# Detection, Measurement and Toxicology of Semiconductor Nanocrystals in *Ceriodapnia dubia*

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## ABSTRACT

We have begun assessing the toxicology of commercially available fluorescent semiconductor nanocrystals that contain toxic metals. We have found that:

1) Fluorescence of the aquatic invertebrate *Cerodaphnia dubia* increased significantly after a 4 hr exposure to 200 ppt nanocrystals. Changes in fluorescence were time and dose dependent.

2) 48-h acute toxicology endpoint assessment of these nanocrystals (using US EPA standard protocol) found no measurable toxicity at concentrations as high as 100 ppb ([Cd] in 100 ppb nanocrystals was >7000 ppb). Measured Cd LC50 in 48-h laboratory exposures was 28.55 ppb.

3) Inductively coupled plasma mass spectrometry (ICP-MS) can be used to detect and measure Cd and Se in organisms exposed to these nanocrystals.

These results suggest that coatings present on nanocrystals provide a protective measure from toxic metals during acute exposures.

## INTRODUCTION

Engineered nanomaterials which differ in size, shape, surface area and chemical composition are currently used in research applications, and, on a larger scale, in industrial and consumer applications. Concerns for environmental effects due to the increased use of nanoparticles are commonly addressed in current literature. The need for proactive research prior to large scale commercial applications, environmental risk assessments, and environmental monitoring have been proposed ([1-3]). Although carbon-nanostructures have received some toxicological characterization ([1 & 4]) few results of research about the toxicology of other nanoscale particles has been reported.

Because of the known toxicity of Cd and Se used in microcrystal nanoparticles, the potential results of exposure to these nanomaterials warrant thorough investigation. These nanoparticles, depending on size and composition, fluoresce at visible wavelengths and are commercially available from a number of sources. They have the distinct advantage over other available fluorophores of providing long-lasting stable fluorescence that is extremely fade resistant. They also have a relatively broad excitation spectrum but can be engineered to produce a relatively narrow emission spectrum. Because of these advantages and the ease with which the surface of the nanoparticle can be modified, these nanoparticles are finding wide spread use in applications, such as *in vivo* and *in vitro* markers, labels and trackers.

We describe the results of a series of studies designed to characterize the acute toxicity of semiconductor nanocrystals using a standard test organism, *Ceriodaphnia dubia*.

## METHODS

### 2.1 Organisms

*Ceriodaphnia dubia* (Arkansas State University Ecotoxicology Research Facility, Jonesboro, AR, USA) were grown in accordance with US EPA 2002 standards [5]. Moderately hard water (MHW, hardness = 90 mg/L as $CaCO_3$). was used for daphnia maintenance and solutions. Organisms were exposed to ambient light and a temperature of ~22$^o$ C was maintained. Organisms were fed daily (1.5 ml per 100 ml water/15 organisms) with a combination of lab produced algae and yeast-cereal leaves-trout chow diet (YCT) for *C. dubia*.

### 2.2 Nanocrystals

Qdot® 545 ITK™ Carboxyl Quantum Dots (Fisher Scientific; Fisher part no Q21391MP) were used in all experiments. These semiconductor nanocrystals contain a Cd/Se crystalline core (~4 nM dia) that is coated with a shell of ZnS. This shell is coated with an organic polymer that can be treated so that active residues are available for linking to other molecules (e.g. antibodies, lectins and nucleic acids). The nanocrystals used in these experiments had available carboxyl groups on their surface.

### 2.3 Microscopy

Organisms were exposed to nanocrystals by incubating 12 organisms in 100 ml of MHW containing 200, 400 or 600 ppt nanocrystals. Control organisms were treated identically except that nanocrystals were not added to MHW. Eight organisms were removed for examination 4, 8 and 24 hrs after exposure began. This process was repeated 3 times so that a total of at lest 24 organisms were examined at each time and exposure dose. All images were taken using a Nikon epifluorescence (Nikon Eclipse E800, excitation λ 465-495:



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emission λ 515-555 nm) microscope equipped with equipped with Hg-vapor illumination and a Cascade Photometric digital camera. At each sampling time, 8 organisms were randomly chosen from each solution and placed on a glass slide in MHW. A brightfield image of each organism was taken. To insure consistent focus images in which the post abdominal claw was in clear focus were made. A fluorescent image of was then taken without altering the focus. Images were digitized using Nikon ACT-1© software and stored for further analysis.

