Video Article

Removal of Trace Elements by Cupric Oxide Nanoparticles from Uranium *In Situ* Recovery Bleed Water and Its Effect on Cell Viability

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Abstract

In situ recovery (ISR) is the predominant method of uranium extraction in the United States. During ISR, uranium is leached from an ore body and extracted through ion exchange. The resultant production bleed water (PBW) contains contaminants such as arsenic and other heavy metals. Samples of PBW from an active ISR uranium facility were treated with cupric oxide nanoparticles (CuO-NPs). CuO-NP treatment of PBW reduced priority contaminants, including arsenic, selenium, uranium, and vanadium. Untreated and CuO-NP treated PBW was used as the liquid component of the cell growth media and changes in viability were determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay in human embryonic kidney (HEK 293) and human hepatocellular carcinoma (Hep G2) cells. CuO-NP treatment was associated with improved HEK and HEP cell viability. Limitations of this method include dilution of the PBW by growth media components and during osmolality adjustment as well as necessary pH adjustment. This method is limited in its wider context due to dilution effects and changes in the pH of the PBW which is traditionally slightly acidic however; this method could have a broader use assessing CuO-NP treatment in more neutral waters.

Video Link

The video component of this article can be found at https://www.jove.com/video/52715/

Introduction

Approximately 20% of the US electrical supply is provided by nuclear energy and, based in part on national incentives to increase energy independence, US nuclear capacity is expected to increase [1]. Worldwide growth of nuclear energy also is expected to continue, with much of the growth occurring outside the US [2]. As of 2013, 83% of US uranium was imported, but 952,544 metric tons of reserves exist in the US [3,4]. In 2013 there were 7 new facility applications and 14 restart/expansion applications between Wyoming, New Mexico, and Nebraska [5]. In the US, uranium is predominately extracted through *in situ* recovery (ISR) processes [6]. ISR causes less land disruption and avoids creating tailing piles that can release environmental contaminants [7]. ISR uses water-based oxidizing solutions to leach uranium from the underground ore body, after which the uranium is extracted from the leachate through an ion exchange process [8]. To maintain a negative water balance in the ore body, a portion of the leachate, called production bleed water (PBW), is bled off. A portion of the PBW is decontaminated using reverse osmosis (RO) and re-introduced into the mining process, but PBW also could have beneficial industrial or agricultural uses, if toxic contaminants can be reduced to acceptable levels determined by state regulatory agencies for surface and groundwater [9]. Currently, most ISR uranium facilities use RO to remove contaminants from PBW. However, RO processing is energy intensive and produces toxic waste brine, which requires regulated disposal.

Many water decontamination methods exist, including adsorbents, membranes, and ion exchange. Of these, adsorption is the most commonly used, and recent developments in nanoparticle synthesis has enhanced the capabilities of adsorbent-based water decontamination processes [10]. Cupric oxide nanoparticles (CuO-NPs) previously had not been extensively studied on uranium ISR PBW, but in recent studies of contaminant removal from groundwater, CuO-NPs were found to have unique properties, including not requiring pre- or post-water treatment steps (*e.g.*, adjusting pH or redox potential) and performing well in different water compositions (*e.g.*, in different pHs, salt concentrations, or competing ions) [11]. In addition, CuO-NPs are easily regenerated by leaching with sodium hydroxide (NaOH), after which the regenerated CuO-NPs can be reused. Details of CuO-NP trace metal filtering capabilities from natural waters have been previously published [11–14].

Although useful for water treatment, metal oxide nanoparticles can be toxic to living organisms, but the extent of the toxicity depends, in part, on nanoparticle characteristics and constituents [10,15,16]. Therefore, it is important to study simultaneous contaminant removal and nanoparticle toxicities before field applications. The current study determined the capability of CuO-NPs to remove PBW priority contaminants (including arsenic, selenium, vanadium and uranium), and assessed the effect of CuO-NP treatment on PBW cytotoxicity.

PBW was collected from an active ISR uranium facility and utilized to determine the efficacy of CuO-NP treatment in priority contaminant removal. PBW cytotoxicity before and after CuO-NP treatment also was assessed. PBW is a complex geological (industrial/environmental) mixture and both the National Institute of Environmental Health and Science (NIEHS) and the Agency for Toxic Substances & Disease Registry (ASTDR) are placing emphasis on studying the toxicity of environmentally relevant mixtures, including mixtures as they exist in nature or industrial settings, as well as promoting *in vitro* testing to prioritize chemicals for further *in vivo* testing [17–19]. Studies of chronic, low-dose mixture exposures are challenging because chronic exposure to a low dose mixture not produce obvious effects, at least not in the short time frame of most laboratory studies. Similarly, most *in vitro* studies of chemical mixtures expose cells to a defined lab-made mixture of 2 or more metals [20,21]. These studies provide baseline information, but simplified mixtures do not replicate the complex antagonistic and synergistic interactions that may occur in a native, environmental sample, where the full range of mixture components are present.

The goals of this study were to examine alternate contaminant removal processes for PBW and to evaluate the effect of (CuO-NP) treatment on PBW cytotoxicity using cultured human cells. The results could benefit the uranium industry through the development of more efficient or environmentally friendly methods for contaminant removal. This study provides the first evidence that reduction of priority contaminants in PBW by CuO-NPs reduces cytotoxicity in mammalian cells [22].

Protocol

All samples were collected at the uranium liquid processing building of a uranium ISR facility in Wyoming.

1. Production Bleed Water (PBW)

1. Collect two types of water samples from an ISR uranium facility: PBW and reverse osmosis (RO) water. Collect PBW from a monitoring tap after the ion exchange process but before reverse osmosis decontamination. Collect RO samples after the PBW is decontaminated by reverse osmosis treatment.
    NOTE: Lixiviant is transported in pipelines from multiple well fields to the uranium liquid processing building, where it is collected in a column and prepared for ion exchange. Approximately 1-3% of the lixiviant after ion exchange is removed from the circuit and termed production bleed water (PBW). PBW is re-used in the mining processes or decontaminated/demineralized with RO filtration.
2. Collect water samples in high density polyethylene (HDPE) bottles with zero head space according to standard operating procedures for sample collection and analysis of the Wyoming Department of Environmental Quality (WYDEQ) [23].
3. Measure temperature and pH on-site and transport samples on ice to keep them cool.
4. Store PBW at 4 °C. Keep the PBW solution cool until after the concentrated Eagle's minimum essential media (EMEM-10x) is added during media preparation as instructed in the following protocol.
    NOTE: PBW is an oxidized solution that will precipitate if allowed to freeze or warmed to room temperature. After dilution the PBW solution is sufficiently dilute that it will not precipitate when heated to 37 °C before application to cells and during incubation.

