Quantum dots enhance Cu\(^{2+}\)-induced hepatic L02 cells toxicity

Yuxia Zhao, Kuangfei Lin*, Wei Zhang, Lili Liu

State Environmental Protection Key Laboratory of Environmental Risk Assessment and Control on Chemical Process, Shanghai 200237, China.

E-mail: maryzys@163.com

Received 07 January 2010; revised 07 April 2010; accepted 16 April 2010

Abstract

As a new class of xenogenous nanoparticle, quantum dots (QDs) possess the potential to co-exist with Cu\(^{2+}\) in human liver. The combined toxicity is thus concerned. Considering QDs and Cu\(^{2+}\) are known ROS (reactive oxygen species) inducer, we investigated the combined oxidative stress and corresponding protective strategy using human hepatic L02 cells. The results demonstrated that the presence of a small amount of MPA-CdTe QDs (2 µg/mL) in a Cu\(^{2+}\) solution (2.5–20 µg/mL) resulted in a higher toxicity with up to 8-fold cell viability decrease, which was accompanied by cell morphology changes. The combined toxicity was then confirmed as ROS associated oxidative stress with up to 300% and 35% increase of the intracellular ROS level and glutathione S-transferase (GST) activity, respectively. N-acetylcysteine (NAC) can also provide almost complete protection against the induced toxicity. Therefore, the ROS associated oxidant injury might be responsible for the QDs-Cu\(^{2+}\)/Cu\(^{2+}\) induced toxicity and could be balanced through cytoprotective antioxidant enzyme GST.

Key words: quantum dots; hepatic L02 cell; reactive oxygen species; glutathione S-transferase

DOI: 10.1016/S1001-0742(09)60350-8

Introduction

Semiconductor quantum dots (QDs) have been extensively used in the last several years due to its unique physicochemical properties (Hardman, 2006). Structurally QDs consist of a metalloid crystalline core and a cap (Jamieson et al., 2007). The release of these core metal ions could induce reactive oxygen species ROS generation once QDs are exposed to some oxidative environment (Lewinski et al., 2008). Also, QDs could transfer energy to nearby oxygen molecules to induce the generation of ROS (Clapp et al., 2004). Copper can also induce oxidative stress on various organisms and tissues leading to oxidative damage of lipids, proteins, and nucleic acids (Jorge and Gonsebatt, 2009). Some reports assumed that the ROS generation induced by Cu\(^{2+}\) or Fe\(^{3+}\) was enhanced significantly with the addition of ultrafine carbon black (UfCB) (Wilson et al., 2002). The possible combined oxidant injury involvement of QDs and Cu\(^{2+}\) herein is therefore worth considering.

Many environmental pollutants engage signaling pathways that could be activated in response to oxidative stress (Jorge and Gonsebatt, 2009). To understand the pathways leading to the induction of antioxidant responses will enable development of strategies to protect against oxidative damage (Jorge and Gonsebatt, 2009). The glutathione S-transferase (GST) is a major group of detoxifying enzymes especially for environmental contaminants, and it is known to be regulated by the promoter regions of GST genes (Pi et al., 2008). Control of GST enzyme activity maybe a key to change the fate of the cell exposed to QDs and/or Cu\(^{2+}\), survival or death.

The primary human body exposure to QDs nanoparticles arise from the emerging biomedical applications including in vivo imaging, targeting, and diagnostics (Hardman, 2006). QDs have been proven to be systemically distributed in human body and primarily in liver (Yang et al., 2007). Liver is also a known target organ for Cu\(^{2+}\) accumulation (Luza and Speisky, 1996). The primary exposure pathway for copper is by ingestion. A hepatic copper concentration was also found beyond 6 mg/g dry weights in children (Bhave et al., 1982). Environmental concentration of copper has been found in excess of 1 mmol/L (Rössner, 1998). Moreover, QDs have been used for labeling HeLa cells and targeting Her2 epitopes on breast cancer cells at 100 and 4 µg/mL, respectively (Jaiswal et al., 2003; Wu et al., 2003). Therefore, the accumulation of QDs and Cu\(^{2+}\) at µg/mL level in liver adopted in this study is plausible and a human hepatic cell line (L02) as a cellular model was applied in this study.

The objective of this article was to investigate the effect of QDs on Cu\(^{2+}\) induced L02 cells toxicity and the possible toxicity mechanisms, focusing on the individual and combined L02 cell toxicity of QDs and Cu\(^{2+}\). The L02 cell related toxicity was confirmed through three aspects: cell viability decrease, the oxidative injury mechanism, and the extent of NAC protection. The rationale of the present
study was that up regulation of GST enzyme or ROS level, diminishing cellular toxicity as well as improving NAC protection together to resist the oxidative related toxicity.

