Supporting Information

Nanometals induce stress and alter thyroid hormone action in amphibia at or below North American water quality guidelines

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Content

Supplementary Experimental Section

Particle Characterization. The zeta potential of each NP was determined using a PALS Zeta Potential Analyzer Ver. 3.57 (Brookhaven Instruments Corporation, Holtsville, NY). Particle size and distribution information was provided by J. Dinglasan (ViveNano) and was further assessed by transmission electron microscopy (TEM) and dynamic light scattering (DLS), respectively. NP metal content was measured for the specific lots by ViveNano using inductively coupled plasma (ICP) analysis.

Using TEM, we also confirmed NP sizes in the context of tissue culture medium. Ten nM solutions of nanosilver, QDs, and nanozinc oxide were prepared in the medium used for tail fin biopsy culture. The samples were vortexed but not sonicated. Each solution was placed on one of two polymer grids and dried for approximately 20 min in a low-temperature oven to remove solvent. Samples were then scanned using a Hitachi H-7000 transmission electron microscope with an AMT 2k X 2k CCD camera. This instrument provides a resolution sufficient to be able to assess whether or not a particle is less than 10 nm in diameter.

Isolation of Total RNA and Quantitation of Gene Transcripts. Mechanical disruption utilized 300 μl TRIzol reagent, a 1 mm diameter tungsten-carbide bead, and safe-lock Eppendorf 0.5 ml microcentrifuge tubes in a Retsch MM301 Mixer Mill (Fisher Scientific, Ottawa, ON) at 20 Hz two times for 1.5 min with the chambers being rotated in between the cycles. Twenty μg of glycogen (Roche Diagnostics, Laval, PQ) were added prior to isopropanol precipitation to maximize RNA yield. Isolated RNA was subsequently resuspended in 10 μl diethyl pyrocarbonate (DEPC)-treated RNase-free water and stored at -70ºC. cDNA was synthesized from 5 μl (~0.5 μg) total RNA as per the manufacturer's protocol using the RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas, Burlington, ON) with minor modifications: RNA was first annealed with 200 ng random hexamer primer in the presence of dNTPs and then cDNA was synthesized by adding the mixture of reaction buffer, ribonuclease inhibitor and RevertAid H Minus M-MuLV reverse transcriptase and incubating at 25ºC for 10 min and then at 42ºC for 1.5 h. The cDNA products were diluted five-fold prior to PCR amplification and stored at -20ºC. The steady-state levels of the *TRβ*, *RLKI*, *HSP30*, *CAT*, and ribosomal protein L8 (*rpL8*) transcript were analyzed using a MX3005P real-time quantitative PCR system (Stratagene, La Jolla, CA). Each 15 μl amplification reaction contained 10 mM Tris-HCl (pH 8.3 at 20ºC), 50 mM KCl, 3 mM $MgCl_2$, 0.01% Tween 20, 0.8% glycerol, 40,000-fold dilution of SYBR Green I (Molecular Probes Inc., Eugene, OR), 200 μM dNTPs, 69.4 nM ROX reference dye (Invitrogen), 2 μl of diluted cDNA, and one unit of Hot Start Taq DNA polymerase (Fermentas), and a gene-specific primer pair including 5 pmol of *rpL8*, *RLKI*, *HSP30*, or *CAT* primers or 10 pmol of *TRβ* primers; (*TRβ*: up 5'-AGCAGCATGTCAGGGTAC and down 5'-TGAAGGCTTCTAAGTCCA; *rpL8*: up 5'-AGGGGACAGAGAAAAGGTG and down 5'-TGAGCTTTCTTGCCACAG; *RLK I*: up 5'-GTTGGCGTTGGTGTTAGCGG and down 5'-GGCACTGCTTCTTGCAACTTG; *HSP30*:

up 5'-GCCTCCACCAGACT and down 5'-GTCTCCTTCCTTCCG; *CAT*: up 5'-GAATGGTTACGGCTCACA and down 5'-GCAATGGCTTCATACAGA). The thermocycle program included an initial enzyme activation step at 95ºC (9 min) followed by 40 cycles of 95ºC denaturation (15 sec), 55ºC (*rpL8*, *TRβ*, and *RLKI*), 60°C (*CAT*) or 61°C (*HSP30*) annealing (30 sec), and 72ºC elongation (45 sec). Controls lacking cDNA template and an inter-plate standard containing a mixture of cDNA were included to determine the specificity of target cDNA amplification as well as the quality of each QPCR run. The integrity of amplification reactions was confirmed by the presence of a single DNA product following gel electrophoresis and by amplicon sequencing. Additionally, the efficiency of the target amplifications across the gene-specific primer sets used was validated to be approximately equal allowing the use of the comparative Ct method (1). Quadruplicate reactions were performed for each sample and data were averaged and normalized to the expression of the invariant control gene encoding the *rpL8*.