2. Preparation of CuO Nanoparticles (CuO-NPs)

1. Combine a pure ethanolic solution containing 250 ml of 0.2 M $CuCl_2 \cdot 2H_2O$, 250 ml of 0.4 M sodium hydroxide (NaOH), and 5 g polyethylene glycol (PEG) in a round-bottom flask with six mm borosilicate glass balls.
2. Place the solution in a modified microwave oven and allow it to react under reflux at ambient air pressure for 10 min at 20% power (intervals of 6 sec on, 24 sec off).
3. Cool the solution to room temperature (20 °C), then decanted into 50 ml conical tubes, leaving the glass balls.
4. Centrifuge the solution in the 50 ml conical tubes at 1,000 x g for 30 min, decanted, and then wash the CuO-NPs with a sequence of 300 ml hot water (60-65 °C), 100 ml ethanol, and 100 ml acetone.
5. Dry the CuO-NPs to room temperature (20 °C) in the 50 ml conical tubes.
6. Scrape the CuO-NPs out of their tubes into a mortar. Cover the CuO-NPs with tin foil and heat the CuO-NPs to 110 °C in an oven to remove the remaining liquid. Combine CuO-NPs into one batch and weigh the CuO-NPs.
    NOTE: The preparation of CuO-NPs and CuO-NP treatment of PBW were conducted in Water Quality Laboratory of Ecosystem Science and Management, University of Wyoming. CuO-NP synthesis followed the procedure of Martinson and Reddy (2009) [11].

3. Treatment of PBW with CuO-NPs

1. Add 50 mg (1 mg/ml) of CuO-NP to a 50 ml conical tube followed by 50 ml of PBW. Seal the tube and reacted for 30 min on a bench top orbital shaker at 250 rpm.
2. Centrifuge sample tubes at 250 x g for 30 min and then filter the supernatant using a 0.45 µm syringe filter. Alter the centrifuge speed and time can depend on the nanoparticle to ensure the CuO-NPs become compact in the centrifuge tube.

4. Elemental Analysis

1. Prepare Untreated (control) and CuO-NP-treated PBW samples for elemental analysis as follows.
2. Acidify aliquots (40 ml) of CuO-NP-treated and untreated PBW with trace metal grade nitric acid to a pH of 2.0. Analyze acidified PBW aliquots for cations by inductively coupled plasma-mass spectroscopy (ICP-MS) as described in Reddy and Roth (2012) [13].

3. Prepare unacidified aliquots (20 ml) of CuO-NP-treated and untreated PBW and analyze the unacidified aliquots for anions by ion chromatography (IC) as described in Reddy and Roth (2012) [13].
    NOTE: Aliquots were analyzed by the Wyoming Department of Agriculture Analytical Services, Laramie WY 82070. A description of the IC and ICPMS procedure can be found in Reddy and Roth, (2012) [13].

5. Preparation of Cell Culture Media Using PBW

1. Use two control (EMEM-1x and RO+media) and eight PBW test media solutions (four concentrations each of untreated PBW and CuO-NP-treated media) in the viability studies. Overviews of the solutions are as follows:
    1. For EMEM-1x control, purchase Eagle's minimum essential media (EMEM-1x) with L-glutamine and sodium bicarbonate already added. Add fetal bovine serum (FBS) and antibiotics per manufacturer's instructions.
        NOTE: EMEM-1x is purchased diluted to the proper concentration for cell growth and containing L-glutamine and sodium bicarbonate. EMEM-1x requires the addition of fetal bovine serum (FBS) and an antibiotic mix of penicillin and streptomycin (50 I.U./ml penicillin and 50 µg/ml streptomycin). EMEM-1x is used as a control media because it is the manufacturer's recommended growth media for both cell types used in this study. Concentrated EMEM-10x is diluted with RO water from the facility or untreated or CuO-NP-treated PBW to produce the test solutions. Concentrated EMEM-10x when purchased does not contain L-glutamine or sodium bicarbonate so these are added in addition to the fetal bovine serum (FBS) and an antibiotic mix of penicillin and streptomycin.
    2. For the RO control solution use RO water collected from the ISR facility. Use the same protocol as the PBW test media only substitute 100% RO water from the ISR facility in place of PBW. To dilute the untreated and CuO-NP-treated solution use RO or ultrapure water from the laboratory.
    3. Dilute untreated PBW into four test concentrations before mixing with the cell culture media components. Prepare the four different concentrations of untreated PBW solutions by mixing untreated PBW with RO (from the laboratory) in the following combinations: 100% (pure PBW + no RO water), 75% (187.5 ml of PBW + 62.5 ml RO water), 50% (125 ml of PBW + 125 ml of RO water) or 25% (62.5 ml of PBW + 187.5 ml of RO water).
    4. Dilute CuO-NP-treated PBW into four test concentrations before mixing with the cell culture media components. Prepare the four different concentrations of CuO-NP-treated PBW solutions by mixing PBW (pre-treated with 1 mg/ml CuO-NP for 30 min) with RO (from the laboratory) in the following combinations: 100% (pure CuO-NP-treated PBW + no RO water), 75% (187.5 ml of CuO-NP-treated PBW + 62.5 ml RO water), 50% (125 ml of CuO-NP-treated PBW + 125 ml of RO water) or 25% (62.5 ml of CuO-NP-treated PBW + 187.5 ml of RO water).

