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Probing metabolic stability of CdSe nanoparticles: Alkaline extraction of free cadmium from liver and kidney samples of rats exposed to CdSe nanoparticles

Zikri Arslan[a,*], Mehmet Ates[a], Wanaki McDuffy[a], M. Sabri Agachan[a], Ibrahim O. Farah[b], W. William Yu[c], Anthony J. Bednar[d]

[a] Jackson State University, Department of Chemistry and Biochemistry, PO Box 17910, Jackson, MS 39217, USA
[b] Jackson State University, Department of Biology, PO Box 18540, Jackson, MS 39217, USA
[c] BioScience Research Collaborative, Rice University, MS 602, 6500 Main Street, Houston, TX 77030, USA
[d] US Army Engineer Research and Development Center (ERDC), Waterways Experiment Station, Vicksburg, MS 39180, USA

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ABSTRACT

Cadmium selenide nanoparticles (CdSe NPs) exhibit novel optoelectronic properties for potential biomedical applications. However, their metabolic stability is not fully understood because of the difficulties in measurement of free Cd from biological tissues of exposed individuals. In this study, alkaline dissolution with tetramethylammonium hydroxide (TMAH) is demonstrated for selective determination of free Cd and intact NPs from liver and kidney samples of animals that were exposed to thiol-capped CdSe NPs. Aqueous suspensions of CdSe NPs (3.2 nm) were used to optimize the conditions for extracting free Cd without affecting NPs. Nanoparticles were found to aggregate when heated in TMAH without releasing any significant Cd to solution. Performance of the method in discriminating free Cd and intact NPs were verified by Dogfish Liver (DOLT-4) certified reference material. The samples from the animals were digested in 4 mL TMAH at 70 °C to extract free Cd followed by analysis of aqueous phase by ICP-MS. Both liver and kidney contained significant levels of free Cd. Total Cd was higher in the liver, while kidney accumulated mostly free Cd such that up to 47.9% of total Cd in the kidney was free Cd when NPs were exposed to UV-light before injection.

1. Introduction

Nanotechnology has been revolutionizing the human life by invention of novel nanoparticles (NPs) of intriguing physical and chemical properties for technological and medicinal applications. However, this revolution is not worry-free due to the potential health risks associated with the production and use of products containing nanoparticles [1–5]. Colloidal semiconductor nanoparticles of cadmium selenide (CdSe), are probably among the most concerned NPs because of their cytotoxicity and potential adverse effects on human and environmental health. Nanoparticles of CdSe exhibit bright, photo-stable and size-tunable emission (fluorescence) that make them optimal fluorophores and potential alternatives to traditional dyes, such as Rhodamin Green, for in vivo biomedical imaging and diagnostics [6–8]. The toxicological issues have been reviewed in several articles [1,9,10]. Nevertheless, there is still little known about the pharmacokinetics (accumulation, distribution, metabolism and elimination) of CdSe NPs and other similar nanoparticles. In vitro studies provide useful information about the toxicity issues and health risks but are not sufficient to fully address the safety issues on human and environmental health.

The major hurdle in addressing the safety issues of semiconductor nanoparticles is the lack of biosafety data mainly because of the fact that toxic effects vary substantially depending on physicochemical properties and environmental conditions, such as size, charge, outer coating, concentration, physical stability and solubility [1,5,11,12]. For instance, CdSe NPs could exhibit adverse effects even if the NPs remain stable in the body [12]. The effects become more detrimental if cadmium ions (Cd^{2+}) are released to the body as outer coating intended to enhance stability and biocompatibility undergoes metabolic degradation. Both Cd and Se are toxic to humans causing hepatic, renal, and neurologic toxicities [13–17]. Cadmium also interferes with DNA repair and metabolic proteins, and substitutes for Zn^{2+}. Initially it accumulates in the liver with a half life of 15–30 years and is gradually mobilized to kidney causing nephrotoxicity. The cadmium–metallothionein complex is also

* Corresponding author. Tel.: +1 601 979 2072; fax: +1 601 979 3674.
E-mail address: zikri.arslan@jsums.edu (Z. Arslan).

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toxic to the kidney since most chelating agents that remove Cd^{2+} from the liver are excreted by the kidney [18–20]. Thus, accurate tracing of CdSe NPs and their species in the body is important to explore their physiological fate and safety.

Studies attempting to elucidate the effects of CdSe NPs have attributed their cytotoxicity to the formation free Cd ions from NPs [21–23]. Derfus et al. [21] for instance reported that untreated CdSe NPs were innocuous to liver hepatocyte, but cell viability increased when NPs were exposed to UV light, which was explained by the oxidation of cap and consequently liberation of free Cd ions. Similarly, the toxic effects were attributed to poisoning from Cd ions in the growth medium when a freshwater organism, *Daphnia magna*, were exposed to mercaptoundecanoic acid (MUA) coated CdSe NPs [22]. In the same study, polyetheylene oxide (PEO) coated CdSe NPs also induced acute toxicity on *D. magna*, though no significant Cd was detected in the growth medium, indicating a different mode of action from intact NPs [22]. Citrate stabilized CdSe NPs were also reported to release Cd ions to solution during the exposure of planktonic culture, *Pseudomonas aeruginosa*, and the NPs exhibited a dose dependent toxic effects [23]. At low dose, NPs induced similar effects to that of Cd ions, but were more toxic than Cd ions at higher doses [23]. These studies support the fact that the effects of CdSe NPs will not only vary among species, but also with routes of synthesis and coating materials used. Moreover, Cd ions are released from CdSe NPs if the core is not protected properly, which could infact confound the actual effects of NPs.