1 Materials and methods

1.1 Chemicals

All reagents used in the present research including 6-carboxy-2',7'-dichlorodihydrdofluorescein (CDCFH), 3-(4,5-dime-thylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and anhydrous cupric chloride were obtained from Sigma Chemical Corporation (USA). Unless indicated otherwise, all other reagents and chemicals used were of the highest grade available. MPA-CdTe quantum dots were kindly provided by Prof. Xinhua Zhong from Chemical Engineering School of East China University of Science and Technology. Red mercaptopropionic acid coated CdTe quantum dots (2r = 3.7 nm) employed in this study were synthesized as described in a previous study (Zou et al., 2008). The molar concentration is 5.8 x 10^-6 mol/L and the mass concentration is 0.64 mg/mL. After synthesis, the QDs were collected by centrifugation and purified by propanol. The QDs were proven to be stable in pH range of 6 to 12 and no aggregation was observed in serum-free cell culture.

1.2 Cell culture conditions, QDs, and Cu^{2+} treatments

Human hepatic L02 cells were obtained from the Cell Bank of the Chinese Academy of Science. The cells were cultured in RPMI-1640 medium (GIBCO BRL, Grand Island, USA) and 10% fetal bovine serum (FBS) (GIBCO BRL, Grand Island, USA) at 37°C in a humidified atmosphere of 95% air and 5% CO2. RPMI 1640 medium was phenol red-free and contained 1% penicillin-streptomycin. For spectrofluorometric and colorimetric assays, the cells were cultured in 96-well plates (Sarstedt, Montreal, Canada) at an initial density 10^4 cells/cm²; for intracellular Cu^{2+} concentrations assay. A density 10^6 cells/cm² in 12-well plates was used. One hour prior to the treatments, the serum-contained medium was aspirated, and the cells were replaced with fresh serum-free medium.

Cells were first treated with QDs (2–160 µg/mL) and/or CuCl2 (2–320 µg/mL) for 24 hr to confirm the IC_{50} values of the QDs and Cu^{2+} using MTT assays. The IC_{50} value being the concentration that causes 50% cell mortality after 24 hr incubation. QDs (IC_{10} value) 2 µg/mL and Cu^{2+} (IC_{10}–IC_{40} value) 2.5, 5, 10, 20 µg/mL were selected for other experiments. The combined toxicity was measured by cell viability and cell morphology changes. The ROS levels, MMP decrease and the protection effect of NAC were employed to test the oxidative stress. GST enzyme activity was measured to determine the oxidative stress related protective strategy. QDs and CuCl2 solutions were diluted from stock solutions of 10 mg/mL QDs and 30 mg/mL CuCl2 with serum-free cell culture medium and sterilized ultrapurified water, respectively. The cells were incubated with chemicals for 24 hr before biochemical analysis.

NAC was dissolved in phosphate-buffered saline (PBS 400 mmol/L), and diluted to a final concentration of 10 mmol/L in serum-free medium. NAC was added to the culture medium 2 hr before the treatment, then replaced by fresh medium containing various concentrations of QDs-Cu^{2+} and Cu^{2+} and incubated for 24 hr, followed by other similar measurement of ROS, MMP, and MTT.

1.3 MTT assay

Colorimetric MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide, Sigma, USA) assays were performed to assess the metabolic activity of the cells treated as described above. After 24 hr treatment, the media were removed and replaced with drug-free and serum-free media (200 µL/well). L02 cells were then incubated for 4 hr at 37°C using 10 mmol/L MTT solution. The media were then removed, and the blue MTT formazan precipitate was dissolved in 100 µL DMSO and measured on a microplate reader model 550 (Bio-Rad, USA) at the absorbance wavelength of 570 nm and reference wavelength of 630 nm. All measurements were done in three independent experiments.

1.4 Intracellular ROS level assays

Assay of intracellular ROS level was carried out in various QDs-Cu^{2+}/Cu^{2+} treated samples as mentioned above. Cells were rinsed three times with PBS (pH 7.4) and further incubated with 10 µmol/L CDFCH solution for 30 min. Membrane-impermeable CDFCH was absorbed by cells and oxidized into highly fluorescent CDFCH by ROS. The supernatant containing CDFCH were aspirated, and 100 µL PBS (pH 7.4) was added to the well-plate wells. The fluorescence intensity measured by a fluorescence microplate reader of CytoFluor 2350 (Millipore, USA) at excitation wavelength of 485 nm and emission wavelength of 538 nm. The relative fluorescence over control was calculated as the measured ROS levels.