2. Prepare 250 ml of RO+media, untreated PBW+media and CuO-NP-treated PBW+media concentration by adding 25 ml of concentrated EMEM-10x to 190 ml of the 100% RO and the 100%, 75%, 50% or 25% of the premade untreated or CuO-NP-treated PBW concentrations created in step 6.1.3 and 6.1.4.
3. Adjust the pH of each solution to 7.4 with NaOH or HCl.
4. Supplement each concentration of untreated and CuO-NP-treated PBW as well as RO+media with the following standard components: 25ml (10%) fetal bovine serum (FBS), 2.5 ml L-glutamine, 0.55 g NaHCO$_3$ and 1.25 ml Pen/Strep (50 I.U./ml penicillin and 50 µg/ml streptomycin).
5. Adjust the osmolality of each concentration of untreated PBW+media, CuO-NP-treated PBW+media and RO+media to 290-310 mOSM/kg by adding RO water and measure using an osmometer.
6. Filter each solution using a 0.22 µm vacuum filter unit, and store at 4 °C.
    NOTE: Due to slight variations in the amount of RO water used to adjust osmolality, vary final media concentrations within a 5% range, with untreated PBW+media concentrations at 56%, 44%, 29% and 16.5% and CuO-NP-treated PBW+media concentrations at 53%, 45%, 30% & 17%.

6. Cell Viability

NOTE: Given that kidney and liver are target organs of heavy metal toxicity, employ cultured human embryonic kidney (HEK293) cells (HEK) and human hepatocellular carcinoma (HepG2) cells (HEP) testing methods [24–26].

1. Prepare a culture of HEK and HEP cells 2-3 days before plating the 96-well plates used in the experiment per manufacturer's instructions.
2. Measure cell viability using the 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) assay.
    NOTE: The MTT assay protocol was modified from Meerloo *et al*. (2011) [27].
    1. Obtain MTT in powder form. Add phosphate buffered saline (PBS) to make up a stock concentration of 50 mg/ml. Agitate the solution for 2 hr and then filter with a 0.45 µm syringe filter and aliquot into 1.5 ml freezer safe tubes. Protect tubes from light and store at 4 °C.

3. Remove HEK and HEP cells from their culture dishes using trypsin, centrifuge at 1,000 x g for 5 min and decant the trypsin. Add 5 ml of PBS and mix cells to obtain a single cell solution. Then, apply 20 µl of the single cell solution to a hemocytometer to obtain a cell count per milliliter of solution. Centrifuge the cells again at 1,000 x g for 5 min and decant the PBS used to rinse the cells. Add the appropriate amount of EMEM-1x to adjust the concentration of cells to 500 cell/100 µl (100 µl/well).
4. Fill the perimeter wells of the plate with 200 µl PBS to control for evaporation.
5. Seed cells at a density of 500 cells/well adding 100 µl to each well, except for the perimeter wells (which are not plated with cells).
    NOTE: Seeding density for HEK and HEP cells is based on experimental growth curves that allow the peak of growth to occur around days 4-5. Prepare growth curves for all cell lines to estimate seeding density.
6. Incubate cells for 24-hr at 37 °C allowing them to recover (form tight adhesions to the plate) before performing baseline MTT readings of cell density.
7. Perform baseline MTT readings of cell density by removing the seeding media from the first column (not including the perimeter) and adding 100 µl of MTT (5 mg/ml in media) to the wells for 1 hr.
8. After one hour, remove the MTT and add 100 µl of dimethyl sulfoxide (DMSO) to dissolve the MTT-formazan produced by viable cells (20 min).
9. Read the optical density (OD) of the first column at an absorption wavelength of 570 nm to obtain a baseline reading.

1. Use baseline readings to ensure all plates were seeded correctly and that cells are growing consistently between plates. Remove the DMSO from the column being tested before incubating for the next 24 hr.
   NOTE: If DMSO is left in the plate overnight it pulls moisture from the adjacent column, causing a reduction in the media volume.

10. Warm the test solutions (*i.e.*, the EMEM-1x, RO, untreated PBW and CuO-NP-treated PBW media solutions) to 37 °C in a water bath.
11. Remove the seeding media from the rest of the plate (not including the perimeter or the first column which was used for the baseline reading) and replaced with 100 µl of EMEM-1x, RO+media, untreated PBW+media concentrations or CuO-NP-treated PBW+media concentrations (one solution per plate). Incubate cells in their test concentrations or control solutions for a total of seven days (Days 2-8).
    NOTE: There 10 plates total: 1 EMEM-1x, 1 RO+media, 1 of each untreated PBW+media concentration (56%, 44%, 29% and 16.5%) and one plate of each CuO-NP-treated PBW+media concentration (53%, 45%, 30% & 17%) per experiment per cell line.
12. Each day following baseline MTT reading, remove the control and test solutions (listed in the note under 6.11) from the next column of their respective plate (*e.g.* Day 2 test and control media are removed from row 3, wells B-G; Day 3: row 4, wells B-G *etc.*) and repeat the MTT protocol as described in steps 6.7-6.9 above.
13. Repeat the protocol every day for seven days. Average the OD results for each row (6 wells) and reported against time to generate a seven-day growth curve.
14. To assess the effect of copper chelation on cell viability in CuO-NP-treated PBW+media follow the same procedure as above, except add 100 µM of D-penicillamine to control and test solutions before adding the solutions to their respective plates. Perform data analysis using scientific graphing software.

7. Geochemical Modeling

1. Download Visual MINTEQ version 3.0/3.1 a freeware from the following website http://www2.lwr.kth.se/English/Oursoftware/vminteq/.
   NOTE: Visual MINTEQ is a freeware chemical equilibrium model for the calculation of metal speciation, solubility equilibria, sorption *etc.* for natural waters. In addition it is used to predict ion speciation, ion activities, ion complexes and saturation indices which is compared to the concentration of elements before and after treatment (mass spectroscopy results) to examine possible mechanisms of element removal [28].
2. Open the program and input the mass spectroscopy data from step 4, including pH, alkalinity and the concentrations of different elements, into the program.
   NOTE: Given that groundwater is oxidized during *in situ* uranium extraction process, use oxidized species of arsenic, vanadium, and uranium for input.

8. Inhibitory Concentration 50 (IC_{50})

1. Calculate the IC_{50} for the untreated and CuO-NP-treated PBW+media concentrations by first averaging the viability (OD averages) on day 5 of three separate runs.
2. Subtract day five viability averages of the untreated and CuO-NP-treated PBW+media concentrations from day five viability averages of EMEM-1x to calculate viability differences. Then divide the viability differences by the average viability on Day 5 in EMEM, and multiply by 100 to get percent inhibition.
3. Subtract the percent inhibition from 100 (EMEM-1x viability) to get the percent viability for each untreated and CuO-NP-treated PBW+media concentration.
4. Input into scientific graphing software by setting EMEM-1x at a concentration of one and a percent viability of 100; transform all concentrations into log scale (X = Log (X)) and perform nonlinear regression with least square fit analysis.