As occurred in vitro, CdSe NPs may degrade in vivo releasing Cd ions that accumulate through the body and mediate the effects of exposure. Thus, detection of total Cd in certain organs of exposed individual, without any knowledge of Cd ions and intact NPs, is not sufficient to fully explain their metabolic stability, toxicology and mechanism of action. To date, few in vivo studies [11,24–27] investigated the accumulation and kinetics of NPs on animal models, but were not able to provide a map of the species distribution and free Cd levels in the tissues. In most cases, localization of NPs was targeted in tissues and cells by using the fluorescence microscopy without any information about the levels of free metal ions [24–27]. This is mainly because of the lack of methodology to achieve separation of free Cd and intact NPs from soft tissues. The extraction of Cd from tissue samples requires acid treatment, but this strategy is not a viable method for selective determination of free Cd since intact CdSe NPs are also decomposed to Cd and Se [21]. Contrary to this, detection of free Cd in culture studies do not require aggressive chemical treatment, and therefore is a relatively straightforward task since free Cd ions in the growth medium can be separated from NPs by means of physical methods (e.g., dialysis or ultrafiltration). Nonetheless, the information does not reflect the free Cd levels accumulated by the cells or microorganism.

Detection of free metal ions from the tissues of individuals that are exposed to NPs is a challenging task without altering the composition of intact NPs. Yet, the development of robust and reliable methods will provide indispensable tools to address the concerns associated with environmental and human health effects of nanoparticles. For this purpose, we have investigated the experimental conditions for selective determination of Cd ions and CdSe NPs from biological samples. The specific objectives of this study are (1) to develop a method for quantitative separation of Cd ions and intact NPs originating from degradation of thiol-capped CdSe NPs, (2) to apply the procedure to samples collected from rats exposed to CdSe NPs to determine the distribution of the Cd ions and intact CdSe NPs. Thiol-capped CdSe NPs were synthesized, purified and used without further modification or additional ZnS coating. We opted for bare thiol-cap as it provides minimal surface protection for NP core, and therefore, is ideal to delineate the metabolic stability of the NPs and the fates of degradation products in vivo.

2. Experimental

2.1. Materials and solutions

Cadmium chloride ($CdCl_2{\cdot}2H_2O$ 98%, Sigma–Aldrich), selenium powder (99%, Acros Chemicals), thioglycolic acid (98%, $d = 1.325\ g\ mL^{-1}$, Sigma–Aldrich) and $NaBH_4$ (98% Sigma–Aldrich) were used for synthesis of CdSe QDs. Tetramethyl ammonium hydroxide (TMAH) solution (99.99%, $d = 1.016\ g\ mL^{-1}$) was purchased from Alfa Aesar. Trace metal grade nitric acid (HNO_3) and hydrochloric acid (HCl) were used. Teflon tubes (Savillex, Minnetonka, MN) were used for heat-assisted extraction and dissolutions. Stock Cd(II) solution (1.0 M) was prepared by dissolving 9.15 g of $CdCl_2$ in 50 mL water. Selenium stock solution (0.5 M) was made by dissolving 3.95 g Se powder in sufficient volume (ca. 4 mL) of concentrated HNO_3. Upon dissolution, the solution was heated to get rid of excess HNO_3 and then diluted to 100 mL with 10% HCl to reduce from Se(VI) to Se(IV). Thioglycolic acid was used directly from the bottle. $NaBH_4$ solution (10% w/v) was prepared freshly in 0.1% NaOH solution. Multi-element solutions and calibration standards were prepared from 10 $\mu g\ mL^{-1}$ multi-element solution (Spex Certiprep).

2.2. Apparatus

UV–vis absorption spectra were recorded by using a HP 8453 UV-visible spectrometer. Fluorescence spectra were taken by Horiba Jobin Yvon benchtop FluoroMax-2 spectrofluorometer. A Fisher Scientific Model 100 Sonic Dismembrator equipped with titanium alloy probe (12.5 cm long, 3.1 mm wide tip) was used for ultrasonic agitation of samples. The transmission electron microscopy (TEM) images of the NPs were acquired by JEOL-1011 TEM instrument. The resolution of JEM-1011 is 0.2 nm lattice with magnification of 50 to 1×10^6 under the accelerating voltage of 40–100 kV. The colloidal solution in water was dropped onto 50 Å thick carbon-coated copper grids and allowed to dry. A 48-well digestion block (Digiprep MS, SCP Science, Champlain, NY) was used for extractions and dissolutions. Elemental measurements were performed by Varian 820-MS ICP-MS instrument (Varian, Australia), which was equipped with a peltier-cooled glass concentric spray chamber, micromist nebulizer (400 $\mu L\ min^{-1}$), standard one-piece, low flow, ball-and-socket connection torch quartz, standard Ni sampler and skimmer cones, patented Collision Reaction Interface (CRI), a unique 90 degree ion mirror delivering exceptional sensitivity, all-digital detector; Discrete Dynode Electron Multiplier (DDEM, Model AF250, ETP Australia) providing nine decades of linear dynamic range. Samples were introduced via Varian autosampler (Model SPS3). The ICP-MS instrument was optimized by using 5 ppb Ba, Be, Ce, In, Mg, Th, for sensitivity, doubly charged ions and oxides. Data were collected using ^{111}Cd and ^{114}Cd isotopes at peak hopping mode using manufacturers recommended settings. The results for Cd are reported as average of ^{111}Cd and ^{114}Cd signals. Internal standard correction was performed with ^{103}Rh that was added to the sample stream on-line.