1.5 Mitochondrial membrane potential assays

Rhodamine 123 were dissolved in dimethyl sulfoxide (DMSO) and diluted to a final concentration of 1 µmol/L in serum-free medium. The uptake of the cationic fluorescent dye, Rhodamine 123, has been used for the estimation of mitochondrial membrane potential as mentioned by Andersson (1987). In this study we employed a revised method using Rhodamine 123. Briefly, cells were cultured at 96-wells plate for 24 hr then were separated from the incubation medium by centrifugation at 3000 r/min for 5 min. The supernatant were aspirated carefully and the deposited cells were washed twice with PBS buffers, then 1 µmol/L Rhodamine 123 was added and incubated at 37°C for 30 min. The amount of Rhodamine 123 remaining in the incubated L02 cells were measured fluorimetrically using a Shimadzu RF5000U fluorescence spectrophotometer (Japan) set at 490 nm excitation and 520 nm emission wavelengths. The capacity of mitochondria to take up the Rhodamine 123 dyes was calculated as the relative fluorescence incorporated with Rhodamine 123 over control.
1.6 GST enzyme activity

1-Chloro-2,4-dinitrobenzene (CDNB) was used as the substrate for GST. The GST activity was determined according to the method of Zhu et al. (2005), and was expressed as nmol of CDNB-GSH conjugate formed per min per mg of cellular protein (Zhu et al., 2005).

1.7 Statistical analysis

All results represent as means ± standard deviations from triplicate experiments performed in a parallel manner unless otherwise indicated. Significance was tested among and between groups using the one-way analysis of variance (ANOVA) followed by Dunnett’s posthoc test. Statistically significant data sets were indicated by a star “*” for p < 0.05. All the significances represent comparisons of QDs-Cu²⁺ co-exposed cells to individual Cu²⁺ treated groups.

2 Results

2.1 Effect of QDs on Cu²⁺-induced toxicity: cell viability decrease and cell morphology changes

IC₅₀ value of 2 µg/mL QDs and IC₁₀–IC₄₀ value of Cu²⁺ (2.5, 5, 10, 20 µg/mL) were selected in this study, the individual Cu²⁺ and QDs-Cu²⁺ induced cytotoxicity have been measured in our previous study (unpublished data) using MTT assay. The results showed that the addition of QDs improved Cu²⁺ induced L02 cell toxicity with up to 8-fold cell viability decrease (2 µg/mL of QDs just induced a 10% or so cell viability decrease over control) (Fig. 1). Both QDs-Cu²⁺ and Cu²⁺ induced toxicity showed dose-depend trend.

The morphology changes of L02 cells treated with QD₃-Cu²⁺/Cu²⁺ were observed using phase contrast microscopy through Hematoxylin-Eosin (HE) stains as shown in Fig. 2. Normal L02 cells were characteristic fibrous bearing intact nuclei. Also the adherence state was very good. The cells got a slight morphology changes after treated with 5 µg/mL Cu²⁺, while a significant decrease cell numbers and cytoplasmic shrinkage occurred with the addition of 2 µg/mL QDs, which is consistent with the MTT results. Much serious characteristic cytotoxicity could be observed for L02 cells treated with 20 µg/mL of Cu²⁺/QDs-Cu²⁺, both treated cells displayed condensed nuclear morphology with an obvious decrease of nuclear diameter.

NAC (10 µmol/L) was 2 hr early added to cells and then aspirated followed by the same concentrations of QDs-Cu²⁺/Cu²⁺ treatment. The morphology of L02 cells pretreated with NAC were obviously recovered, revealing the cytotoxicity induced by Cu²⁺/QD-Cu²⁺ were ROS associated oxidative damage.

2.2 QDs enhance Cu²⁺-induced intracellular ROS level

ROS associated oxidant stress is the common pathway for QDs and Cu²⁺ to induce cell toxicity. The ROS generation, MMP decrease as well as protection effect of NAC...
were measured to test whether the QDs-Cu²⁺/Cu²⁺ mediated toxicity occurred through ROS associated oxidant injury. ROS production was observed in culture medium using a fluorescence microplate reader after 24 hr of incubation. QDs-Cu²⁺ treated cells were characterized by a remarkable fluorescence compared to that of Cu²⁺, indicating the enhanced oxidative stress with addition of QDs as shown in Fig. 3a. The ROS production was enhanced by up to 3 folds with addition of QDs (2 μg/mL of QDs alone just induced 39% ROS increase over control). And both the QDs-Cu²⁺ and Cu²⁺ induced ROS generation were according to dose-depend manner which was consistent with MTT results, reflecting the ROS associated oxidative stress would responsible for L02 cells toxicity.