9. Data Analysis

1. Compare concentrations of elements in untreated and CuO-NP-treated PBW with a two-tailed, paired, Student T-test.
2. Calculate the areas under the curve (AUC) by using the growth curve data collected over seven days and analyze the variance with repeated measures analysis of variance (ANOVA), followed by Tukey's post hoc comparison between all groups (n = 3).
3. Compute the IC_{50} by using data from day five of the growth curve for both untreated and CuO-NP-treated PBW+media solutions (described above). P values of <0.05 are considered significant.
   NOTE: For the purpose of statistical analysis, mass spectroscopy values of half the detection limit was assigned to ions concentrations levels below that limit [29].

Representative Results

PBW component concentrations and pH in untreated and CuO-NP-treated PBW are reported in **Table 1**. Martinson and Reddy (2009), reported that the point of zero charge of the CuO-NP is estimated at 9.4 ± 0.4. Given that the pH of PBW was 7.2-7.4, in these conditions, water donates protons to CuO-NPs, causing the nanoparticle surface to be positively charged allowing for the adsorption of negatively charged species. CuO-NP treatment removed priority contaminants from PBW, including arsenic, selenium, uranium and vanadium (**Table 1**). The average arsenic concentration was reduced by 87% [from 0.0175 to 0.002 mg/L (two-tailed paired t-test, *p* <0.0001)]. CuO-NP treatment also significantly reduced selenium (30%), uranium (78%), vanadium (92%), and phosphate (85%) (*p* <0.05).

Speciation modeling results, reported in **Table 2**, support the analytical results: 99% of total dissolved arsenic in PBW is present as $HAsO_4^{2-}$ and $H_2AsO_4^-$ and 94% of total dissolved selenium in PBW is present as SeO_4^{2-}. These species are negatively charged, hence capable of adsorbing to CuO-NPs. Speciation modeling predicted that 99% of vanadium species in PBW are negatively charged, also promoting adsorption to CuO-NPs. However, speciation modeling predicted only 35.5% of the uranium species are negatively charged, which would limit adsorption to CuO-NPs. Analysis of saturation indices predicted that no species of arsenic-, selenium-, uranium- or vanadium-containing minerals were near saturation (*i.e.*, mineral precipitation) levels, supporting adsorption to CuO–NPs, versus precipitation.

To assess if expected concentrations of priority contaminants are in the media made from untreated and CuO-NP-treated PBW, samples of undiluted control media (EMEM-1x), 56% untreated PBW+media and the 53% CuO-NP-treated PBW+media were analyzed by ICP-MS. Undiluted control media (EMEM-1x) is a commercial product supplied with L-glutamine and sodium bicarbonate (pre-added). Copper and selenium concentrations in control EMEM-1x were slightly elevated as expected because they are essential for cell growth, but arsenic, uranium and vanadium were negligible, reported in **Table 3**. Preliminary studies showed that, arsenic, selenium and vanadium concentrations were reduced by CuO-NP treatment and that the decrease was represented in the concentrations in the CuO-NP-treated PBW+media. The measured concentration of uranium in the CuO-NP-treated PBW+media was decreased compared to untreated PBW, and this decrease was more pronounced than predicted by Visual MINTEC v.3 modeling. Copper levels rose in CuO-NP-treated media as expected.

To determine the ability of CuO-NP treatment to ameliorate cytotoxicity of PBW on mammalian cells, viability was assessed in cells exposed to solutions of PBW+media before and after CuO-NP treatment. Both HEK (**Figure 1A**) and HEP (**Figure 1B**) cells were exposed to different concentrations of untreated or treated PBW+media for up to seven days. In cells grown in untreated PBW+media, viability was impaired in a concentration-dependent manner, whereas CuO-NP treatment improved cellular viability in both cell lines. The integrated AUC in **Figure 1C** shows that HEK cells grown in CuO-NP-treated PBW+media were more viable compared to untreated PBW+media at the three highest concentrations (29%, 44% and 56%). HEP cells showed slightly different viability: only the two highest concentrations of untreated PBW+media (44% and 56%) showed impaired viability compared to CuO-NP-treated PBW+media (**Figure 1D**). The more dilute concentrations of PBW were less toxic to HEP cells, and cell viability less affected by treatment. The viability of both HEK and HEP cells grown in 16.5% untreated PBW +media was not significantly different from cells grown in 53% CuO-NP-treated PBW+media ($p < 0.05$). Thus, CuO-NP treatment appeared to ameliorate the cytotoxicity of PBW, with viability near control levels. As discussed above, CuO-NP treatment of PBW is associated with an increase in copper concentrations. The increase was expected, based on earlier results by Reddy and Roth (2012), in which they used CuO-NPs to remove arsenic from groundwater. The increase in copper is dependent on the specific water chemistry of the PBW, but remained below EPA MCL of 1.3 mg/L. However, it was important to rule out that the increase in copper concentrations contributed to improved viability (*i.e.*, in addition to, or instead of, the decrease in priority contaminants). Accordingly, the copper chelator D-penicillamine was added to EMEM-1x control, RO+media control, untreated and CuO-NP-treated PBW+media solutions, and then MTT viability growth curve were generated, as described above. Copper chelation did not significant affect viability of either HEK or HEP cells incubated in RO+media control, untreated and CuO-NP-treated PBW+media (results not shown).