2.3. Preparation of CdSe NPs

Procedure for synthesis of water soluble CdSe NPs was adapted from the protocols described by Gaponik et al. [28] and Chen et al. [29]. In a typical synthesis 0.5 mL of 1.0 M $CdCl_2$ solution and 0.5 mL of thioglycolic acid ($HSCH_2COOH$) as capping agent were added to 250 mL deionized water in a three-neck flask under stirring. The solution was initially turbid because of the incomplete solubility of Cd–thiolate complex, but clear solution formed at around pH 9–9.2 by adding appropriate volume of 1.0 M NaOH. Selenium solution (10 ml of 0.5 M Se(IV) in 10% HCl) was placed in another three neck

flask which was connected to the first flask containing Cd–thiolate complex via a glass transfer tubing). Both flasks were fitted with a septum and deaerated for 30 min by bubbling N_2 gas under stirring. Hydrogen selenide gas (H_2Se) was generated by adding 10 mL of 10% $NaBH_4$ in 0.1% NaOH into Se(IV) solution. The H_2Se gas was immediately passed onto to Cd–thiolate solution with slow nitrogen flow for 30 min. CdSe NPs formed at this stage indicated by a color change in solution from clear to yellow/orange at room temperature. The solution was heated until the color of the solution turned orange. At this point, N_2 gas flow stopped and the solution was refluxed overnight. The color of the solution turned into bright red. The NPs were precipitated in dimethylsulfoxide solution (DMSO) which is a nontoxic solvent commonly used as a carrier in cell culture studies. To purify, 25 mL of DMSO was placed onto 25 mL NP solution in 50 mL polypropylene tube and centrifuged at 7000 rpm for 1 h. The supernatant was discarded and the precipitate was agitated in 10 mL acetone, and then re-centrifuged again for 30 min. After discarding the supernatant, the precipitate was washed with acetone twice and then redispersed in 50 mL deionized water. Residual acetone was readily evaporated by mild heating the NP the aqueous suspension. As synthesized, the CdSe NPs were stable for months when kept in dark at 4 °C.

2.4. Testing effects of ultrasounds on NP stability and extraction efficiency

It is critical to maintain the stability of the NPs in the solution or sample while attempting to separate free Cd from intact CdSe NPs. Initial experiments to test the stability of the NPs were conducted by means of ultrasonic agitation via titanium probe. Aqueous suspensions of thiol-capped CdSe NPs (3 mL, $n = 3$) were diluted to 5 mL with deionized water, 25% TMAH and 2.5% (v/v) HCl yielding NPs in water, 10% TMAH and 0.5% (v/v) HCl, respectively. All suspensions were exposed to ultrasounds for 3 min at 80% P (80 W). The UV–vis spectra of the sonicated suspensions were recorded and compared with that of the control. The control suspension was the same as those prepared in water, but not exposed to ultrasounds.

To determine Cd concentration released from NPs to the solution after sonication, 1.0 mL of each sonicated solution was precipitated in 1.0 mL acetone by centrifuging for 1 h at 12,000 rpm. The acetone layer was evaporated at room temperature, and then 0.25 mL of the aqueous layer from each solution, including the controls, were pipetted out, diluted to 2 mL with 1% (v/v) HNO_3 and analyzed by ICP-MS for Cd. The concentrations were from ICP-MS were corrected for the dilution.

2.5. Testing effects of heating in TMAH on NP stability and extraction efficiency

Tetramethyl ammonium hydroxide (TMAH) is a water soluble strong base that complexes and stabilizes metal ions in solution. It has been used for alkaline dissolution of biological samples for determination of a number of trace elements [31–36]. Unlike strong acid decomposition, treatment in TMAH does not result in mineralization of organics, but rather leaches organically bound metals to solution. Within this context, TMAH offers an alternative pathway for extraction of free Cd from the tissues containing CdSe NPs provided that TMAH does not lead to the decomposition of NPs to free ions. To examine the stability in hot TMAH, 3 mL NP suspensions ($n = 3$) were diluted to 5 mL with water and 25% TMAH in polypropylene tubes and heated at 70 °C for 1 h. The spectra of the treatments were recorded and compared with the control suspension which was not heated. In another experiment, recoveries for spiked Cd solution were examined, where 3 mL NP suspensions ($n = 3$) were first diluted to 5 mL with 25% TMAH, then spiked to 50 μg L^{-1} Cd and heated at 70 °C for 1 h. Measurement of free Cd was performed similarly as described in Section 2.4 after re-precipitation in acetone.

2.6. Method validation

Prior to application to the liver and kidney samples collected from the rats, the performance of the method was verified by analysis of Dogfish Liver (DOLT-4) certified reference material. This reference material is produced from pulverized dogfish liver tissue and certified for a number of trace metals, including Cd (24.3 ± 0.8 μg g^{-1}), for method development and quality control in determination of trace metals in similar samples. Samples (ca. 50 mg, $n = 4$) from the bottled material were weighed into screw-capped Teflon tubes and solubilized by heating in 4 mL of 25% TMAH at 70 °C for 2 h. Another set of samples (50 mg, $n = 4$) were measured into Teflon vessels and spiked with 59.7 μL (1.215 μg Cd) of 100-fold diluted CdSe NP suspension (31.56 μg mL^{-1} CdSe NP) and heated in 4 mL 25% TMAH similarly. The mass of the spiked Cd was equal to the mass of Cd in 50-mg DOLT-4 sample (1.215 ± 0.004 μg).

