Kidney Toxicity and Response of Selenium Containing Protein-glutathione Peroxidase (Gpx3) to CdTe QDs on Different Levels

Lining Zhao,* Wansong Zong,† Hao Zhang,‡,§,1 and Rutao Liu*,1

*School of Environmental Science and Engineering, Shandong University, China-America CRC for Environment & Health, Shandong Province, Qingdao, Shandong 266237, P. R. China; †College of Population, Resources and Environment, Shandong Normal University, Jinan 250014, P. R. China; ‡Laboratory of Immunology for Environment and Health, Shandong Analysis and Test Center, Qilu University of Technology (Shandong Academy of Sciences), Jinan 250014, China; and §Hubei Provincial Key Laboratory of Occurrence and Intervention of Rheumatic Diseases, Affiliated Hospital of Hubei University for Nationalities, Enshi 445000, China

1To whom correspondence should be addressed at School of Environmental Science and Engineering, Shandong University, China-America CRC for Environment & Health, Shandong Province, 72# Jimo Binhai Road, Qingdao, Shandong 266237, P. R. China. Fax: +86-531-88364868; E-mail: rutaoliu@sdu.edu.cn and Fax: +86-531-82605433; E-mail: zhanghao0911@yeah.net.

ABSTRACT

The toxic mechanism of cadmium-quantum dots (Cd-QDs) to organisms is still debating. In this paper, it was found that Cd-QDs could induce adverse effects to kidney by entering into cells in a time and dose manner and disturbing the redox balance in vivo. As a selenium containing protein, glutathione peroxidase3 (Gpx3) plays a crucial role in maintaining the balance of redox system. The decrease in Gpx3 activity might be related to the imbalance of redox system. Similar to the animal results, it was demonstrated that Gpx3 activity is also inhibited by Cd-QDs in vitro. To investigate the underlying mechanism of Cd-QDs on conformational and functional changes of Gpx3, systematical measurements including calorimetric, multispectroscopic studies, and molecular model studies were carried out on molecular level. Results showed that Cd-QDs binds to Gpx3 via Van der Waals’ force and hydrogen bonds, resulting in structural changes with increasing contents of $\alpha$-helix. By interacting with Glu136 in the cavity of Gpx3 as well as Phe132, Pro130, and Van129 surrounded, Cd-QDs changes the microenvironment of fluorophore and further reduce the activity of Gpx3.

Key words: Cd-QDs; kidney toxicity; redox balance; glutathione peroxidase3; molecular mechanism.

Due to unique material properties, nanoparticles (NPs) have more extensive development in biological applications (Alivisatos, 2004; Beloglazova et al., 2015; Ito et al., 2005). Semiconductor quantum dots (QDs), as a member of the nanoscale material, are widely used in bio-labeling, biomedicine, optoelectronics, and other medical applications (Su et al., 2010; Wang et al., 2010, 2013). To date, cadmium-quantum dots (Cd-QDs) has increasingly captured the attention of scientists partially because of the core/shell basic structure (Lovrić, Bazzi, et al., 2005; Sun et al., 2014). The composition of a Cadmium containing core and a biocompatible shell can reduce the inherent toxicity and enhance its chemical stability (Su et al., 2010). However, studies have revealed that intracellular environment can make some nanoparticles, such as QDs, to degrade to toxic components and further reduce cell viability (Chang et al., 2006; Derfus et al., 2004). Safety of Cd-QDs remains a major concern for its application in vivo due to the nonspecific interaction and the potential to be degraded (Rzigalinski and Strobl, 2009; Santra, 2012; Wang, Liu, et al., 2016). The induction of reactive oxygen species (ROS) and a series of markers of oxidative stress...
are responsible for the observed cytotoxicity of Cd-QDs. There is much evidence suggesting the involvement of oxidative stress in QD-induced toxicity (Brodin and Davis, 2017; Yaghini et al., 2014). We have studied the interactions of QDs with important antioxidant enzymes such as catalase and superoxide dismutase and demonstrated that QDs adversely affect the structure and activity of these enzymes (Sun et al., 2014, 2015). Glutathione peroxidase3 (GPx3) also plays a crucial role in maintaining the redox balance and defending the oxidative stress in organisms. Limited data are available on the biological and toxic effects of Cd-QDs to Gpx3.