The half maximal inhibitory concentration (IC_{50}) was calculated from day five growth of HEK and HEP cells grown in untreated PBW+media (**Table 4A**) and CuO-NP-treated PBW+media (**Table 4B**). For HEK cells grown in untreated PBW+media, the IC_{50} value was 1.264 (log % PBW). Thus, the untreated PBW+media would have to be diluted to 18.38% to get to a 50% decrease in viability. For HEK cells grown in CuO-NP-treated PBW+media, the IC_{50} value was 2.744 (log % PBW). This result suggests that theoretically the cytotoxicity of the solution was reduced to the extent that treated PBW+media would need to be concentrated by 500% (log % PBW = 2.744) to produce a similar 50% decrease in viability. For HEP cells grown in untreated PBW+media, the IC_{50} was 1.243 (log % PBW). This would require a dilution of the PBW+media to 17.5% to produce a 50% decrease in viability. In contrast, for HEP cells grown in CuO-NP-treated PBW+media, the IC_{50} was 5.327 (log % PBW). This value likely was so large, because the viability of the cells in CuO-NP-treated PBW+media was not significantly different from cells grown in EMEM-1x (control). Bright field imaging, illustrated in **Figure 2**, of both HEK and HEP cellular growth on day five. Cell number and attachment in the CuO-NP-treated PBW+media (**Figure 2E, F**) were improved compared to untreated PBW+media (**Figure 2C, D**).
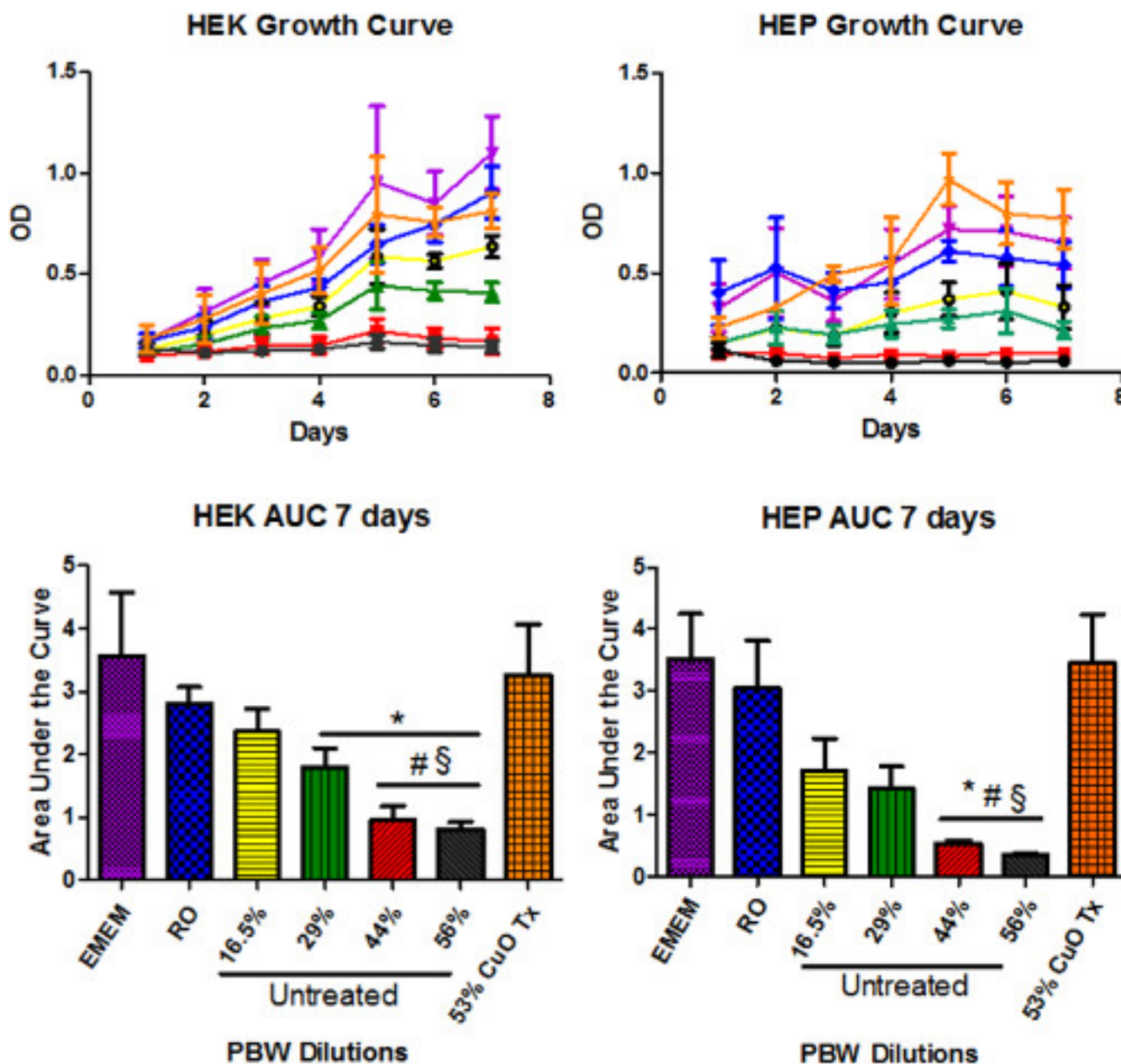
Figure 1: Growth Curves. Growth curves were used to assess the viability and growth of the cultures during treatment. Growth curves for HEK (A) and HEP (B) cells grown in four dilutions of PBW+media compared to 53% CuO-NP-treated PBW+media (upper panels). EMEM-1x control (EMEM) ▽, RO ◆, 53% CuO-NP-treated ★, 16.5% untreated PBW ◯, 29% untreated PBW ▲, 44% untreated PBW ■, 56% untreated PBW ●. Area under the curve (AUC) analysis of HEK (C) and HEP (D) 7 day growth curve data (lower panels). *$p < 0.05$ compared to EMEM control, #$p < 0.05$ compared to RO control, §$p < 0.05$ compared to 53% CuO NP-treated PBW-media. (Compared using a two-tailed ANOVA with Tukey's post hoc analysis, n = 3.)
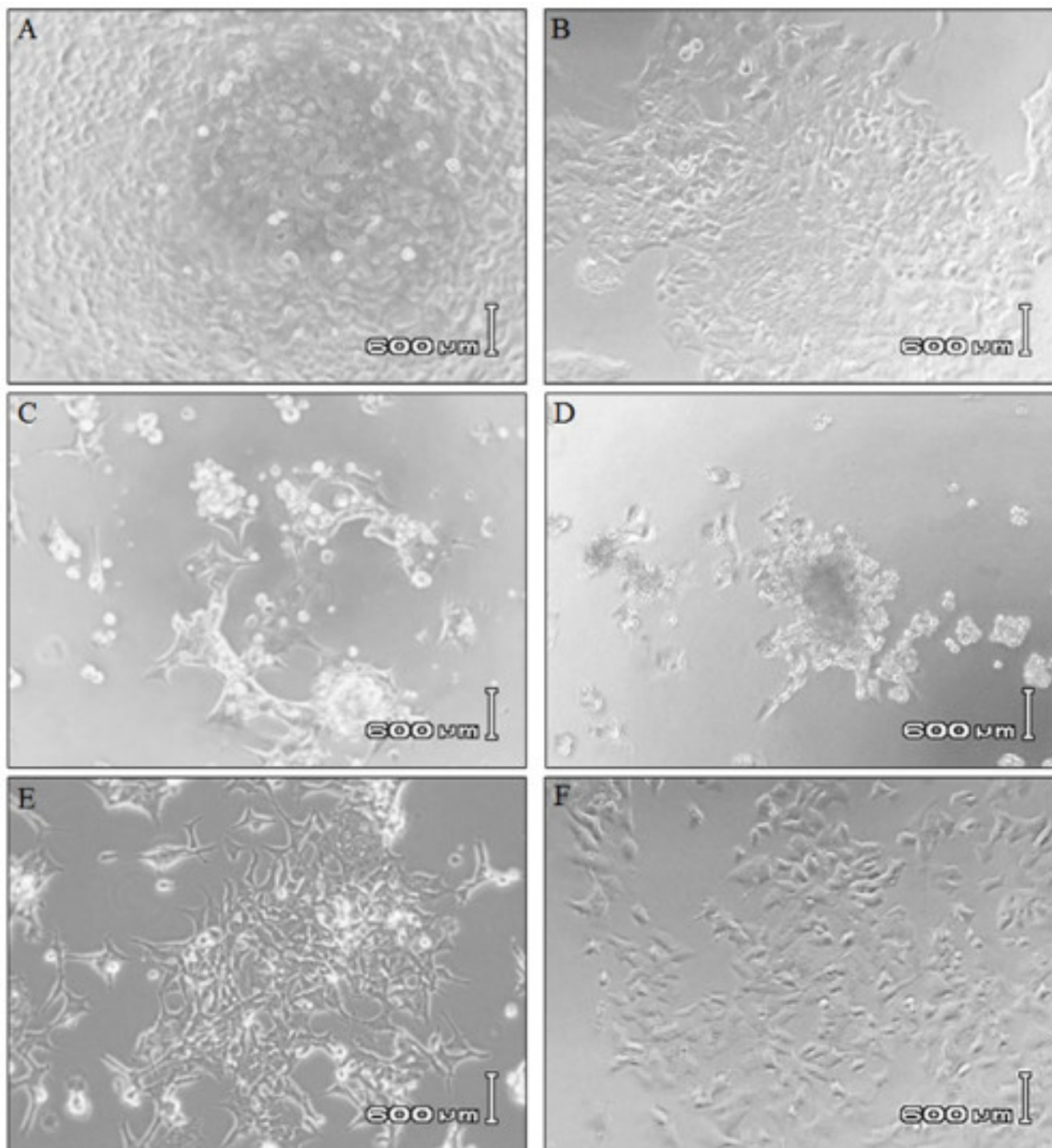
Figure 2: Cell morphology before and after CuO-NP treatment. Bright field microscopy (20X) of HEK (left column) and HEP (right column) cells at day 5, grown in: EMEM-1x control (EMEM) (**A**, **B**), 56% untreated PBW+media (**C**, **D**) and 53% CuO-NP-treated PBW+media (**E**, **F**) was used to examine cell morphology. HEK and HEP cells grown in EMEM-1x control (EMEM) (**A**, **B**) show healthy, near-confluent growth. HEK and HEP cells grown in untreated PBW+media have reduced numbers and appear detached (**C**, **D**). HEK and HEP cells grown in CuO-NP-treated PBW+media show better attachment and healthy, more confluent cells (**E**, **F**).