The solutions from the dissolution were further processed with and without centrifugation to determine (a) if tissue Cd was quantitatively extracted into solution, (b) if the method was able to discriminate between free Cd (e.g., tissue Cd) and CdSe NPs. To do this, the digest from unspiked samples were first completed to 10 mL with deionized water. Then, 1 mL from each solution was taken and further diluted to 5 mL with 5% (v/v) HNO_3 (no centrifuge set). The remaining 9 mL were centrifuged at 6000 rpm for 1 h to precipitate suspending material, and then 1 mL of the aqueous layer was similarly diluted to 5 mL with 5% (v/v) HNO_3 (centrifuge set). The digests spiked with CdSe NPs were diluted to 10 mL with water and centrifuged 1 h at 6000 rpm similarly. One milliliter from each solution was then diluted to 5 mL with 5% (v/v) HNO_3. All solutions were analyzed for Cd by ICP-MS against aqueous calibration standards in 5% (v/v) HNO_3.

2.7. Samples and animal exposure

Liver and kidney samples were collected from male Sprague Dawley rats (3-weeks old with average body weight 60 ± 5 g). The rats were purchased from Harlan Breeding Laboratories in Indianapolis, Indiana, USA. They were housed in polycarbonate cages and acclimated for 2 weeks under 12-h light/dark cycle, 22 ± 2 °C, 50–60% relative humidity with water free access to food purchased from PMI Feeds Inc. (St. Louis, Missouri). The rats were exposed to CdSe NPs intraperitoneally according to the protocol (No: 08-09) approved by the Institutional Animal Care and Use Committee (IACUC). Before injection, the NP solutions were sterilized by passing through sterile 0.45 μm PTFE membrane filter. This method afforded effective for sterilization of thiol-capped NPs without any significant loss by the membrane. We opted for intraperitoneal injection of NPs, instead of intravenous, to maximize the NP accumulation in the body through multiple injections to achieve measurable differences between the free Cd and intact NPs.

The exposure scheme is summarized in Table 1. The rats were randomly divided into five groups (one control and four treatments; T-1, T-2, T-3 and T-4) with six rats in each group. The animals in T-1 and T-2 received 1.26 and 5.05 mg CdSe NP, respectively. To affect the NP stability, a portion of the NP solution was exposed to UV light for 2 h under a 100 W long-wave (365 nm) UV lamp at a distance of approximately 15 cm. The animals in T-3 and T-4 received 1.26 and 5.05 mg of UV-light exposed CdSe NPs, respectively. All injections were made in saline (0.9% w/v NaCl as carrier). Controls received saline solution. Occasionally, trace metal impurities may be introduced from food and saline during long period of exposure involving multiple injections. The control group was used primarily to ensure that Cd concentrations detected in the organs of the

Table 1
Experimental design for exposure of Sprague Dawley rats to CdSe NPs. Injections were made in saline (0.9% NaCl) intraperitoneally. In each administration, 0.5 mL solution (saline + NP) was delivered that contained either 80 or 320 μL of the sterile CdSe NPs solution (3156 μg mL^{-1} CdSe). Injections were made on the 1st, 4th, 7th, 10th, and 13th days of exposure. Each rat received 0, 1.26 or 5.05 mg NP after five injections.

	Control(n = 6)	No UV treatment		UV-exposed NPs	
		T-1(n = 6)	T-2(n = 6)	T-3(n = 6)	T-4(n = 6)
Volume of NP solution (μL)	0	80	320	80	320
Injection volume (mL)	0.5	0.5	0.5	0.5	0.5
Number of injections	5	5	5	5	5
Total NP mass (mg)	0	1.26	5.05	1.26	5.05

rats were due to the exposure to the NPs. A total of five injections (0.5 mL) were made intraperitoneally to each rat over the course of 15 days, once every three days (1st, 4th, 7th, 10th, and 13th day). On the 15th day, the rats were sacrificed under 100% carbon dioxide. The organs were collected and stored in phosphate buffered formalin until analysis.

2.8. Dissolution of animal tissues

To extract free Cd, liver and kidney samples were (ca. 0.25 g wet weight) were weighed into Teflon tubes and digested with 4 mL of 25% (m/v) TMAH (99.999%) at 70 °C for 2 h. The contents were diluted to 10 mL with deionized water and then centrifuged at 6000 rpm for 30 min. A portion (2 mL) of the centrifuged solution was transferred to 2 mL micro-centrifuge tube and re-centrifuged at 12,000 rpm for 1 h to completely separate the suspended tissue and intact NPs from the solution. At the end 0.5 mL of the final solution was diluted to 2 mL with 5% HNO_3 and analyzed by ICP-MS. Approximately, 50 mg sub-samples of Dogfish Liver Certified Reference Material (DOLT-4) were digested in 25% (m/v) TMAH and processed similarly as the liver and kidney samples.

For determination of total Cd content, the liver and kidney samples were also digested in concentrated nitric acid. In this case, approximately 0.25 g samples from each liver and kidney were digested in 3 mL of concentrated HNO_3 for 1 h at 120 °C. Samples of DOLT-4 were also digested in 3 mL HNO_3. All acid digests were diluted to 10 mL with water, and analyzed for Cd in 10-fold diluted solution by ICP-MS.