As a tetramer selenoprotein, GPx3 has been known to catalyze the reduction of H₂O₂ to water or organic hydro-peroxides containing the redox balance and defending the oxidative stress in Glutathione peroxidase3 (GPx3) also plays a crucial role in maintaining the redox balance and defending the oxidative stress in organisms. Limited data are available on the biological and toxic effects of Cd-QDs to Gpx3.

Characterization of the CdTe QDs
The physical characterization of CdTe QDs was carried out on transmission electron microscopy (TEM; Hitachi, Japan) and the Zetasizer Nano instrument (ZS Nano, Malvern, New York). UV-visible absorption (UV-2700, Shimadzu, Japan), fluorescence spectroscopy (RF-6000, Shimadzu, Japan), and infrared spectroscopy (Nicolet 155, Thermo Scientific) were also utilized to investigate the photochemical characteristics of CdTe QDs.

MATERIALS AND METHODS

Chemicals
The CdTe QDs capped with carboxyl groups were bought from Sigma Co. Ltd. Dulbecco’s Modified Eagles Medium (DMEM), penicillin/streptomycin and fetal bovine serum were all purchased from Thermo Fisher Scientific. CM-H₂DCFDA bought from Thermo Fisher Scientific, Inc was stored at –20°C. Annexin V-APC/7-AAD apoptosis kit was purchased from Lianke Biotechnology Co Ltd (China) and stored at 0–4°C and protected from light. For molecular experiments, Gpx3—an Escherichia coli based expressed protein which contains residues from Gly 73 to Lys 226, was bought from Cloud-Clone Corp, and activity of these enzymes (Sun et al., 2014, 2015). Glutathione peroxidase3 (GPx3) also plays a crucial role in maintaining the redox balance and defending the oxidative stress in organisms. Limited data are available on the biological and toxic effects of Cd-QDs to Gpx3.

Kidney, as the mainly bio-accumulated organ of Cd-QDs, plays an important role in metabolism, oxidative-reductive, and immunologic processes with numerous functions. In this study, kidney toxicity induced by Cd-QDs was investigated (such as cell viability, intracellular Cd-QDs level, ROS level, and cell apoptosis level), and Gpx3 activity was examined. In addition, the direct interactions of Cd-QDs with Gpx3 were also carried out to detect changes of Gpx3 activity in vitro. Calorimetric, multispectroscopic methods, and molecular model studies were adopted to further investigate the underlying mechanism of this system. Our study will contribute toward a better understanding of Cd-QDs-caused damages to Gpx3 at both cellular and molecular levels and provide new strategies on the inhibition and treatment of damages caused by Cd-QDs.

Mouse Experiment

Isolation of the primary cells. Male C57BL/6J (B6) mice were purchased from the School of basic medicine sciences, Shandong University (Jinan, China). The primary kidney cells were obtained as describe in our previous study (Wang et al., 2017). Cd-QDs was dissolved in complete medium to prepare a stock solution and diluted before use. The isolated cells were cultured in complete medium and exposed to different concentrations of QDs in 96-well plates for other assays.

ROS and apoptosis level assays. Preliminary experiments were performed to determine the expected means and sample distributions of the control and experimental groups. CM-H₂DCFDA probe was utilized to detect the production of intracellular ROS levels (Erusalimov and Kusmartsev, 2010; Jing et al., 2016; Testa et al., 2011; Vincent et al., 2004). After exposure for 1 h, cells were washed and resuspended with PBS buffer. Meanwhile, a negative control without a probe and the positive control with 100 μM H₂O₂ were performed to clearly distinguish fluorescence of ROS signals in cells.

To detect the ROS level in vivo, group of 4-week-old C57BL/6 mice was fed with 100 μM QDs or control fed with DI water for a month. The weight of the animals was measured every other day. One month later, the animals were sacrificed and the kidney cells were isolated as described in 2.3.1. All mice used in this study were of the same genetic background and assigned to groups by randomization. Although the environmental exposure of CdTe QDs is much less than the utilized concentration in the literature, Cd can be released and accumulated in organisms through the bio-concentration of food chain after long-term exposure. In addition, the order of Cd-QDs concentration in this study is in accordance with the naturally occurring level of Cd-QDs in the aquatic organism and studies which utilized Cd-QDs as a stimulus (Hauck et al., 2010; Su et al., 2010; Ye et al., 2012).

