Sulfidation as a Natural Antidote to Metallic Nanoparticles Is Overestimated: CuO Sulfidation Yields CuS Nanoparticles with Increased Toxicity in Medaka (*Oryzias latipes*) Embryos

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S Supporting Information

ABSTRACT: Sulfidation is considered as a natural antidote to toxicity of metallic nanoparticles (NPs). The detoxification contribution from sulfidation, however, may vary depending on sulfidation mechanisms. Here we present the dissolution–precipitation instead of direct solid-state-shell mechanism to illustrate the process of CuO-NPs conversion to CuS-NPs in aqueous solutions. Accordingly, the CuS-NPs at environmentally relevant concentrations showed much stronger interference on Japanese medaka (*Oryzias latipes*) embryo hatching than CuO-NPs, which was probably due to elevated free copper ions released from CuS-NPs, leading to significant increase in oxidative stress and causing toxicity in embryos. The larval length was significantly reduced by CuS-NPs, however, no other obviously abnormal morphological features were identified in the hatched larvae. Co-introduction of a metal ion chelator [ethylene diamine tetraacetic acid (EDTA)] could abolish the hatching interference induced by CuS-NPs, indicating free copper ions released from CuS-NPs play an important role in hatching interference. This work documents for the first time that sulfidation as a natural antidote to metallic NPs is being overestimated, which has far reaching implications for risk assessment of metallic NPs in aquatic environment.

INTRODUCTION

Over the past decade, CuO-NPs have been widely used in industrial products and processes due to their extraordinary electric and catalytic properties.^{1–4} Also, CuO-NPs display enhanced antimicrobial activity toward a broad spectrum of microorganisms, leading to their use in consumer products.^{5,6} Most, if not all, of these uses would increase the potential for their widespread release into the waters, and bring potential environmental risks by CuO-NPs.^{7,8}

Metallic NPs readily undergo transformations in the natural environment, which dramatically affects their toxicity as well as ecological risks in return.^{9–16} Among the transformations, sulfidation as a natural antidote to metallic NP toxicity has drawn a great deal of attention over the past years.^{17–24} There is a general consensus that the use of metallic NPs would not cause a safety problem for natural environment due to the detoxification contributions from sulfidation.^{18,19} For instance, Ag₂S-NPs, formed through a direct solid-state-shell pathway, could greatly reduce the toxicity of Ag-NPs to organisms, i.e., zebrafish, killifish, nematode worm, least duckweed, and *Escherichia coli* because of the great reduction in the silver ions released due to the limited Ag₂S-NPs dissolution compared to Ag-NPs.^{19–22} CuS particles were reported to be much less cytotoxicity than CuO particles due to the low

solubility of CuS in cell culture media [i.e., Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum (FBS)].²⁵

Nevertheless, previous study²⁵ was conducted in cell culture media with 5 mg/L particles rather than aqueous solutions where aquatic organisms are indeed daily exposed to the particles at environmentally relevant concentrations. Also, even if CuS released copper ions, due to the presence of phosphate in cell culture media, the formation of copper phosphate with lower solubility than CuS could mitigate the cytotoxicity, which would overestimate the detoxification contributions from sulfidation. Alternatively, copper has a higher affinity for sulfide than that of silver, indicating that CuO-NPs might undergo sulfidation by a different mechanism than Ag-NPs, which accordingly results in variant toxicological implications. Thus, understanding how CuO-NPs sulfidize in aqueous solutions and toxicological responses of the sulfidized particles at environmentally relevant concentrations to aquatic organisms

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is essential to accurately evaluate the detoxification contributions from sulfidation.

Here we present a dissolution–precipitation mechanism to illustrate the process of CuO-NP conversion to CuS-NPs in aqueous solutions with varied molar ratios of S/CuO. Accordingly, we document for the first time that the CuS-NPs have much stronger interference on Japanese medaka (*Oryzias latipes*) embryo hatching than CuO-NPs. Furthermore, we investigated the potential mechanism for the stronger hatching interference of CuS-NPs than CuO-NPs. Collectively, these findings are very important to comprehensively understand the implications of metallic NPs sulfidation in the aquatic environment.