3. Results and discussion

3.1. Characterization of CdSe NPs

The thiol-capped CdSe NPs exhibited an absorption maximum at 559 nm with a strong fluorescence maximum at 621 nm as shown in Fig. 1A and B, respectively. A typical TEM image of the NPs is shown in Fig. 1C indicating that shape of the CdSe NPs is close to regular spherical nanocrystals. Averaged particle size was determined as 3.2 nm using the absorption maximum (559 nm) as described by Yu et al. [30]. A portion of the suspension (0.1 mL) was digested in 2 mL HNO_3 and analyzed for Cd and Se content. Cadmium and selenium concentrations were calculated as 2035 μg mL^{-1} Cd (18 mM) and 1396 μg mL^{-1} Se (16.5 mM), respectively, with Cd:Se molar ratio of 1.1:1. The molar concentration of CdSe was assumed to be equal to that of Se (16.5 mM) yielding 3156 μg mL^{-1} CdSe NPs.

3.2. Effects of ultrasounds on NP stability and extraction efficiency

The physical and chemical changes resulting from ultrasonic agitation of the NPs are illustrated in Fig. 2A. Nanoparticles were not affected from ultrasounds in water and alkaline TMAH. No significant changes were observed in the color of these solutions (Fig. 2A

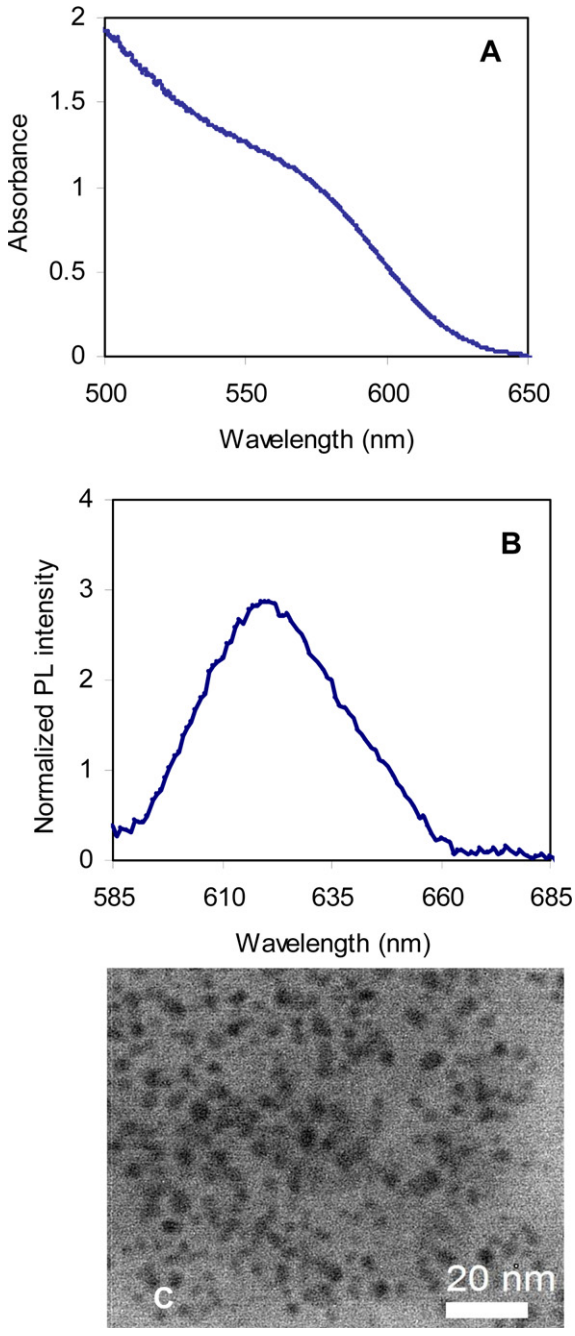

Fig. 1. (A) UV–vis absorption spectrum of thiol-capped CdSe NPs in water. (B) Fluorescence spectrum of the CdSe NPs. (C) TEM image of the thiol-capped CdSe NPs. Average particle size is 3.2 nm based on the absorption maximum of 559 nm.

inserted photograph). The UV–vis absorption spectra of these treatments overlapped with that of the control indicating that ultrasonic agitation did not significantly affect the stability of thiol-cap or the NP size. Conversely, NPs in 0.5% (v/v) HCl rapidly precipitated out of the solution (Fig. 2A inserted photograph), due to the removal of thiol coating in the acidic medium. The characteristic absorption maximum at 559 nm was completely lost for all treatments in 0.5% (v/v) HCl.

Free Cd concentrations determined by ICP-MS from the aqueous phase of the NP suspensions are illustrated in Fig. 2B as the average of triplicate treatments. The values were 2.2 ± 0.3, 3.2 ± 1.2, 2.7 ± 0.8 and $1475 \pm 52\ \mu g\ L^{-1}$ for control, water, 10% TMAH and 0.5% (v/v) HCl, respectively. Substantial amount of Cd was released from the NPs to solution in HCl, which is indicative of decomposition of NPs during precipitation. On the other hand, NPs did not exhibit any significant degradation under ultrasounds in water and 10% TMAH ($p = 0.054$). Based on these results, ultrasonic agitation of tissues in TMAH could enable separation of free Cd from intact NPs provided that free Cd in the tissue is extracted to solution quantitatively. The efficiency of this approach to extract free Cd from tissues was examined with Dogfish Liver (DOLT-4) certified reference material. Samples of DOLT-4 were sonicated 3–4 min at 80% P in 4 mL of 25% TMAH. The homogenates were centrifuged and aqueous phases were analyzed by ICP-MS. Unfortunately, it was not feasible to achieve quantitative extraction of tissue Cd in TMAH by ultrasonic agitation. The recoveries were less than 5% in all treatments.