In consideration of the above reason, we choose these concentrations (from 0.1 to 100 μM) as the CdTe QDs exposure concentration in this study to provide representative results.

Apoptosis level was measured by the method described by Koopman et al. (1994). Harvested cells (about 5×10⁶ cells per milliliter) were washed with sodium chloride and resuspended in 100 μl binding buffer prepared in distilled water. Cells were incubated for 15 min in dark after adding 7-AAD (10 μl) and Annexin V-APC (5 μl) to each tube, and analyzed by flow cytometry.

Intracellular Gpx3 activity assays. Gathered cells (2.2.1) were ultrasonically lysed for 20 times (6 s each) in PBS on ice and centrifuged to obtain supernatants. Glutathione peroxidase3 activity was measured according to the protocols (Griffith, 1990; Shi and Zhou, 2010). Activity of blank control without CdTe QDs was set...
as 100% (Wang, Wang, et al., 2016). The in vivo Gpx3 activity was also determined in this study.

Analysis of Functional and Structural Changes for Gpx3 Molecule

Gpx3 activity assays in vitro. Samples were prepared as follows: 10% PBS (0.2 M), 30 μl Gpx3 solution (10⁻⁶ M), and different volume of Cd-QDs solutions were added in 1-ml centrifuge tubes and diluted to 1 ml by adding ultrapure water.

Isothermal titration calorimetry measurement. The measurement was conducted on Microcal ITC 200 to investigate the thermodynamic parameters of the binding process between Gpx3 and CdTe QDs. After filtered with 0.22 μm membranes, 40 μl Gpx3 was put into the syringe and 280 μl Cd-QDs was loaded into the sample cell, respectively. After the thermal equilibration and the initial delay finished, 14 serial drops of Gpx3 (the first drop of 0.4 μl and 13 drops of 3 μl) were titrated into Cd-QDs continuously with the stirring speed of 1000 rpm.

UV-visible absorption and circular dichroism measurements. A quartz cuvette with a path length of 10 cm was used to measure the UV-visible absorption spectra on a UV-2450 spectrophotometer (Shimadzu, Japan) from 190 to 350 nm. The samples were prepared as 2.4.1 and the control group was prepared without Gpx3. For circular dichroism (CD) spectra measurement, spectra were recorded on a J-810 CD spectrometer (Shimadzu, Japan) from 190 to 350 nm. Scanning speed was set at 200 nm min⁻¹. Each spectrum was the average of three successive scans. CDpro software was used to calculate the secondary structure contents of Gpx3 (Sreerama and Woody, 2000).

Fluorescence spectra measurements. Fluorescence spectra were measured with the wavelength ranging from 290 to 450 nm. Parameters were set as follows: the excitation wavelength: 278 nm, scanning speed: 1200 nm min⁻¹, the voltage of photo multiplier tube: 800 V, the excitation and emission slit widths: 5.0 nm. Synchronous fluorescence spectra were measured at Δλ = 15 nm and Δλ = 60 nm.

Molecular docking studies. We adopted Molecular Operating Environment (MOE) (Chemical Computing Group, Inc, Canada) to study the possible binding site of Gpx3-Cd-QDs complex. RSCB Protein Data Bank (https://www.rcsb.org/) was used to download the crystal structure of Gpx3 (PDB code: 2r37). A module of MOE was used to build the structure of Cd-QDs. Water molecules were removed at first and essential hydrogens and gaster charges were added. The parameters were set as follows: Placement: Triangle Matcher; Rescoring 1: London dG; Refinement: Forcefield.

In this paper, statistical data are expressed as the mean ± standard error of the mean (SEM) of independent experiments. One-way analysis of variance was used to evaluate the multiple comparisons of blank control with Cd-QDs treated groups.

