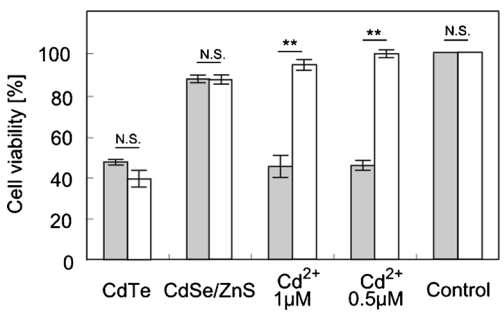

Figure 2 Viability of cadmium-resistant cells (*open bar*) and its parental cells (*closed bar*) treated with QDs samples (10 μg/mL), with $CdCl_2$ aqueous solutions and control (without chemicals) for 24 h. Values represent mean ± SD ($n=3$).

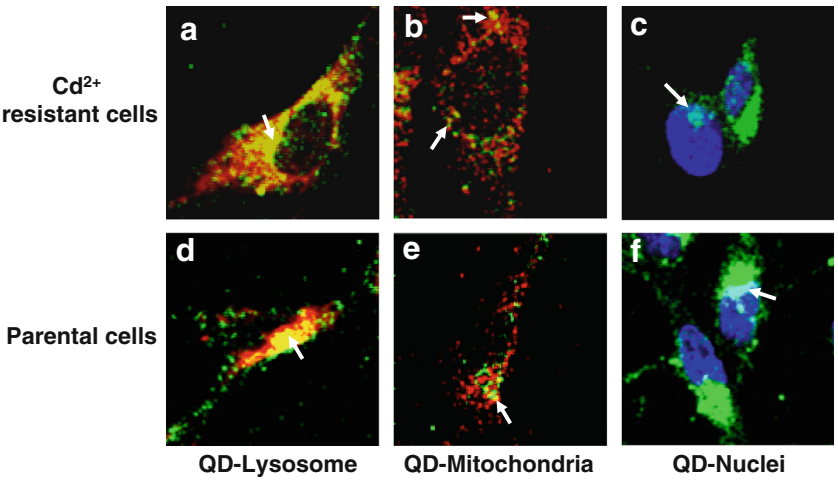

Figure 3 Confocal fluorescent microscopic image of the Cd^{2+}-resistant cells (*a–c*) and parental cells (*d–f*) treated with CdTe QDs (10 μg/mL) for 6 h. *a* and *d* show colocalization (*yellow*) of QDs (*green*) and lysosome (*red*); *b* and *e* show colocalization (*yellow*) of QDs (*green*) and mitochondria (*red*); *c* and *f* show colocalization (*light blue*) of QDs (*green*) and nuclei (*blue*). The *arrows* point colocalized regions of QDs and each organelle.

cadmium ions, QDs lead to cell death in naive and in cadmium-resistant cells by activation of the HSP70B′ promoter, a well-established marker for the heat shock-induced cellular impairments.

In vitro cytotoxicity of QDs. In the first stage of our investigation, we evaluated the cytotoxicity of CdTe and CdSe/ZnS QDs conjugated with cysteamine by the WST-1 assay. The CdTe QDs led to a highly significant reduction of NIH/3T3 cells viability. We hypothesized that the cytotoxic effect caused by CdTe QDs is largely due to the release of free cadmium upon degradation of their outer surface in the cell culture medium. In contrast, CdSe/ZnS, are much more stable and free cadmium is barely detectable in the extracellular medium or inside the cells.