2.3 Effect of QDs on Cu²⁺-induced MMP decrease

A direct role for ROS in mitochondrial malfunction has been suggested (Shidoji et al., 1999). ROS production may be accompanied by the reduction of ΔΨ₂ (Foster and Torres, 2006). ROS can be generated in a wide variety of metabolic pathways that catalyzed by mitochondrial respiratory chain enzymes, cytochrome P-450 systems, nitric oxide synthase, NADPH oxidase and lipoygenase (Turrens, 1997; Liu et al., 2001; Forman et al., 2001; Droge, 2002). Among them Mitochondria appear to be the most sensitive organelles as the first to be affected (Shidoji et al., 1999). We thus investigated the MMP potential to confirm the ROS generation source. The ΔΨ₂ results showed slightly significant decrease with the addition of QDs. However, the extent of decreased ΔΨ₂ is not consistent with corresponding ROS results. Both the QDs-Cu²⁺ and Cu²⁺ induced ΔΨ₂ drop did not show dose-depend trend as the case of ROS (Fig. 3b), suggesting that the resource of ROS generation may be not only from mitochondria malfunction. ROS may be generated according to the combined pathways containing both mitochondria and some other metabolic pathways, which was not consistent with the results of Pourahmad and O’Brien (2000), who concluded that the Cu²⁺ and Cd²⁺ induced cytotoxicity as a result of mitochondrial toxicity.

2.4 NAC prevents QDs-Cu²⁺/Cu²⁺ induced cellular oxidative damage

N-Acetylcysteine, a strong antioxidant containing a mercapto groups, has been reported to attenuate QDs induced ROS generation. NAC was shown to maintain mitochondrial oxidative metabolism improving cell survival in response to various insults (Ratan et al., 1994). Also was able to abolish ROS-generation and preserve normal mitochondrial morphology after QDs treatment (Cotgreave, 1997). NAC was used in the present study as antioxidant agent protective against oxidant damage and assess the relations of cell damage with ROS production. These results can further confirm QD-Cu²⁺ and Cu²⁺ cytotoxicity evaluated by ROS and MMP.

Cells that pretreated with NAC showed very weak nuclear staining, as shown in Fig. 3. In addition to provide effective protection against ROS generation associated oxidative stress, NAC can also get mitochondrial membrane potential and cell morphology normalized, indicating the ROS associated oxidative stress herein is responsible for the QDs-Cu²⁺/Cu²⁺ induced toxicity. The results were consistent with the effective protection of NAC against QDs-Cu²⁺/Cu²⁺ induced cell viability decrease (data not shown). The mechanisms regarding the protective effect of NAC maybe ascribed to the following: (1) NAC can reduce the concentration of ROS in the cell culture medium; (2) NAC can induce the synthesis of glutathione, the most abundant and effective cellular antioxidant; (3) NAC could improve QDs surface passivation, thereby leading to less damage to the mitochondrial redox system (Cotgreave, 1997).

![Fig. 3](image-url)
2.5 Effect of QDs on Cu\(^{2+}\)-induced GST enzyme activity

GST induction is known to occur through xenobiotic response element (XRE) or ARE (Prince et al., 2009). The GST induction of L02 cells treated with QDs-Cu\(^{2+}\)/Cu\(^{2+}\) showed dose dependent manner. Addition of QDs improved Cu\(^{2+}\) induced GST activity by 13% to 35% (2 \(\mu\)g/mL QDs alone just induced a 10% increase of GST over control). The GST level induced by Cu\(^{2+}\) was elevated with the addition of QDs as expected, and was parallel to the decrease of the cell viability. These phenomena are consistent with the notion of QDs-Cu\(^{2+}\) enhancing GST enzyme via possible up-regulation of GST encoded genes to resist intracellular oxidative injury.

![Fig. 4](image)

Fig. 4 Effect of QDs on Cu\(^{2+}\) induced up-regulation of GST enzyme activity. GST enzyme activity in L02 cells treated with Cu\(^{2+}\)/QDs-Cu\(^{2+}\) for 24 hr. The results represent the mean ± SD of values obtained from three samples in each group. GST activity is expressed as % of control. Values with an asterisk differ significantly from individual Cu\(^{2+}\) treated group (*p < 0.05).