MATERIALS AND METHODS

Sulfidation of CuO-NPs. Sulfidation of CuO-NPs was conducted in 50 mL centrifuge tubes (polypropylene) by mixing Na₂S (0.4 M) of NaNO₃ (10 mM) solution with CuO-NPs (12.5 mM) in the presence of dissolved oxygen (DO) at room temperature, which is comparable to the Ag-NPs sulfidation.^{19,21,22} Briefly, adding CuO-NPs (40.0 mg, Sigma-Aldrich, U.S.A.) to 10 mM of NaNO₃ solution and subjecting the suspensions to ultrasonication for 10 min, and then designed volumes of Na₂S were added to change molar ratio of S/CuO. Before mixing, free copper ions released from CuO-NPs were removed by centrifugation (9500 rpm for 30 min) and washed with 10 mM of NaNO₃ for three cycles. The solutions were stirred (120 rpm at room temperature) for a designed time at pH 10.0 ± 0.2 to allow the reaction to occur. Afterward, the final solutions were centrifuged (9500 rpm for 30 min) and washed with ultrapure water three times followed ethanol washing one time. Finally, the deposits from the final wash were dried at room temperature to obtain the solid sulfidized particles. The influence of natural organic matter (NOM, humic acid from Sigma-Aldrich) on CuO-NPs sulfidation was carried out in the presence of NOM (0–100 mg/L) to represent typical surface, groundwater, and wastewater concentrations.^{26–28}

Physicochemical Characterization of CuO-NPs and Solid Products of CuO-NPs Sulfidation. The ξ potential and hydrodynamic diameter of particles were determined using a Malvern Zetasizer Nano Series dynamic light scattering (DLS) instrument. Stock solutions with concentration of 50 mg/L were sonicated (5 min) before samples (10, 100, and 200 μ g/L) preparation for DLS measurement, and three independent measurements were taken with each measurement consisting of 11 runs. The lattice fringe of samples was determined on a JEOL JEM-2100F high-resolution transmission electron microscope at an accelerating voltage of 200 kV, and the elemental composition of imaged objects was established using the energy-dispersive X-ray (EDX) detector (Oxford Inca). The morphology of particles was observed using transmission electron microscopy (TEM) on a HITACHI-H7500 at an accelerating voltage of 80 kV. The TEM samples were prepared by depositing 60–100 μ L of NPs suspension (~50 mg/L) on carbon-coated nickel grids, followed by drying at room temperature overnight. The X-ray photoelectron spectroscopy (XPS) spectra of the samples were recorded using a Kratos Axis Ultra DLD spectrometer (Kratos). Crystal structures of the samples were measured by X-ray diffraction (XRD) using a PANalytical X'Pert PRO diffractometer with Cu K α radiation (λ : 0.154 nm). It should be noted that the XRD pattern from the standard CuS particles (JCPDS reference

code: 00–001–1281) was used as CuS standard reference spectrum. Data were collected with continuous scanning 2 θ from 20° to 80° at 0.0263° per step.

Exposure of Medaka (*Oryzias latipes*) Embryos and Adults to NPs. Japanese medaka (*Oryzias latipes*) were cultured at the Research Center for Eco-Environmental Sciences (Chinese Academy of Sciences, Beijing) and housed in medium-hard water at 25 °C and a photoperiod of 14 h light and 10 h dark. In general, adults were fed twice daily a diet of live brine shrimp. The embryos were collected, counted, and rinsed several times in ultrapure water to remove any residue on the embryo surface. Twelve healthy embryos [~4 h postfertilization (hpf)] were incubated in each well of 24-well transparent plates (NEST Biotech, U.S.A.). Three mL of CuSO₄ or Cu(NO₃)₂ or NPs suspension at designed concentrations (10, 100, and 200 μ g/L) was added to each well. Three replicate trials were conducted in this study. Throughout the whole exposure period (8 d), copper salts and NPs solutions were changed every 24 h, and the development status of the medaka embryos was observed under an inverse microscope (×4–100, Olympus BX41, Japan) to assess embryos mortality, hatching rate and morphological abnormalities. The end points used to assess developmental toxicity included embryo survival and hatching rates. Malformations were described and documented among the larvae from both the control and treatments.

In addition to embryos, Japanese medaka adults (6-month age) were also exposed to CuO-NPs, CuSO₄ or sulfidized products of CuO-NPs to determine their actual toxicity (48 h). Briefly, each 5 L glass jar containing 8 medaka adults were housed in medium-hard water at 25 °C and a photoperiod of 14 h light and 10 h dark. The medaka adults were exposed to CuSO₄ or NPs solutions at the concentrations of 0, 10, 25, 50, 100, 200, 500, and 2000 μ g/L, and the solutions were changed every 24 h. Meanwhile, the number of deaths was recorded to calculate the LC₅₀.