The aim of this study was to provide new insights in the origin of QD cytotoxicity in the intracellular environment through the use of cadmium-resistant cells, an approach that has not been reported as yet, although it is highly relevant in this situation. In our experiments, the viability of both parental and cadmium-resistant decreased upon treatment with CdTe QDs (10 μg/mL, 24 h). On the other hand, both cell types

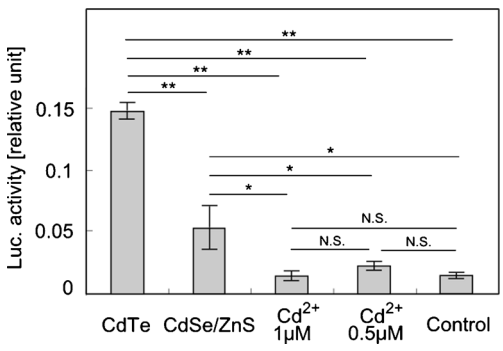

Figure 4 HSP promoter activity in cadmium-resistant cells treated with QDs samples (10 μg/mL), and $CdCl_2$ solutions for 12 h as measured by luciferase reporter gene assay. The luciferase activities are indicated as relative values and represent mean ± SD (n = 3).

demonstrated substantial resistance against CdSe/ZnS (10 μg/mL, 24 h), implying that these QDs were not contaminated with cadmium ion prior to use and, more importantly, that they did not release cadmium ions in the cellular environment, at least within the time frame of this measurement. Furthermore, QDs were most often found at lysosome, in agreement with previous findings by Cho et al. 2007. Hence, the QD-induced cytotoxicity is expected to be initiated within these organelles. Moreover, Cho et al. determined intracellular cadmium ion at the QDs exposure condition (Cho et al. 2007). For CdTe QDs (10 μg/mL, 24 h) exposure, the intracellular cadmium concentration is similar level to cadmium chloride (1 μM, 24 h) exposure. On the contrary, there was no cadmium ion for CdSe/ZnS QDs (10 μg/mL, 24 h) exposure. Our data strongly support these previous findings.

Evocation of heat shock stress by QDs. Normally, HSP70B′ is induced by cadmium ion (Wu et al. 1986; Feder and Hofmann 1999). We reasoned that if the QDs do not release cadmium within cellular organelles or the cytosol, HSP70B′ could not be activated. We observed that both CdTe and the CdSe/ZnS QDs induced the promoter activity. This result suggests the dependence of the CdTe Qds-induced cytotoxicity on the intracellular free cadmium ions possibly released from lysosome. However, CdSe/ZnS do not release the cadmium ion in intracellular environment, as described by Cho et al. 2007. It is known that the expression of HSP70B′ is induced by cadmium ions, but it is also regulated by other cytotoxic stimulations (Schlesinger 1990; Kiang and Tsokos 1998), such as protein denaturation (Welch 1992) or ROS (Ma et al. 1998; Gorman et al. 1999). Actually, there is no denying that ROS can be generated from the QDs surface (Winnik and Maysinger 2013). The generated ROS from the QDs surface possibly induces heat shock stress. Recent research reports point to the fact that nanoparticles, other than QDs, can affect protein conformation/folding (Shang et al. 2007), protein aggregation/fibrillation (Linse et al. 2007; Chen et al. 2012),