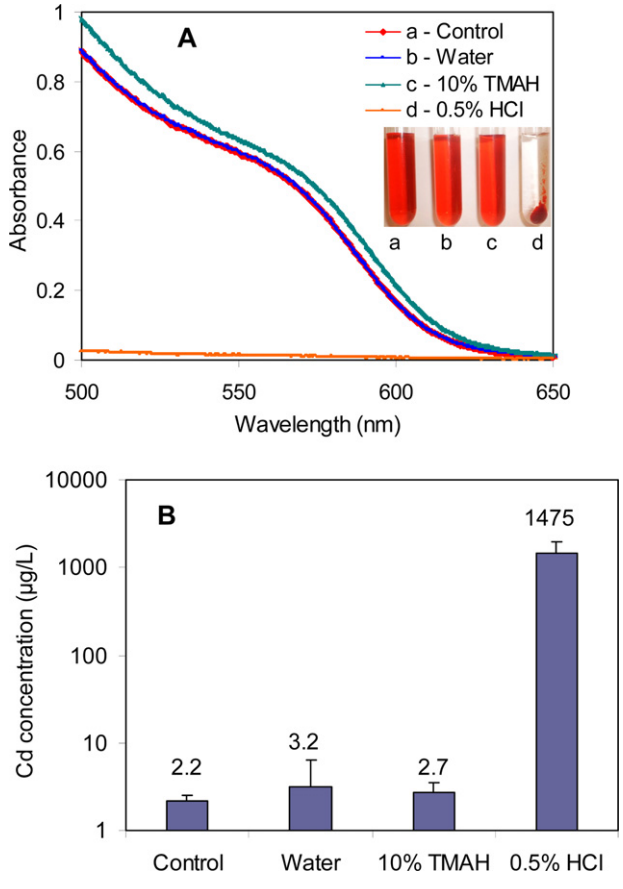

Fig. 2. Effects of ultrasonic agitation on the stability of CdSe NPs. (A) UV–vis absorption spectra for CdSe NPs exposed to ultrasounds in different solvents. Inserted photograph illustrates the stability of the NPs in each particular solvent. (B) Cadmium concentration resulting from degradation of CdSe NPs under ultrasounds. Values are average ± standard deviation for three replicates for each group ($n = 3$).

3.3. Effects of heating in TMAH on NP stability and extraction efficiency

The UV–vis absorption spectra of the solutions are illustrated in Fig. 3A. The NPs aggregated when heated in 10% TMAH solution as occurred in 0.5% (v/v) HCl, but those heated in water showed no sign of aggregation. The absorption maximum at 559 nm disappeared as the NPs precipitated out of the solution indicating that heating in TMAH resulted in the removal of thiol coating (Fig. 3A embedded photograph). Interestingly, this precipitation did not induce any significant disintegration of NPs as opposed to that occurred in 0.5% (v/v) HCl. Free Cd concentration in the TMAH treatments ($1.9 \pm 0.4\ \mu g\ L^{-1}$) was not statistically different from those heated in water ($1.8 \pm 0.6\ \mu g\ L^{-1}$) and control suspensions ($1.5 \pm 0.4\ \mu g\ L^{-1}$) (see Fig. 3B, $p = 0.068$).

Cadmium concentration from the NP suspensions spiked with $50\ \mu g\ L^{-1}$ Cd was measured as $46.2 \pm 5.3\ \mu g\ L^{-1}$ (%Recovery = 92.4 ± 10.6) (Fig. 3B last bar). This result points to the fact that the method was able to discriminate between free Cd (e.g., spike) and CdSe NPs when heated in TMAH. Within this context, the results demonstrate an added advantage of TMAH to selectively separate free metal ions and NPs from biological samples. Such a methodology affords an indispensible tool in elucidating the health

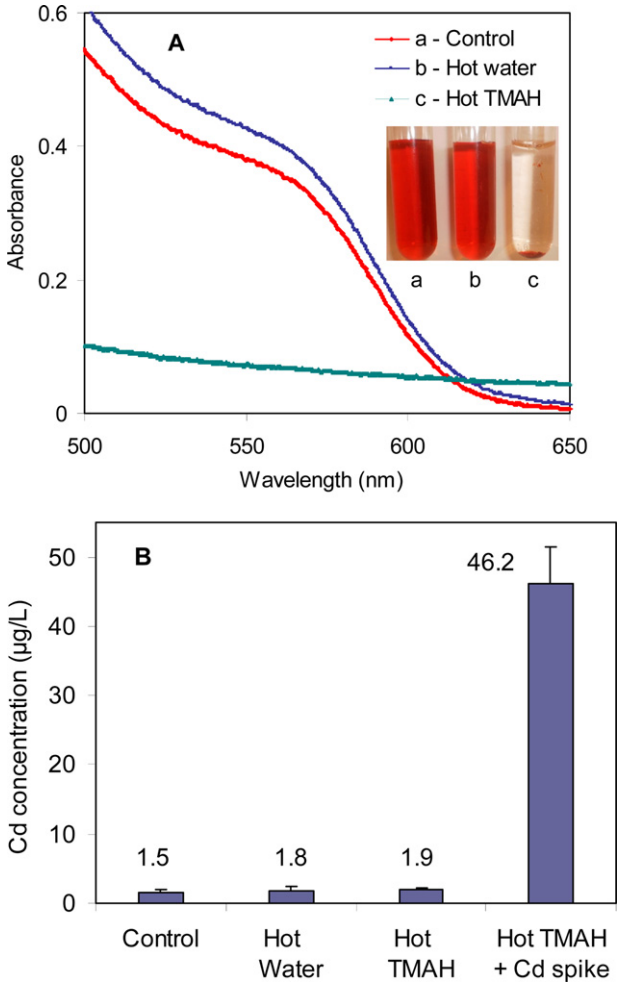

Fig. 3. Effects of heating in TMAH on thiol-capped CdSe NPs. (A) UV–vis absorption spectra from CdSe NPs after heating in water and TMAH. Inserted photograph illustrates the stability of the NPs in hot water and hot TMAH. (B) Free cadmium concentration measured in controls and NP samples heated in water, 10% TMAH. Spiked Cd concentration in NP solution is $50\ \mu g\ L^{-1}$. Values are average ± standard deviation for three replicates for each group ($n = 3$).