Dissolution of NPs. The dissolution of CuO-NPs and solid products of CuO-NPs sulfidation was determined by quantifying free copper ions in solution with an initial concentration of total copper (100 mg/L). In order to investigate the effect of DO on the NPs dissolution, we conducted the dissolution experiments in the presence and absence of DO respectively; a group of samples were conducted using ultrapure water with ~6.1 mg/L DO, and another group of samples, using N₂-purged (12 h) deoxygenated ultrapure water, were conducted in a glovebox with N₂ headspace. The initial pH of these samples was changed to 7.2 ± 0.2. All the samples were stirred (100 rpm) at room temperature. Three mL of each sample was collected from the reactors at designed time (from 0 to 168 h), and particles were separated from the supernatant by using Amicon Ultra 3 kDa MWCO centrifuge tubes. Recovery efficiencies for the processing of 5 mg/L and 10 mg/L solution of dissolved copper (CuSO₄) yielded 95.6 ± 1.9% and 103.7 ± 2.6%, respectively, presenting that negligible loss of the analyte occurred during the ultrafiltration step. Copper ion concentration of the supernatants was determined using an inductively coupled plasma optical emission spectrometry (ICP-OES, Thermo, U.S.A.). Copper ion release (%) was calculated as percentage of the starting copper concentration.

Electron Paramagnetic Resonance (EPR) Analyses. The X (9.8 GHz)-band EPR analyses using an EMX-plus CW spectrometer (Bruker Biospin) and the spin-trap 5,5-dimethyl-

Figure 1. Characterization of CuO-NPs used in the present study. (A) Low-resolution TEM was used to show the size and shape of CuO-NPs. Image analysis revealed that CuO-NPs had an average size of 36.1 nm with a standard deviation of 6.4 nm. (B) Size distribution of CuO-NPs ($n = 120$). (C) High-resolution TEM image of the CuO-NPs showing the lattice fringe of 0.261 nm. (D) XRD spectrum was used to confirm the crystalline structure of CuO-NPs.

1-pyrroline-*N*-oxide (DMPO, Sigma-Aldrich, U.S.A.) could show a relative reactive oxygen species (ROS) intensity by observing peak height. The analyses were performed following the method as previously described by Wang et al.²⁵ Briefly, 10 mg/L copper particles or CuSO_4 were mixed with DMPO (100 mM) and H_2O_2 (1 mM) in buffer for 20 min. The reaction was triggered by the addition of H_2O_2 . Afterward, the solution was injected into a 50 μL capillary (0.9 mm OD) which was subsequently inserted into a quartz EPR capillary tube (3 mm OD $\times$ 250 mm L $\times$ 0.5 mm wall). Microwave power, 20 mW; scan width, 100 G; sweep time, 60 s; center field, 3511 G; modulation amplitude, 1 G; Time constant, 20.48 ms.

Assessment of ROS Accumulation and Total Glutathione (Reduced) Content in Medaka Embryos. Formation of ROS in medaka embryos was measured spectrophotometrically using an oxidation-sensitive fluorescent probe, 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) as previously described.²⁹ Briefly, the embryos (~ 4 hpf) were treated with various forms of copper, that is, CuO-NPs, CuSO_4 and sulfidized CuO-NPs, respectively. After 48-h exposure, the embryos were transferred into 96-well plates and treated with DCFH-DA solution (50 μM), and the plates were incubated for 1 h in the dark at room temperature. After incubation, the embryos were rinsed in ultrapure water and then the fluorescence was measured using a microplate spectrofluor-

ometer (Thermo, U.S.A.) with excitation and emission wavelengths at 485 and 530 nm, respectively. After medaka embryos were exposed to the various forms of copper for 48 h, the reduced glutathione (GSH) levels were measured by Glutathione Assay Kit (Promega, U.S.A.) following the operation procedures in the specifications. The fluorescence was evaluated by luminometry (Thermo, U.S.A.).