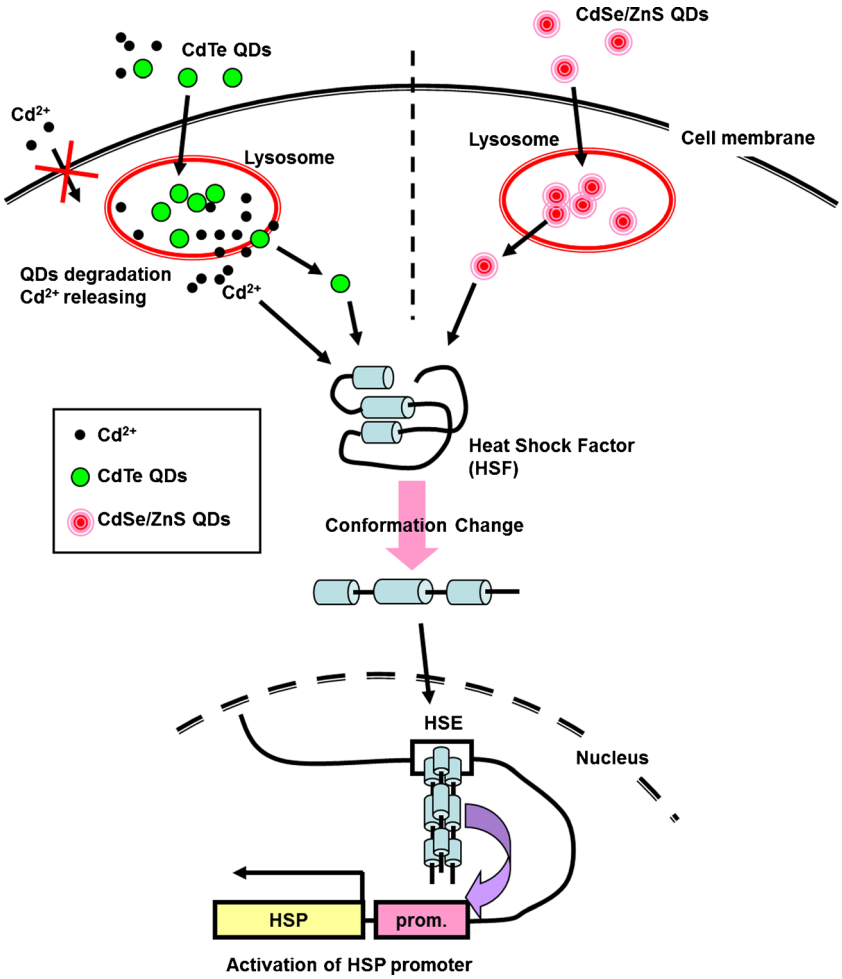

Figure 5 Schematic representation of mechanistic pathways for HSP70B′ expression by QDs. HSP70B′ expression is evoked following conformational change of heat shock factor (HSF) caused by both cell-incorporated QDs or/and Cd^{2+} released from QDs.

and production of ROS (Uchino et al. 2002; Li et al. 2003). Hence, our observations may be taken as an indication that CdSe/ZnS QDs, themselves, can trigger similar in vitro reactions as a consequence of their size and that the HSP activity observed is the outcome of this effect.

Mechanism for cytotoxic effect of QDs. Based on the results from these and previous studies, we propose the following tentative mechanism to account for the QD-triggered effect in cadmium-resistant cells (Fig. 5). Cadmium-resistant cells cannot uptake extracellular cadmium ion because the ZIP8 transporter is suppressed. However, the viability of cadmium-resistant cells decreased similarly to parental cells, when cells are treated with CdTe QDs. Therefore, it can be considered that the cytotoxicity towards cadmium-resistant cells is induced by CdTe QDs internalized by the cells. The incorporated CdTe QDs are localized within the lysosomes. They degrade under the oxidative cellular conditions and release cadmium ions. The released cadmium ions induce protein denaturation, and triggers HSP70B′ promoter activation. On the other hand, CdSe/ZnS QDs do not release cadmium ion. Nevertheless, they induce marked cell death following HSP70B′ expression, indicating that QDs may also induce protein denaturation or/and conformational changes. Hence the cytotoxic effect of QDs, related to the heat shock signal, is generated not only by released cadmium ions, but also QD nanocrystals themselves.

Conclusions

In this study, we investigated the reduction of cell viability by free cadmium ions of QDs and revealed the mechanism involved in QD-induced cytotoxicity using cadmium-resistant cells. CdTe QDs significantly reduced cell viability, whereas, CdSe/ZnD treatment decreased slightly. Cytotoxicity of CdTe QDs in cadmium-resistant cells supported the internalized QDs degradation and cadmium ions release. Cytotoxicity of CdTe QDs was markedly greater than CdSe/ZnS QDs, but both QDs activated HSP70B′ promoter. QDs themselves are likely to contribute to HSP70B′ promoter activation and induced cytotoxic stress in the cells, because CdSe/ZnS QDs do not release sufficient cadmium to activate this promoter.

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