Element (mg/L)	Average, St. Dev. & Significance		
	Before Treatment	After Treatment	
Arsenic	0.018 ± 0.001	0.002 ± 0.0	***
Selenium	1.8 ± 0.07	1.3 ± 0.05	**

Copper	0.0015 ± 0.001	0.93 ± 0.43	*
Calcium	102 ± 82	106 ± 15	
Strontium	3.3 ± 1.1	1.5 ± 0.4	*
Magnesium	44 ± 2.1	47 ± 1.7	
Sodium	610 ± 0.0	627 ± 27	
Uranium	0.98 ± 0.03	0.21 ± 0.03	***
Barium	0.037 ± 0.02	0.019 ± 0.01	
Potassium	12 ± 0.0	12 ± 0.8	
Silicon	12 ± 0.7	12 ± 0.5	
Vanadium	1.3 ± 0.07	0.1 ± 0.02	***
Phosphate	0.35 ± 0.07	0.05 ± 0.0	***
Sulfate	805 ± 21	807 ± 15	
Conductivity	3125 ± 143	3190 ± 62	
pH	7.31 ± 0.09	7.36 ± 0.05	

Table 1: Analysis of cations and anions before and after CuO-NP treatment. Average element concentrations before and after treatment with CuO-NP. Significance between the concentration of CuO-NP-treated and untreated PBW are designated as * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$. A blank cell indicates no significant difference. Chloride concentrations ranged between 46.5 ± 0.707 and 55.25 ± 8.180. Aluminum, boron, and molybdenum concentrations were low and showed no significant change due to CuO-NP treatment. Manganese concentrations were not consistent.

Components	% of total concentration	Species
Arsenic	58.7	$HAsO_4^{2-}$
	41.2	$H_2AsO_4^-$
Uranium	64.1	$Ca_2UO_2(CO_3)_3$ (aq)
	32.2	$CaUO_2(CO_3)_3^{2-}$
	0.03	$UO_2(CO_3)_2^{2-}$
	3.5	$UO_2(CO_3)_3^{4-}$
	0.09	$Ca_2UO_2(CO_3)_3$ (aq)
	0.02	$CaUO_2(CO_3)_3^{2-}$
Selenium	94.3	SeO_4^{2-}
	5.6	$CaSeO_4$ (aq)
Vanadium	2.1	HVO_4^{2-}
	95.7	$H_2VO_4^-$
	2.1	$H_2V_2O_7^{2-}$
	0.01	$HV_2O_7^{3-}$
	0.01	$V_4O_{12}^{4-}$

Table 2: Species modeling using Visual MINTEQ ver. 3.0 software. Visual MINTEQ ver. 3.0 software (KTH Royal Institute of Technology, Valhallavägen, Sweden) was used to calculate metal speciation of the PBW components listed in **Table 1**. (aq) = aqueous as opposed to the solid form of that species.