RESULTS AND DISCUSSION

CdTe QDs Characterization

Size distribution carried out with dynamic light scattering analysis (Figure 1A) showed that the diameters of CdTe QDs range from 2.7 to 5.6 nm, and the TEM imaging illustrated a favorable unity and homogeneity of CdTe QDs. To study the photochemical properties of CdTe QDs, the UV-visible spectra, fluorescence spectra and infrared spectra were tested. Results showed that the characterized peak of carboxyl groups around 1500 nm and fluorescence peak at 570 nm were observed, indicating the good optical features that CdTe QDs possess.

Damage in Kidney Cells Induced by Cd-QDs

Cell viability and intracellular Cd-QDs detection. First, we evaluated the cytotoxicity of Cd-QDs in the kidney cells by using 7-AAD assay after 6/24 h exposure, respectively. When the concentrations of Cd-QDs were under 10 μM, the cell viability did not reduce significantly. Under 50–100 μM of Cd-QDs, the cell viability changed significantly as shown in Figure 2A. The decrease in cell viability was dose- and time-dependent, probably related to the uptake of Cd-QDs and the produce of ROS (Su et al., 2010; Wang et al., 2013).

Previous studies revealed that QDs enters into cells via endocytosis (Akiyoshi Hoshio et al., 2004; Derfus et al., 2004). Compared with the traditional detection method on concentrations of Cd-QDs which digests cells into clear solutions, we detected the relative cellular Cd-QDs contents in situ. Other than destroy the samples, this measurement could keep the origin states of kidney cells reflect the actual Cd-QDs content at single cell level (Chen et al., 2008; McRae et al., 2009; Wang, Liu, et al., 2016; Yang et al., 2007). In Figure 2B, after incubation for 6/24 h, the proportion of kidney cells containing QDs significant increased with the increased concentration of QDs in a dose- and time-dependent manner. About 38.9%/83.8% (6/24 h) cells contained Cd-QDs when the concentration reached 100 μM.

ROS and apoptosis level assays. After detected cell viabilities in each group, the intracellular ROS generation in animals (I) and cells (II) were measured. In Figure 3A, ROS generation rose slightly in treated group compared with the control group. From the
cellular (II) prospective, with the increased Cd-QDs concentration, the number of kidney cells with higher fluorescence intensity (a higher ROS level) increased, especially when the concentration was greater than 50 μM. Exposure of Cd-QDs to kidney cells resulted in an increase in ROS level, suggesting that the redox equilibrium was disrupted particularly at the cellular level which are in accordance with the research of Lovrić/C21, Cho, et al. (2005).

Cell apoptosis and necrosis are the two main basic types and mechanisms of cell death (Buja et al., 1993; Swynghedauw, 1999). The percentage of apoptosis induced by Cd-QDs for 6/24 h is shown in Figure 3B. Especially when kidney cells were exposed to 100 μM Cd-QDs for 24 h, the percentages of living cells, early apoptosis, late apoptosis, and necrotic cell were up to 4.53%, 6.89%, 55.9%, and 32.7%, respectively. Studies showed that ROS (such as hydrogen peroxide) could chemically degrade Cd-QDs in cells (Mancini et al., 2008). We speculate that when kidney cells were exposed to Cd-QDs, the following reactions exist: (1) Cd-QDs was digested by enzymolysis and induce ROS production in organisms which have demonstrated by the former studies (Shaikh et al., 1999; Tanimoto et al., 1993; Thévenod and Friedmann, 1999). (2) excessive ROS will further degrade Cd-QDs to be released, eventually leading to cell apoptosis (Luo et al., 2013; Nel et al., 2006).

Intracellular Gpx3 activity changes. The above results suggest a toxic effect of Cd-QDs to kidney cells that can decrease cell viability and cause redox state disorders in cells. As a selenoprotein secreted in kidney, Gpx3 functions as a redox buffer for the discrimination between irrelevant and serious inflammatory stimuli (Brigelius-Flohé and Maiorino, 2013). The intracellular Gpx3 activity changes when Cd-QDs exposed to kidney cells were further detected. Treated group of mice that fed with 100 μM QDs for a month were sacrificed, and Gpx3 activity results are shown in Figure 4A. Comparing with the blank control group, the Gpx3 activity of the treated group decreased by 25%. Alterations of Gpx3 activity indicate the interruption of redox balance in organisms. We further detected the Gpx3 activity on cellular level. In Figure 4B, when higher concentrations of Cd-QDs exposed to kidney cells, Gpx3 activities decreased in a dose-dependent manner. When the concentration of Cd-QDs reached to 100 μM, the Gpx3 activity decreased to 69.4%, suggesting that Gpx3 had no capacity of returning to the normal level.