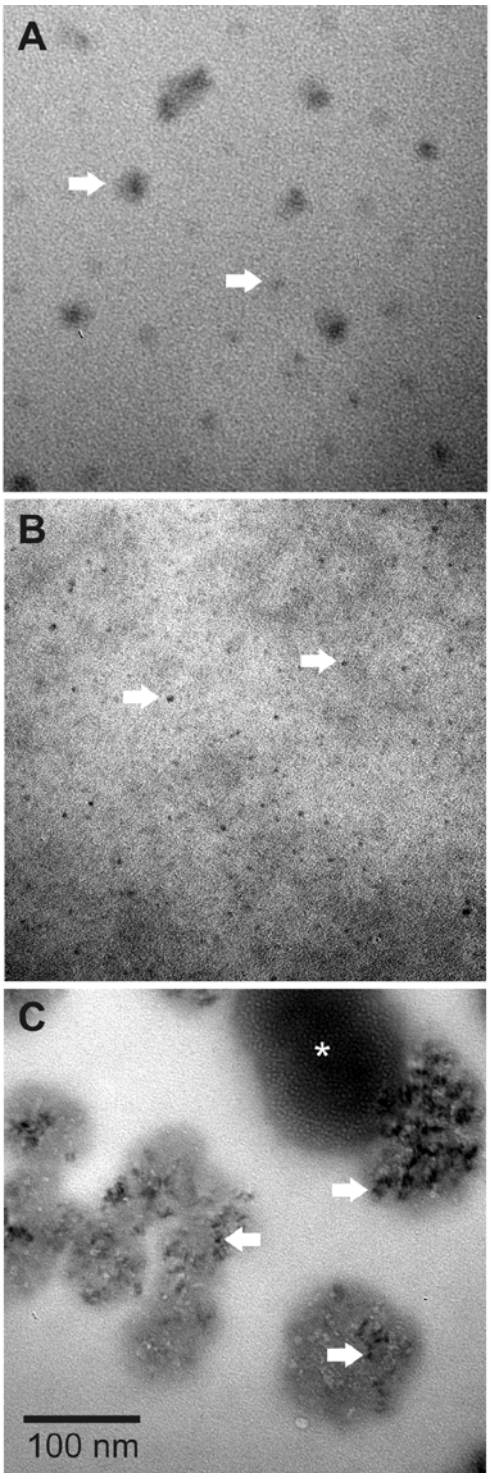
Determination of Acute Toxicity. The biological test method was adapted from the Environmental Protection Series Biological Test Methods: Acute Lethality Test Using Rainbow Trout Report EPS 1/RM/9 July 1990 with amendments in May 1996 and May 2007 and Reference Method for Determining Acute Lethality of Effluents to Rainbow Trout Report EPS 1/RM/13 Second Edition-December 2000 with amendment in May 2007.

The acute toxicity studies used green QDs from ViveNano at a stock concentration of 20 mg/mL and nanosilver at a stock concentration of 1.5 mg/mL. Ten tadpoles per 30 L tank were exposed to nanosilver or silver nitrate in triplicate at 22°C. Five concentrations and a well water control were used for the nanosilver and silver nitrate LC_{50} tests. A 5-fold dilution series was used to test nanosilver, including 0.0024,

0.012, 0.06, 0.03 and 1.5 mg/L nanosilver particles (equivalent to 0.7 µg/L - 0.44 mg/L silver). The silver nitrate tests were conducted using a standard toxicological logarithmic dilution series of 0.1, 0.18, 0.3, 0.56, 1.0 mg/L silver. Due to scarcity of material, tadpoles were exposed to a single maximum possible concentration of 100 mg/L green QD particles (equivalent to 10.6 mg/L cadmium). These tests were conducted using 3 animals per 3 L vessel in triplicate at 22°C.

The toxicity data was analyzed statistically using CETIS™ Version 1.1.2 (Comprehensive Environmental Toxicity Information System, Tidepool Scientific Software, McKinleyville, CA) in accordance with Environmental Protection Series: Guidance Document on Statistical Methods for Environmental Toxicity Tests EPS 1/RM/46 March 2005 with June 2007 amendments. The LC_{50} and its 95% confidence limits were derived by statistical analysis of mortalities using Probit analysis. The LC_{50} is the median lethal concentration (i.e. the concentration of material in effluent that is estimated to be lethal to 50% of test organisms).

Supplementary Figure 1. Transmission electron micrographs of the nanosilver, quantum dots, and nanozinc oxide samples used in the present study. Ten nM solutions of (**A**) nanosilver, (**B**) quantum dots, and (**C**) nanozinc oxide were prepared in 70% Leibovitz L-15 medium and scanned using a Hitachi H-7000 transmission electron microscope at 150,000X magnification. All samples showed electron-dense regions corresponding to the presence of metals (examples are indicated by the arrows). The region indicated by an asterix is due to matrix organics. Due to differential flattening artifacts that can occur during drying of the samples onto the polymer matrix that can make features appear larger than they are in solution and the limit of resolution of the instrument, an exact measurement of the NP dimensions was not feasible below ~10 nm. However, we were able to confirm that nanosilver, QDs, and nanozinc oxide particles had diameters in the low nanometer range consistent with the manufacturer's information provided. Nanosilver contained particle sizes from <10 nm to approximately 20 nm in diameter (A). Green QD particle sizes were <10 nm (B) and the nanozinc oxide appeared as aggregates of multiple <10 nm particles (C).



Literature Cited

(1) Livak, K. J.; Schmittgen, T. D., Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **2001,** *25*, 402-8.