3 Discussion

ROS includes the superoxide (O\(_2^−\)), hydroxyl radicals and hydrogen peroxide (H\(_2\)O\(_2\)) (Oh et al., 2004). The ROS associated toxicity has been confirmed in many investigations (Prince et al., 2009). The natural, intracellular equilibrium between ROS formation and degradation may be disturbed by intracellular located nanoparticles (Limbach et al., 2007). Oxidative stress arises from a serious imbalance between the production of reactive oxidizing species and their degradation by the antioxidant defense system in organisms.

Our results show that MPA-CdTe QDs can improve Cu\(^{2+}\) mediated cytotoxic effect to human hepatic L02 cells. Addition of 2 \(\mu\)g/mL QDs enhance Cu\(^{2+}\) induced L02 cells viability decrease up to 8-fold. The corresponding cells reveal various degrees of morphology changes. The increased ROS levels (maximum by 3-fold) and the protection effect of NAC further confirm the toxicity mechanism of ROS associated oxidative damage. The enhanced GST enzyme activity seemed to provide effective ROS degradation strategies, implying ARE system mediated GST enzyme induction countering QDs and/or Cu\(^{2+}\) induced oxidative stress.

Toxic metals represent a major class of environmental sources of chemical toxin, and many metal elements are famous ROS inducer. It is known that GST induction can occur through the Nrf2/ARE system to defense against oxidative stress (Jorge and Gonsebatt, 2009; He et al., 2008; Prince et al., 2009). Moreover, Shinkaia et al. (2009) also reported that GST isozymes as a transporter has been proved to be responsible for facilitation of iAs(III) excretion extracellularly via catalyze GSH conjugation formation, reducing cellular accumulation of arsenic and thus inhibit iAs(III)-mediated cytotoxicity in hepatocytes. In this regard, the increased GST enzyme activity here may be a key adaptive response in cellular defense against QDs and/or Cu\(^{2+}\) induced oxidative damage. In addition to catalyze GSH conjugation of ROS, GST stimuli may be responsible for possible Cd\(^{2+}\) release from QDs and Cu\(^{2+}\) excretion extracellularly in L02 cells to reduce cell toxicity.

Some metal nanoparticles are more readily transported to cell membrane or into living cells by bypassing its barriers to “normal”-sized metal, and then releasing metal ions that damage cell machinery (Limbach et al., 2007; Balogh et al., 2001; Moore, 2006). The similar carrier role of QDs nanoparticles to take attached Cu\(^{2+}\) into cells was thus plausible due to the interaction of Cu\(^{2+}\) with similar MSA-CdTe QDs confirmed by the applications of QDs for trace Cu\(^{2+}\) analysis (Zhang et al., 2008). The QDs might have played the Trojan horse role by allowing more Cu\(^{2+}\) accumulate into living cells followed by the subsequent release of these metals leading to the higher ROS levels, and thus the enhanced toxicity.

In summary, the addition of QDs enhanced Cu\(^{2+}\)-induced oxidative damage significantly as evaluated by cell viability decrease, changes of cell morphology, ROS and GST enzyme activity as well as protection effect of NAC. In addition to counteract ROS, GST stimuli may also be responsible for possible Cd\(^{2+}\) and Cu\(^{2+}\) excretion. The results will provide potential protection method to counteract combined toxicity involvement of the new typical xenobiotic QDs nanoparticles. A clear understanding in this area is expected to lead to significant developments in cancer chemoprevention and inflammatory lung disease. Considering the increasing environmental release and the potential co-existence of QDs nanoparticles with some other accumulated pollutants, the combined toxicity involvement of QDs that possible occurred in realistic natural environment and human exposure is worth serious considering.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (No. 40871223, 40901148, 20677015), the National High-Tech Research and Development Program (863) of China (No. 2007AA06Z331), the
Fundamental Research Funds for the Central Universities (No. WB0911011, WB0914041), the National Environmental Protection Public Welfare Science and Technology Research Program of China (No. 200909089), the Shanghai Educational Development Foundation “Chenguang Project” (No. 2007CG39), the Shanghai Natural Science Fund (No. 09ZR1407700), and the State Key Lab of Urban Water Resource and Environment, Harbin Institute of Technology (No. ES200902). We thank Master Mingcang Chen for carrying out many experimental runs and Master Zheng Fang for supplying the MPA-CdTe QDs.

References