Inductively Coupled Plasma Mass Spectrometry (ICP-MS) Measurement. To determine the concentration of copper in medaka embryos, ICP-MS analysis was performed on embryos (~ 4 hpf) exposed to 200 $\mu\text{g/L}$ of CuO-NPs, CuSO_4 , and sulfidized products of CuO-NPs for 48 h. Meanwhile, we also measured the copper concentration of medaka embryos coexposed to ethylene diamine tetraacetic acid (EDTA) and the copper particles or CuSO_4 as described above. Each exposure group included 12 embryos which were thoroughly washed three times in ultrapure water before being subjected to acid digestion. The copper concentrations were measured using ICP-MS (Agilent 8800, U.S.A.) and expressed as $\mu\text{g/embryo}$. To achieve reliable statistical analysis, three replicates were used for each group.

Statistical Methods. In this study, results were statistically analyzed using two-side student's *t* test. A $p < 0.05$ was considered statistically significant. Moreover, data are shown as

Figure 2. Characterization of the solid products from sulfidation of CuO-NPs with Na₂S solution. (A) XRD spectra of sulfidized CuO-NPs generated from initial S/CuO molar ratios varying from 0.125 to 4. CuO and CuS references are shown in A for comparison. (B) Percentage of CuS as a function of initial S/CuO molar ratio. The data were obtained from the semiquantification of XRD spectra. (C) TEM image of sulfidized particles generated from initial S/CuO ratio of 1. (D) HRTEM image of circular ring part (red in color) of C. (E) HRTEM image of quadrangle part (red in color) of C. (F) EDX mappings of sulfidized particles generated from initial S/CuO ratio of 4. (G) HRTEM image of sulfidized particles generated from initial S/CuO ratio of 4.

Figure 3. Toxicity of CuO-NPs, sulfidized CuO-NPs, and copper salts in medaka embryos. (A) The survival and hatching ratios of medaka embryos (12 embryos per group) exposed to the above-mentioned copper particles or salts, respectively. (B) Images of medaka embryos exposed to the above-mentioned copper particles or salts at 200 $\mu\text{g/L}$. * $p < 0.05$ compared with embryos treated with CuO-NPs. The pH of NPs solution for exposure was 7.1 ± 0.1 , and error bars represent the standard deviations from average values.

Table 1. Determination of the Length of Medaka Larvae Hatched from Embryos Exposed to CuO-NPs, Sulfidized CuO-NPs (without or with EDTA), Cu^{2+} (CuSO_4), and Control (Water)

	length of medaka larvae (mm)			
	10 ug/L	100 ug/L	200 ug/L	
			without EDTA	with EDTA
S/CuO: 4	46.6 $\pm$ 0.6	40.6 $\pm$ 1.1 ^b	N.A. ^a	45.8 $\pm$ 0.6
S/CuO: 2	46.2 $\pm$ 0.5	42.6 $\pm$ 1.4 ^b	N.A. ^a	45.9 $\pm$ 0.3
S/CuO: 1	46.5 $\pm$ 0.5	45.5 $\pm$ 0.5	45.2 $\pm$ 0.8	
S/CuO: 0.5	46.5 $\pm$ 0.7	46.2 $\pm$ 0.4	46.5 $\pm$ 0.7	
CuO	46.8 $\pm$ 0.4	46.0 $\pm$ 0.5	46.1 $\pm$ 0.8	
Cu^{2+}	46.5 $\pm$ 1.0	41.0 $\pm$ 1.2 ^b	N.A. ^a	46.3 $\pm$ 0.3
EDTA	45.6 $\pm$ 0.6			
control	46.0 $\pm$ 0.8			

^arepresents the embryos could not hatch at all. ^b $p < 0.05$ compared with embryos in the control (water).

the mean $\pm$ standard deviation from at least three separate experiments.

RESULTS

Sulfidation of CuO-NPs. The CuO-NPs used in this study are spherical in shape with an average diameter of 36.1 ± 6.4 nm (Figure 1A,B). Moreover, the CuO-NPs show lattice fringe of 2.6 Å (Figure 1C) and their special spectrum of XRD (Figure 1D).