Gpx3 activity assays in vitro. The changes of Gpx3 activity can be inhibited by not only the imbalance of redox system, but also to direct exposure of Cd-QDs. Therefore, purify Gpx3 was selected to study the alteration of molecular activity and structure after Cd-QDs exposure. With the increase in Cd-QDs concentration, Gpx3 activity was inhibited (inhibitory rate was 28.2% of the concentration of 100 μM), which was in accordance with the intracellular results. We initially reached a conclusion that Gpx3 activity could also be impacted by the direct exposure of Cd-QDs. Structural changes of enzyme could influence the activities, we further studied the molecular mechanism of Gpx3 with Cd-QDs. Calorimetric, multispectroscopic, and molecular docking studies were adopted in the following investigation (Figure 5).
Isothermal titration calorimetry assays. Isothermal titration calorimetry assays were performed as the stoichiometry to provide the detailed information of ligand-protein interactions and better understanding of the binding mechanism (Canterbury et al., 2017; Damian, 2013; Hrushikesh Joshi et al., 2004; Huang and Lapitsky, 2013; Wang, Yang, et al., 2016). The titration curve was fitted with a single set of binding sites. Thermodynamic parameters—binding constant ($K$), entropy change ($\Delta S$), and enthalpy change ($\Delta H$) could be obtained from the curve-fitting (Wang, Yang, et al., 2016). In Table 1, the changes of Gibbs free energy ($\Delta G$) were calculated by using the equation: $\Delta G = \Delta H - T\Delta S$, where $T$ is the thermodynamic temperature. A negative value of $\Delta G$ indicates that the interaction was spontaneous (Stroobants et al., 2014). The interaction was an exothermic process and the major driving force was the Van der Waals’ force and hydrogen bonds which were demonstrated by the negative values of $\Delta H$ and $\Delta S$ (Ross and Rekharsky, 1996; Ross and Subramanian, 1981). Different from the strong binding interactions ($K$ value normally ranges from $10^7$ to $10^8$), the lower $K$ value ($1.23 \pm 0.14 \times 10^5 \text{ M}^{-1}$) indicated a relatively moderate binding affinity of this system. Despite the binding is moderate, the structure of Gpx3 could be altered with the activity of Gpx3 changed by Cd-QDs (Wang et al., 2017) (Figure 6). The result of blank control group is shown in Supplementary Figure 1.

**Table 1. Thermodynamic Parameters for Binding of Gpx3 to Cd-QDs at 298 K**

<table>
<thead>
<tr>
<th>$K$ ($\times 10^5 \text{ M}^{-1}$)</th>
<th>$\Delta H$ ($\times 10^5 \text{ cal/mol}$)</th>
<th>$\Delta S$ ($\times 10^5 \text{ cal/mol K}$)</th>
<th>$\Delta G$ ($\times 10^5 \text{ cal/mol}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$1.23 \pm 0.14$</td>
<td>$-4.43 \pm 0.157$</td>
<td>$-1.46$</td>
<td>$-7.92$</td>
</tr>
</tbody>
</table>

**Figure 6.** Isothermal titration calorimetry result for Gpx3 interaction with Cd-QDs. Conditions: $c(\text{Gpx3}) = 2.0 \times 10^{-5} \text{ M}$; $c(\text{Cd-QDs}) = 1.0 \times 10^{-4} \text{ M}$. pH 7.4; $T = 298 \text{ K}$.