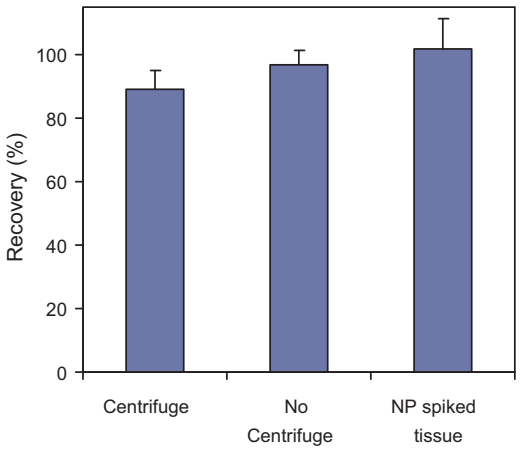

Fig. 4. Recoveries for Cd in Dogfish Liver Certified Reference Material (DOLT-4) by TMAH digestion at 70 °C for 2 h. Digests were analyzed for Cd before and after centrifugation to determine the extraction efficiency of Cd into solution ($n = 4$). Third column shows the recovery from DOLT-4 samples spiked with CdSe NP solution to yield Cd concentration equivalent to that in tissue (1.215 μg). Error bars are standard deviation for four separate measurements.

effects of variety of hazardous NPs, including metal oxides, metallic NPs (e.g., Ag and Cu) and fluorescing quantum dots.

3.4. Method validation

The recoveries for Cd are illustrated in Fig. 4 from dissolution of DOLT-4 samples in TMAH. In all scenarios, the recoveries were quantitative (e.g., >90%). Dry-weight concentrations from the samples were 23.6 ± 1.2 and $21.7 \pm 1.5\ \mu g\ g^{-1}$ before and after centrifugation of the solutions, respectively. Differences between centrifuge and no centrifuge groups were marginal ($p = 0.042$) at 95% confidence interval. The results were, however, not statistically different at 90% confidence level ($p > 0.10$), nor were they significantly different from the certified value ($24.3 \pm 0.8\ \mu g\ g^{-1}$) ($p > 0.10$), demonstrating that Cd in tissues was successfully extracted into the solution.

In the case of DOLT-4 samples spiked with CdSe NPs (e.g., 1.215 μg Cd from NPs), the NPs precipitated out of the solution during the dissolution as occurred in hot TMAH. Almost entire NPs precipitated before complete dissolution of the tissue samples. After centrifugation of the solutions, average Cd concentration was found to be $24.7 \pm 2.4\ \mu g\ g^{-1}$ (%R = 102 ± 9.8; see Fig. 4). Because CdSe NPs remain intact in hot TMAH, this concentration corresponds to the Cd extracted from the tissue, demonstrating the ability of the method to distinguish between the free and NP forms of Cd in a sample.

3.5. Distribution of CdSe NPs and free cadmium in animal tissues

The distribution of free Cd and NPs in the liver and kidney samples of the Sprague Dawley rats is illustrated in Fig. 5 as dry-basis. Detailed values are provided in Table S1 as electronic supplementary material. Total Cd content obtained from nitric acid digestion (dark bars) showed that NPs accumulated in liver to higher levels than kidney. Although not proportionately, the levels in the organs also elevated with increasing NP dose, which could be due to the higher retention of the NPs through peritoneum when NPs are injected intraperitoneally.

In in vivo studies, CdSe NPs and other similar fluorescing NPs are usually administered intravenously [1,11,24], Intradermal administration was also used to examine their penetration through skin [25–27]. Despite the differences in route of administration, liver and kidney are the major end-points of the NPs in this study and others [1,11,24–27], suggesting that the route of exposure has no significant influence on the accumulation profile of NPs in the body, but rather affect the accumulation rate. For instance, the highest Cd level was measured in the spleen (ca. $14\ \mu g\ g^{-1}$ wet weight) followed by the liver and kidney when mice were exposed to quantum dot 705 (CdTe NPs) intravenously [11], where 60 μg CdTe was given to each animal. The concentration of Cd in the liver and kidney samples after two weeks of exposure was approximately $5\ \mu g\ g^{-1}$ and $3\ \mu g\ g^{-1}$ (ca. 20 and $12\ \mu g\ g^{-1}$ dry-weight), respectively. Similar accumulation pattern was found in this study where total Cd levels were higher in the liver for all treatments (see Fig. 5 and Table S1). Yet, the accumulation rate in the organs (e.g., Cd-to-