The sulfidation of CuO-NPs was dramatically dependent on the initial S/CuO molar ratio and the reaction time (Figure 2; Supporting Information, SI, Figure S1). Complete sulfidation needed a great excess of sulfide compared to the theoretical (S/CuO: 1) amount of sulfide for stoichiometrical conversion (Figure 2A,B; SI Table S1); at a S/CuO ratio of 4, $99.6 \pm 0.3\%$ CuO-NPs were converted to CuS-NPs. TEM images showed a number of particles smaller than the original CuO-NPs (Figure 2C; SI Figure S2). These small particles with sizes of 4.6–11.7 nm, estimated using the Scherrer equation, were identified as CuS (Figure 2D–G; SI Figures S3 and S4). The atomic ratio of Cu to S, calculated from the integrated peak intensity of EDX (SI Figure S4), was 1.02, which is reasonably close to the 1.0 that is expected for the CuS. In addition, the copper valence of CuS-NPs was confirmed by XPS Auger (SI Figure S5).

Sulfidation affected surface properties of the particles (SI Table S2 and S3). Furthermore, we found that solution pH (tested 12.2, 10.0, 7.2, 4.5, and 3.0) showed slight influences on the CuO-NPs sulfidation in aqueous solutions (SI Figure S6), but NOM (e.g., 50 and 100 mg/L) showed negligible influences (SI Figures S7 and S8).

CuS-NPs Interfered in Medaka Embryo Hatching. Sulfidation acting as a detoxification pathway to metallic NPs toxicity was widely reported in previous studies.^{18–25} However, here we found that sulfidation resulted in a progressive decrease in the rates of survival and hatching of medaka embryo, in accordance with the extent of CuO conversion to CuS (Figure 3A). In addition, sulfidation also caused an increasing trend in acute toxicity (48 h) of CuO-NPs in medaka adults (SI Figure S9). As shown in Figure 3A, CuS-NPs interferes embryo hatching by directly affecting their viabilities, showing marked reduction in survival and hatching rates. When compared with the CuO-NPs treatment, the hatching rate was significantly ($p < 0.05$) lower than that in the sulfidized CuO-NPs treatment. For instance, embryos exposed to CuS-NPs (designated as S/CuO 4) at a concentration of 100 $\mu\text{g/L}$ showed $25.0 \pm 8.7\%$ hatching rate, which is 33.4% lower than that of CuO-NPs (Figure 3A). Clearly, CuS-NPs showed stronger hatching interference than CuO-NPs. However, the surviving embryos ultimately could hatch, which is why the rate of hatching is

Figure 4. Dissolution of CuO-NPs and the sulfidized CuO-NPs (S/CuO: 4, 2, 1, 0.5, and 0.25) within 168 h at pH 7.2 in the presence and absence of dissolved oxygen (DO) using 100 rpm of rotary stirring at the room temperature. (A) In the presence of DO. (B) In the absence of DO. (C) TEM image of CuS clusters in the supernatant of CuS-NPs passing through a 3 kDa MWCO filter.

comparable to that of the survival (Figure 3A). In addition, no obviously abnormal morphological features were identified in the hatched larvae (Figure 3B), except that the larval length was significantly ($p < 0.05$) reduced by CuS-NPs (Table 1).

Mechanisms for Stronger Hatching Interference of CuS-NPs than CuO-NPs. To understand whether the enhanced hatching interference of CuS-NPs is linked to their dissolution characteristics, we investigated the effect of sulfidation on CuO-NPs dissolution. We found that CuS-NPs indeed showed a greater extent of dissolution than CuO-NPs regardless of the presence or absence of DO (Figure 4A,B). Moreover, the dissolution increased with the increasing extent of CuO conversion to CuS (Figure 4A,B), which is consistent (correlation coefficient: -0.964 , $p < 0.05$) with the trend of embryo hatching interference (Figure 3A). However, we also found that the extent of CuS-NPs dissolution was affected by DO (Figure 4A); dissolution of CuS-NPs gradually reached equilibrium within 108 h, with the highest extent of dissolution to $6.6 \pm 0.1\%$. In the absence of DO, however, dissolution equilibrium could shortly reach within 4 h, showing the highest extent of dissolution to $4.2 \pm 0.2\%$ (Figure 4B).

We further found that cointroduction of EDTA reversed the hatching interference of CuO-NPs, CuS-NPs, and copper salt (CuSO_4), respectively, even at the concentration of $200 \mu\text{g/L}$ (Figure 5A). We then used ICP-MS to determine the total burden for copper in medaka embryos. The total copper content in the embryo exposed to CuS-NPs was five times

higher than that of CuO-NPs (Figure 5B), showing a much higher bioavailability for CuS-NPs than CuO-NPs. In addition, cointroduction of EDTA could significantly reduce the amount of copper in embryos (Figure 5B).