	EMEM Control	Untreated	
		PBW	PBW+media
Arsenic	0.003 ± 0.0	0.017 ± 0.0	0.010 ± 0.001
Copper	0.01 ± 0.0	0.0015 ± 0.001	0.018 ± 0.0
Selinium	0.013 ± 0.002	1.75 ± 0.07	1.15 ± 0.06
Uranium	0.00015 ± 0.0	0.975 ± 0.03	0.71 ± 0.01

Vanadium	0.0015 ± 0.0	1.25 ± 0.07	0.785 ± 0.007
		CuO NP-treated	
		PBW	**PBW+media**
Arsenic		0.0022 ± 0.001	0.0015 ± 0.0
Copper		0.926 ± 0.4	0.81 ± 0.0
Selinium		1.25 ± 0.05	0.855 ± 0.0.02
Uranium		0.208 ± 0.03	0.45 ± 0.01
Vanadium		0.102 ± 0.02	0.0795 ± 0.01

Table 3: Concentrations of contaminants in media. Concentrations of priority contaminants (mg/L) in EMEM-1x control (EMEM), untreated PBW, CuO-NP-treated PBW, untreated PBW+media and CuO-NP-treated PBW+media after adding media components (n=3) were assessed to ensure changes in contaminant concentration due to treatment were represented in untreated PBW+media and CuO-NP-treated PBW+media applied to cells.

A	Untreated PBW+Media	
Concentrations of Untreated PBW (log X)	% Viability (HEK cells)	% Viability (HEP cells)
EMEM	100	100
16.5% (1.217)	51.4	50.8
29% (1.462)	39	33.3
44% (1.643)	19.3	14.7
56% (1.748)	14.5	9.4
IC$_{50}$ Log [PBW]	**1.264**	**1.243**
B	CuO-NP-treated PBW+Media	
Concentrations of CuO-NP-Treated PBW (log X)	% Viability (HEK cells)	% Viability (HEP cells)
EMEM	100	100
17% (1.230)	86.7	119.8
30% (1.477)	75.8	86.7
45% (1.653)	81	92.4
53% (1.724)	70.3	97.5
IC$_{50}$ Log [PBW]	**2.744**	**5.327**

Table 4: Calculation of IC$_{50}$. The IC$_{50}$ represents the concentration of untreated PBW+media or CuO-NP-treated PBW+media that is required for a 50% inhibition of viability. The percent viability on day 5 for HEK and HEP cells exposed to dilutions of untreated PBW+Media (**A**) or CuO-NP-treated PBW+Media (**B**) were used to calculate the half maximal inhibitory concentration (IC$_{50}$).

Discussion

Previous studies reported that CuO-NPs removed arsenic from groundwater [11,13,30,31]. This study supports these previous findings and also reports that CuO-NPs remove additional contaminants from PBW. This study also confirms previous reports that CuO-NPs are effective at arsenic removal, despite the presence of other contaminants and potential competing ions [11]. Speciation modeling predicted that 97% of vanadium species in PBW are negatively charged, allowing for adsorption to CuO-NPs, and batch treatment removed 92% of vanadium.

This is the first study to investigate the effects of removing specific contaminants from PBW using CuO-NP, and then assess the changes in cytotoxicity associated with the removal. The results demonstrate that investigating the changes in cytotoxicity of complex mixtures using an *in vitro* approach may be possible, but these methods are not without limits. PBW could not be used full strength on the cells, because to survive, cultured cells require a defined growth media and specific osmolality. PBW+media could also not be used on the cells without pH adjustment. The pH of the PBW was 7.31 before and 7.36 after treatment however; the addition of growth media components reduced the pH to approximately 6.8, depending on the dilution. Ph adjustment is a normal step in the preparation of cell culture media however; adjusting the pH of the PBW+media may have altered the molecular interactions of the element species with media components. Untreated and CuO-NP-treated PBW were combined with concentrated EMEM-10X growth media in various proportions to obtain the test solutions (PBW+media). ICP-MS analysis was performed on test media to verify that the concentrations of metals significantly affected by CuO-NP-treatment (arsenic, copper,

selenium, uranium, vanadium) were at expected concentrations after dilution by media components and osmolality adjustment. The decrease in arsenic, selenium, and vanadium after CuO-NP-treatment is reflected in the concentration differences between untreated PBW+media and the CuO-NP-treated PBW+media. Uranium concentrations are higher in the CuO-NP-treated PBW+media than predicted. ICP-MS data (**Table 1**) suggests that more uranium was removed from PBW during CuO-NP treatment than predicted by modeling. Speciation modeling (**Table 2**) predicted that at pH 7.3, only 35.5% of the uranium species are negatively charged. The model predicts that the major uranium species, calcium uranyl carbonate ($Ca_2UO_2(CO_3)_3$), is neutral.

The observed 78% removal of uranium was likely due to a combination of uranium adsorption and precipitation (as a calcium uranyl carbonate mineral). Based on the geochemical modeling, the percentage of uranium removed by adsorption is less than calculated allowing for a higher concentration in the CuO-NP-treated PBW+media. The mechanism of uranium removal by CuO-NP-treatment is unclear and requires further investigation. An increase in the concentration of calcium, potassium and magnesium was expected when PBW was added to EMEM-10x however; CuO-NP-treatment did not produce a significant change in these elements so no difference was seen in untreated vs. CuO-NP-treated PBW+media. The technique of combining the actual environmental with media components was successful in representing the changes seen in element concentrations due to treatment; however the oxidized nature of the PBW limited how the PBW+media could be made. In an attempt to increase the maximum concentration of the elements in test media, powdered cell culture media was originally mixed with untreated and CuO-NP-treated PBW to make PBW+media. The powdered media often resulted in precipitation of calcium salts and it increased the osmolality of the PBW+media which required a greater dilution with RO water, producing concentrations close to those obtained with liquid 10x media. These issues are most likely PBW-specific due to its oxidative state and may not be an issue with other less sensitive mixtures.