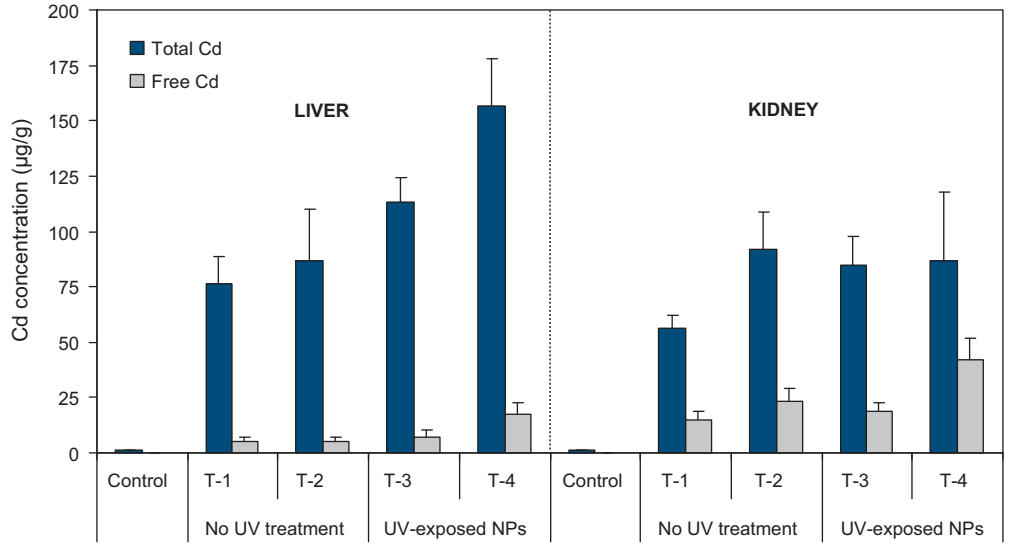

Fig. 5. Distribution of NPs and free Cd in the liver and kidney samples from rats exposed to thiol-capped CdSe NPs. The results are average ± standard deviation (dry-weight) from six rats for each treatment ($n = 6$, control, T-1, T-2, T-3 and T-4). Total Cd levels were higher in the liver than kidney. Free Cd ions accumulated in the kidney. Note that free Cd is present in both liver and kidney even NPs were not exposed UV-light (365 nm). Deliberate exposures of NPs to UV-light elevated the levels of NPs and free Cd in the organs.

NP mass ratio) was relatively low compared with the values from intravenous injection [11], suggesting a substantial loss of NPs.

Thiol compounds are known to be sensitive to UV light. Extensive exposure to light leads to degradation via oxidation of thiol groups. The CdSe NPs capped with thioglycolic acid ($HSCH_2COOH$) were stable for over six months when kept in dark at 4 °C. However, significant differences were detected between the accumulation patterns of the NPs before and after UV light treatment (Fig. 5 and Table S1), indicating a UV-light mediated deterioration of thiol coating. Cadmium levels elevated significantly in the organs when the NPs were injected after illuminating under UV light (365 nm) for 2 h. Total Cd content in the organs of the animals (T-3 and T-4) that received UV-exposed NPs was higher than those (T-1 and T-2) that received UV-unexposed NPs ($p = 0.032$).

Free Cd levels are also summarized in Fig. 5 to delineate the effects of UV-light exposure on NP stability. Unlike high total Cd levels in the liver, free Cd was found to accumulate largely in the kidneys. It is also important to note that there is considerable amount of free Cd in the samples of T-1 and T-2 groups that received UV-unexposed NPs, which points to the metabolic instability of thiol coating and the NPs in the body. Thiol-capped CdSe NPs were reported to be stable in vitro unless NPs were exposed to be UV-light (365 nm) [21]. However, the results in this study suggest that thiol coating was not sufficiently strong in vivo to prevent the release of free Cd to the body even if the NPs were not exposed to UV-light.

The exposure of the NPs to UV-light resulted in significant degradation of NPs as indicated by the elevated free Cd levels, especially in the kidney (Fig. 5). For groups T-1 and T-2 that received UV-unexposed NPs, the highest free Cd concentration was about 6.6% and 26.8% of the total Cd for the liver and kidney, respectively (Table S1). In the case of UV-exposed NPs (T-3 and T-4), free Cd level was relatively similar in the liver (6.0–10.9%), but those in the kidney increased to up to 47.9% of the total Cd (Table S1). These results suggest that exposure of CdSe NPs would be most detrimental to kidney since it appears to be the primary repository for Cd ions. Prolonged exposure may induce severe toxicity on kidney as NPs accumulated in the liver and other organs are gradually mobilized to kidney.

4. Conclusion

An alkaline dissolution procedure with tetramethylammonium hydroxide (TMAH) has been developed and applied to the determination of free Cd ions in the liver and kidney samples of rats exposed thiol-capped CdSe NPs. TMAH is known to be versatile reagent for dissolution of soft tissues. In this study, we have shown that TMAH also offers the capability for quantitative separation of Cd ions from tissues that contain CdSe NPs. The separation relies on the fact that TMAH does not impact the integrity of CdSe core of the NPs while outer thiol coating is readily removed. This method has the potential for assessment of in vivo and in vitro stability of numerous fluorescing nanoparticles and metal oxides, and therefore, affords a powerful and cost-efficient tool for extracting both free metal ions and intact NPs quantitatively from tissues.

It was found thiol-capped CdSe NPs were not fully stable in vivo. Cd ions were released to the body even if the NPs were protected from direct exposure to UV-light. These results raise concerns about common core-shell tactics (e.g., ZnS) and polymeric coatings (e.g., PEG and PEO) formulated to improve the stability of the CdSe core. Possible surface defects (cracks) in the outer shell or polymer coating could yield metabolically unstable NPs such that Cd ions could leak out to the body of the individual being exposed. It is therefore concluded that the experimental studies conducted to date are far from addressing the safety of CdSe NPs completely; extensive research is required to provide answers about their biocompatibility and pharmacokinetics.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhazmat.2011.05.003.

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