CuO-NP toxicity generating from ROS production is attributed to dissolved copper in the solutions.^{25,30,31} Here we used EPR spin-trap techniques to illustrate the mechanism of CuS-NP toxicity by distinguishing particle and ion contributions to ROS production. DMPO with hydrogen peroxide reacting with free copper ions could exhibit a characteristic EPR spectrum.²⁵ CuS-NPs and their clear filtrate (passing through a 3 kDa DWCO filter) had the same peak height (Figure 5C), documenting that all the ROS production by CuS-NPs derived from their soluble fraction. In addition, cointroduction of EDTA could significantly reduce the ROS production by CuS-NPs, which was also confirmed in embryos exposed to CuS-NPs solution containing EDTA (Figure 5D).

Clearly, exposure to CuS-NPs, due to their large amount of copper leaching (Figure 4A), resulted in additional ROS generation relative to the control and CuO-NPs (Figure 5D), which consolidated the stronger hatching interference by CuS-NPs. However, the ROS generation in embryos was significantly reduced in the presence of EDTA (Figure 5D). However, GSH, as an antioxidant preventing cellular damage caused by ROS, was significantly exhausted in CuS-NPs-exposed embryos comparing with the CuO-NPs and control groups (Figure 5E). Co-introduction of EDTA could

Figure 5. Effect of metal chelator (EDTA, 0.125 mM) on the varied forms of copper hatching interference. (A) Protective role of EDTA on the medaka embryo hatching. (B) ICP-MS measurement to determine the copper uptake in the embryos (12 embryos per group, three replicates) exposed to 200 $\mu\text{g/L}$ (48 h) of CuO-NPs, sulfidized CuO-NPs, CuSO₄, and the corresponding copper with EDTA. (C) Hydroxyl radical EPR signal (DMPO spin trap) induced by the various forms of copper. (D) Effect of EDTA on the ROS levels in medaka embryos exposed to the various forms of copper. (E) Effect of EDTA on the GSH levels in medaka embryos exposed to the various forms of copper. It should be noted that control means the noncopper exposed embryos. An additional control named EDTA in the Figures, corresponding to the addition of EDTA in the absence of added copper was also determined in order to account for the effect of EDTA alone. * $p < 0.05$ compared with embryos treated with CuO-NPs. # $p < 0.05$ for pairwise comparisons as shown. Error bars represent the standard deviations from average values.

significantly decrease the depletion of GSH in embryos exposed to CuS-NPs and copper salt (CuSO₄) (Figure 5E). Obviously, the decrease of ROS level and depletion of GSH in medaka embryos exposed to CuS-NPs solution containing EDTA suggested that leached CuS-NPs caused oxidative stress in medaka embryos by disturbing the cellular antioxidant defense system. These analyses, together with the comparison of the copper burden in embryos exposed to CuS-NPs versus the CuS-NP solution containing EDTA, indicated that the free copper ions released from CuS-NPs played an important role in the CuS-NP toxicity in medaka embryos.

DISCUSSION

The theoretical molar ratio of S to CuO is only 1 to fully convert CuO to CuS, while here great excess of Na₂S was needed in this study, which is attributed to the oxidation of HS⁻. In previous studies,^{25,32,33} copper ions and CuS particles have been proven to be able to catalyze the HS⁻ oxidation under oxygen-presence conditions. Here a depletion of DO upon addition of copper ions or CuS-NPs generated from the sulfidation to Na₂S solution was also observed. We found that solution pH influenced CuO-NPs sulfidation, which was

consistent with the previous works on silver and zinc sulfidation.^{23,34} Normally, sulfides are primarily in the forms of HS⁻ and H₂S in the natural environment,³⁵ and the speciation distribution is pH-dependent.³⁶

Our analysis of the sulfidation process of CuO-NPs in solution containing Na₂S documented that a dissolution followed precipitation pathway (namely copper ions released from CuO-NPs form CuS clusters and then form CuS particles) rather than a direct solid-state-shell reaction pathway (namely formation of CuS shell on the CuO-NPs) would be a potential mechanism for the formation of CuS-NPs, which is a completely different pathway compared to Ag-NPs sulfidation.^{12,19–24} CuS clusters (1–3 nm) were observed in the supernatant of CuS-NPs passing through a 3 kDa MWCO filter (Figure 4C). For a S/CuO ratio of 4, moreover, filtrate passing through a 0.22 μm filter after 30 min sulfidation reaction contained a lot of small (<12 nm) CuS particles. Obviously, these particles are far smaller than the original CuO-NPs, indicating that CuS-NPs formed by dissolution followed a precipitation pathway. CuS clusters have been reported as intermediates in the process of copper ion conversion to CuS in aqueous solution.³⁷ Our study visually presented the CuS clusters (1–3 nm) or particles (<12 nm) to evidently support the dissolution–precipitation mechanism.