## 2.3 Image analysis

MetaMorph© 6.10 Meta Imaging Series Environment (Universal Imaging Corporation™) was used to measure average pixel intensity in the fluorescence image of each organism. The outline of each organism (not including the dorsal brood pouch) was traced on the brightfield image of that organism. This outline was transferred to the fluorescence image of that organism (see **Figure 1**). The number and average intensity of the pixels within the outline on the fluorescence image was measured. The number of pixels in each outline was used to verify that the size of the organisms was not affected by exposure dose or time.

## 2.4 Statistics

Average pixel intensity and average number of pixels in each fluorescence image in images from unexposed (control) organisms and exposed organisms were compared (the Students T test, Prism© 4.0 (GraphPad™ Software, San Diego, CA).

## 2.5 Toxicology methods

Forty-eight hour acute toxicity in aqueous samples was assessed with *C dubia* (water flea) using standardized methods [5]. Serial dilutions in [6] MHW resulted in Quantum Dot™ nanocrystal concentrations up to 110 µg/L. Organisms were also exposed to dilutions of CdCl • 2.5 $H_2O$ in MHW to determine 48-hr survival endpoint. Results from the toxicity assays were statistically analyzed using Toxcalc® (version 5.0.25). LC50s were calculated using measured Cd concentrations and corresponding response from exposed organisms. All LC50 determinations and related confidence intervals were determined using Probit analysis. Data were tested using $\alpha = 0.05$ and the normality assumption was tested using Shapiro-Wilk's test. Steel's Many-One Rank test was used to determine significance in survival.

## 2.6 ICP-MS Methods

Cd levels in final dilutions were measured via dynamic reaction cell inductively coupled plasma mass spectrometry (DRC-ICP-MS) EPA method 200.8 [7] (Nebulizer gas flow; 1.0 L/min, RF Power; 1200 W, Analog state voltage; 1800 V, Pulse stage voltage 1100 V). Data were analyzed using ELAN® software ICP-MS version 3.0 Instrument control software for DRC II by Perking Elmer SCIEX.

# RESULTS AND DISCUSSION

## 3.1 Microscopy

Untreated organisms exhibited slight autofluorescence. This was most evident in the digestive tract and was not influenced by incubation time (see **Figure 1**). Treated

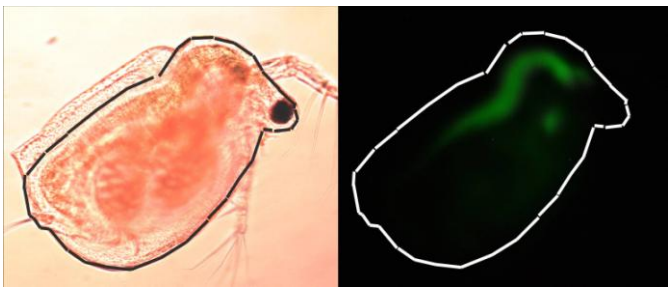

**Figure 1 Images of unexposed daphnid**. Brightfield (right) and fluorescent (left) image of an unexposed daphnia. The outline shows the area within which pixel intensity was measured in the fluorescent image

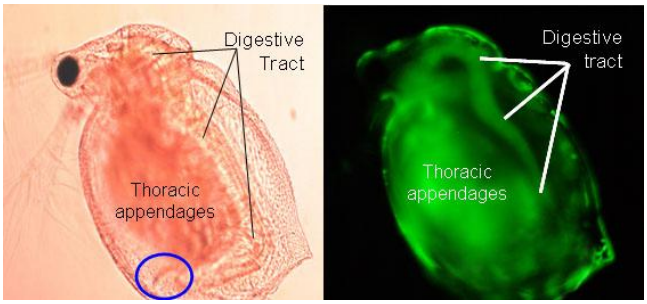


**Figure 2;** Images of exposed daphnid. Brightfield (right) ad fluorescent (left) images of a daphnia that had been exposed to 200 nM Q-dots for 8 hrs. The abdominal claw that was used as a focus point is circled in the brightfield image. Comparison to Figure 1 shows much greater intensity of staining. Note that distribution of Q-dots is not uniform.

organisms showed significant increases in fluorescence (compare fluorescence images in **Figure 1 and 2**). At all exposure concentrations there were significant increases in fluorescence within 4-8 hrs of exposure but by 24 hrs after exposure began, there was no exposure-related significant difference in fluorescence intensity (**Figure 3**). Internal structures were clearly visible and differentially labeled in treated organisms The fluorescence intensity in the digestive tract of exposed organisms was greater than that of unexposed organisms but the greatest increase in fluorescence was in the region of the thoracic appendages (compare **Figures 1 & 2**). Based on measurements of the number of pixels in each fluorescence image there was no significant difference in the

average size of daphnia in any treatment group or at any sampling time..