The MTT assay was chosen to assess cytotoxicity because it is a recognized standard high-throughput assay that evaluates the overall health of cells by measuring mitochondrial activity. This method has advantages and disadvantages. The 96-well format is useful for obtaining multiple data points however; the majority of the cells at day 5 were unhealthy looking, rounded and no longer attached to the plate. The photos in **Figure 2** were taken before the media was removed using a vacuum; suctioning off the media, and then adding the MTT solution may have removed unattached cells or detached poorly adherent cells, contributing to the overall plateau of the MTT signal after Day Two seen with untreated PBW. The assumption is that floating cells are dead or dying and only the attached cells are assessed using this method. It is also important to consider the limitations of the MTT assay with respect to studies using nanoparticles.

Previous studies have reported that, when directly applied to cultured cells, nanoparticles may have inherent toxicity, beyond their base chemical properties, depending on their unique physical characteristics such as size and shape [32,33]. In this current study, we did not apply the CuO-NPs directly onto the cells. Instead, cells were exposed to PBW that had been previously treated with CuO-NPs, centrifuged to remove the majority of the CuO-NPs and then filtered twice to remove more CuO-NPs before the PBW was used to prepare PBW+media. The MS results showed an increase in copper after treatment. This could be copper ions that were dissolved from the nanoparticles during treatment or CuO-NPs that may have passed through the centrifugation/filtration steps to remain in the treated PBW used to make the PBW+media. CuO-NPs range in size from 12-18 nm with a BET measured surface area of 85 ± 1 m^2/g [11] but are known to aggregate and based on the minimal increase in copper concentrations in the treated PBW, most of the copper regardless of the source is removed after centrifugation and filtration. Visual confirmation of improved cell health and confluence support the MTT assay results of improved viability due to CuO-NP treatment of the PBW (**Figure 2**). Future studies using other methods can evaluate (or characterize) similar confounding effects caused by CuO-NPs.

Human Embryonic Kidney (HEK 293) and Human Hepatocellular Carcinoma (HEP G2) cells were chosen for toxicity testing. These are a standard cell lines that are clinically relevant to heavy metal organ toxicity [24,25,34-40]. A low seeding density was used for the MTT assays. Cells were seeded at 500 cells/well, allowed to recover for 24 hr, and then exposed to the test media. The low seeding density was necessary to achieve a growth curve with a log phase around day 5, before becoming over-confluent and stationary on day 6 or 7. Chakraborty *et al.* (2010) reported that in a study of cadmium toxicity on cultured kidney proximal tubule cells (PTC), confluency and proliferation status (proliferating vs. quiescent) affected the response to cadmium exposure: sub-confluent proliferating cells showed more cytotoxicity than confluent (quiescent) cells. HEP and HEK cells exposed to PBW at a higher concentrations (greater confluency) similar to those used for other assay (results not shown) did not show the robust changes in the morphology seen with the MTT assay. Further investigation into the changes in cytotoxicity using non-adherent cell lines or protocols that harvest and collect all cells (*e.g.* flow cytometry) is needed.

Another limitation of the MTT method in studies using nanoparticles is that some types of nanoparticles may interfere with cellular nutrition. Cell culture media typically contain added protein sources, such as fetal bovine serum (FBS), to supplement cell growth. Studies have shown that metal oxide nanoparticle can deplete important growth components in FBS, due to the increased absorption capacity of nanoparticles. Metal oxide nanoparticles have been shown to link to FBS through an interaction with calcium [41]. Depending on the pH of the solution, metal nanoparticles can carry a positive or negative charge. Cytotoxicity studies have shown that metal nanoparticles added to cell culture media adsorb cations, including Ca^{2+}, and then remove FBS/serum albumin through binding of the NP-Ca^{2+} complex to the calcium binding sites on proteins in the FBS. This decreases the concentration of Ca^{2+} and FBS from the media, essentially starving the cells and increasing the cytotoxicity attributed to the nanoparticles [41]. Furthermore, pre-exposure of nanoparticles to FBS/Ca^{2+} coated the nanoparticles, decreasing their cytotoxic effect. However, we did not directly expose the media to CuO-NPs. Also, no significant decrease in Ca^{2+} concentrations were seen in PBW after treatment with CuO-NPs, indicating no significant absorption of Ca^{2+} onto the CuO-NPs priming them to bind with the FBS. However, the concentration of calcium in the PBW is high enough that a nanoparticle-induced decrease may not have been apparent. It is still unlikely that the CuO-NPs used in this study are absorbing large amounts of calcium during processing, because there was no decrease in arsenic absorption capabilities of the CuO-NPs in PBW, which contains high levels of calcium compared to earlier studies with groundwater with a lower calcium concentrations [13].

The data demonstrate that CuO-NPs remove arsenic, selenium vanadium and uranium, and this is associated with improved HEK and HEP cell viability in the MTT assay. The mechanism(s) by which viability is improved has yet to be determined, but could be due to removal of priority contaminants by CuO-NP, among other mechanisms. The current study also demonstrates that standard cell culture methods can be used to assess the efficacy of a nanoparticle ISR water treatment method, potentially allowing a range of mechanistic studies to be completed, before moving into the more costly and time-consuming *in vivo* animal studies. In addition, CuO-NPs may prove to be more versatile for mining processes and for the treatment of metal mixtures than conventional adsorbents like oxides of aluminum, iron, titanium, and manganese, since CuO-NPs do not require pH adjustment or oxidation of water for arsenic removal, and CuO-NPs remove both arsenite and arsenate in the

presence of the competing anions phosphate, silicate and sulfate. Also, CuO-NPs can be regenerated and re-used, reducing reagent costs and the amount of spent treatment waste byproducts in need of disposal [12].

Potential limitations of the MTT protocol include the low cell density at time of exposure, detachment of cells and loss of signal, cell starvation and possible direct exposure of the cells to CuO-NP altering MTT reactivity. Cell density and detachment issues could be addressed by using an alternative test such as flow cytometry, which allows for higher seeding densities as well as the collection of all cells (*i.e.*, both floating and attached). Cell starvation questions could be assessed by measuring growth factor concentrations in the media periodically during treatment. Future work will focus on applying the current protocol to different cytotoxicity assays which will address if possible CuO-NP exposure altered assay activity, measurements of cell starvation during treatment and also testing the ability of CuO-NPs to remove contaminants and affect the cytotoxicity of other types of complex mixtures, such as waste from superfund sites and waste disposal ponds. Such studies would also address whether the methods were robust in various settings.

Disclosures

The authors have nothing to disclose.

Acknowledgements

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