Ag-NPs sulfidation greatly mitigated metallic NPs toxicity in organisms.^{19–22} However, sulfidation of CuO-NPs resulted in a progressive decrease in the rates of survival and hatching of medaka embryo. This is a complete contrast to the general consensus on detoxification contributions from sulfidation, indicating that sulfidation as a natural antidote to metallic NP toxicity is being overestimated.

The primary mechanism of metallic NPs toxicity has been attributed to the dissolution of NPs and their corresponding metallic ions shedding.^{30,38–41} To understand the mechanism for stronger hatching interference of CuS-NPs than CuO-NPs, we first presented that CuS-NPs had a greater extent of dissolution than CuO-NPs, which is in accord with the observation of embryo hatching interference. This is an indirect indication that dissolution characteristics play an important role in the increased toxicity of CuS-NPs relative to CuO-NPs in medaka embryos, which contrasts with previous studies on that sulfidation reduced dissolution of metallic NPs due to the lower solubility of metal sulfides.^{19–22,42} Also, it contradicts the solubility products (K_{sp}) of CuS (8×10^{-37}) and CuO (1×10^{-7}) in solutions. As shown in Figure 4, CuS-NPs show higher extent of dissolution relative to CuO-NPs could be attributed to not only the oxidation dissolution of the formed CuS-NPs, but also the presence of CuS clusters. The oxidation dissolution is supported by the fact that the presence of DO promoted the dissolution extent of sulfidized CuO-NPs along with the time. Aerobic conditions would favor the oxidation of metal sulfides⁴³ which has also been mentioned in a recent work that $3.2 \pm 0.3\%$ of free copper ions released from hollow CuS-NPs within 24 h under oxic conditions.⁴⁴ Here CuS-NPs showed extent of dissolution to $6.6 \pm 0.1\%$ in the presence of DO, with 2.3% higher than the extent of CuS-NPs dissolution in the absence of DO. The second notion was supported not only by the nonzero extent of dissolution with fast equilibrium under DO-absence conditions, but also by the presence of CuS clusters in the supernatant. Rozan et al.⁴⁵ reported that CuS clusters passing through a 3 kDa MWCO filter could account for as much as 60% of the total CuS in river water collected from the Naugatuck and Quinnipiac Rivers in Connecticut, U.S.A. Here,

a large number of CuS clusters (1–3 nm) existed in the supernatant (passing through a 3 kDa MWCO filter), giving reasonable explanation for why the high extent of CuS-NP dissolution was determined even in the absence of DO.

Furthermore, we investigated whether cointroduction of a free metal ion chelator could reduce the hatching interference. Here, cointroduction of EDTA significantly mitigated the hatching interference, indicating the free copper ions released from CuS-NPs play an important role in hatching interference. Clearly, sulfidation of CuO yielded NPs with enhanced toxicity in the medaka embryos due to their increasing dissolution. However, formation of copper-EDTA complex resulted in far less hatching interference than CuS-NPs alone, while it still could trigger oxidative stress and cause slight toxicity. This is why the hatching rate of embryo coexposed to EDTA and CuS-NPs is still lower than that of the control.

Metallic NPs could enter the aquatic environment through runoff or wastewater treatment effluents.⁴⁶ Sulfidation was considered as a very important transformation process for metallic NPs due to its great contribution to detoxification. However, this study provided the first direct evidence that sulfidation could not be completely considered as natural antidote to metallic NPs. Hence, it is important to further consider the ecological risks of other potentially metallic NPs sulfidation in future work.

■ ASSOCIATED CONTENT

● Supporting Information

Additional figures and tables on sample analyses in this study; XRD, EDX, and XPS spectra, HRTEM images, and zeta potential as well as DLS of NPs, and LC50 of medaka adults exposed to sulfidized CuO-NPs, and percentage of CuS obtained from the semiquantification of the XRD spectra. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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