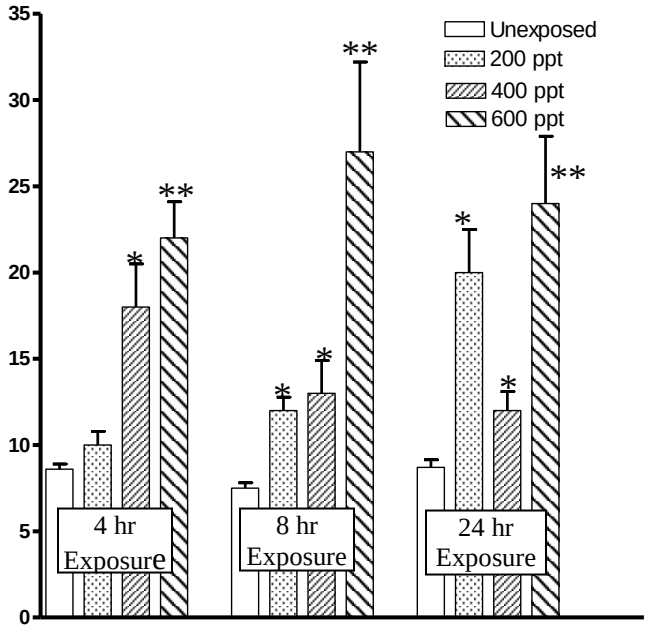


**Figure 3:** Average pixel intensity in images of daphnia exposed to various concentrations of semiconductor nanocrystals. All values are average pixel intensity of 16 organisms. ***** = significantly different from unexposed organisms ($p<0.03$). ** = different from unexposed, 4 & 8 hr exposure ($p<0.05$)

Fluorescence of individual *C. dubia* increased with exposure time.. Internal structures were clearly visible and differentially labeled in treated organisms suggesting that particles were absorbed into body tissues and not simply adsorbed onto the organisms' surface. For example, the heart exhibited no visible fluorescence after 4 hr, while the digestive tract and muscles of the thorax were brightly fluorescent. Thus, this organism may be useful in assessing environmental effects of semiconductor nanocrystals.

## 3.2 Toxicology

Cd 48-h LC50s (as $CdCl \bullet 2.5\ H_2O$) measured through *C. dubia* exposure were 31.91 and 25.19 µg/L. This compares favorably with reported values of 54 - 59 µg/L [8-10]. However, 48-h acute toxicology endpoint assessment of these nanocrystals (using 48-hr US EPA standard test protocol; [5] found no measurable toxicity at concentrations as high as 100 µg/L nanocrystals. ICP-MS was used to measure the nominal concentration of Cd in the CdCl solutions and nanocrystal exposure solutions (Table 1). The measured [Cd] in 100 ppb nanocrystals was >7000 ppb. Organisms were maintained for an additional 48 hrs in nanocrystal solutions, but no lethality was observed after a 98 hr exposure.

**Table 1**. Nominal and measured Cd values in final dilutions used in 48-hr *C dubia* exposures

| Exposure 1 | | | Exposure 2 | | |
|---|---|---|---|---|---|
| Nominal [Cd] | Measured [Cd]* | % Survival | Nominal [Cd] | Measured [Cd]* | % Survival |
| 0.0 | 0.0 | 95 | 0.0 | 0.0 | 100 |
| 20.0 | 23.8 | 75 | 20.0 | 21.4 | 60 |
| 30.0 | 31.4 | 25 | 30.0 | 23.1 | 45 |
| 40.0 | 45.3 | 40 | 40.0 | 33.7 | 50 |
| 60.0 | 62.4 | 20 | 60.0 | 51.1 | 10 |
| 80.0 | 82.1 | 0 | 80.0 | 66.6 | 5 |

* Measured using DRC ICP-MS

Although these nanoparticles also contain Se, the effects of Se were not directly measured. Acute (48 hr) LC50 for Se has been reported as 1864 and 1822 ppb with ambient sulfate concentrations of 55 and 98 mg/L, respectively [11]. Nanoparticle concentrations in this study were suspended in MHW with sulfate concentrations of 81.25 mg/L. so organisms were exposed the Se concentration could also have exceeded its reported 48 hr LC50.

These results indicate that even though semiconductor nanocrystals containing toxic metals can be absorbed by aquatic organisms, the Cd (and Se) in such nanoparticles is not biologically active during acute exposures. This suggests that either the crystalline structure or the outer coating of organic polymer prevents the Cd (and Se) from being available to exposed organisms.

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