**Table 1.** Thermodynamic Parameters for Binding of Gpx3 to Cd-QDs at 298 K.

UV-visible absorption and CD spectroscopy measurements. UV-visible absorption and CD spectroscopy can reflect the information of structural changes and electron transfers of the protein-biomolecules interactions (Fang et al., 2011; Zhang et al., 2016). In Figure 7A, the UV-visible absorption spectra of Gpx3 with different concentrations of Cd-QDs were measured. There are two absorption peaks, the strong one at 208 nm, could reflect the transition of $n \rightarrow p^*$ polypeptide backbone structure (Zhao et al., 2015). With the increasing concentrations of Cd-QDs, the absorption peak decreased with a blue-shift in a dose-dependent manner, indicating a change in the protein skeleton and its secondary structure (Tan et al., 2018; Wu et al., 2007). Figure 7B shows the CD spectra of Gpx3 samples with different Cd-QDs concentrations for $\alpha$-helix structure of protein (Lu et al., 2007; Tabassum et al., 2012). The corresponding secondary compositions analyzed by CD Pro are listed in Supplementary Table 1. The amount of $\alpha$-helix in Gpx3 increased from 75.5% to 80.5%, indicating that Cd-QDs led the proteins to partially denature that may lead to the loss of normal physiological function.

The rather weak absorption peak of UV-visible spectra at around 280 nm, reflect the exposure of the chromophores (Laurent and Assfeld, 2010). No significant shift of the spectra is observed with changes in the concentrations of Cd-QDs. Therefore, we...
speculate that the structural changes of protein are probably due to the fluorescence intensity changes induced by Cd-QDs.

Fluorescence spectra measurements. Fluorescence spectra measurements were utilized due to its high sensitivity and selectivity. Inner filter effect, caused by the ligand absorption during the excitation and emission process, was considered and the results are shown in Supplementary Figure 2. The absorption of the ligand was less than 0.1 at the excitation and emission wavelength meaning that IFE could be negligible here (Lakowicz and Masters, 1983; Wang et al., 2015). In Figure 8, the spectra of Gpx3 were recorded at different concentrations of Cd-QDs. The fluorescence intensity increased gradually with the addition of Cd-QDs, indicating that of Gpx3 structure was altered and fluorophores were changed to be more exposed (Zhang et al., 2016).

Molecular docking studies. Molecular docking studies were carried out to simulate the possible binding sites of Cd-QDs-Gpx3 system and were helpful to clarify the mechanism for structure and activity changes of Gpx3 molecule. Results are presented in Figure 9, Cd-QDs binds with Glu136 in the cavity of Gpx3. The Gaussian contact map superimposed with Cd-QDs is shown in Figure 9B. Beside, some amino acid residues take part in the binding interactions of this system and the region is next to the residue Phe 132. The microenvironment alteration of fluorophore probably leads to the changes of fluorescence intensity. We suppose Cd-QDs molecules entered into the cavity of Gpx3 and bind with the residue Glu 136 together with the surrounding residues, thus the structure of the cavity was changed and became more exposed to substrate, the structure changes of Gpx3 further changed its activity.

CONCLUSIONS
In summary, our research shows that Cd-QDs could induce adverse effects to mouse primary kidney cells. When Cd-QDs enters into cells, excessive ROS are generated, which can lead to cell apoptosis and inhibition on cellular viability. The redox equilibrium was disrupted with function of Gpx3 inhibited. The response of Gpx3 to Cd-QDs and the interaction mechanism of Cd-QDs with Gpx3 were firstly investigated on animals, cellular and molecular levels. The activities of Gpx3 were inhibited and microenvironment and conformation of Gpx3 were altered. Results show that Cd-QDs binds to Gpx3 through Van der Waals’ force and hydrogen bonds. This study examines the potential toxicity of Cd-QDs to kidney cells at different perspectives and is expected to give some important information about the antioxidant enzyme toxicity of Cd-QDs and human health risks in the use of this nanoparticle.

SUPPLEMENTARY DATA
Supplementary data are available at Toxicological Sciences online.

FUNDING
This work is co-supported by NSFC (21277081, 21477067, 21777088, and U1806216), the Cultivation Fund of the Key Scientific and Technical Innovation Project, Research Fund
for the Doctoral Program of Higher Education and Ministry of Education of China (708058, 20130131110016), Science and Technology Development Plan of Shandong Province (2014GSF117027), Natural Science Foundation of Shandong Province (ZR2016YL013), and the Youth Science Funds of Shandong Academy of Sciences (2018QN009) are also acknowledged.

REFERENCES


