Cell Rescue by Nanosequestration: Reduced Cytotoxicity of An Environmental Remediation Residue, $\text{Mg}(\text{OH})_2$ Nanoflake/Cr(VI) Adduct

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Supporting Information

ABSTRACT: Some nanomaterials, such as $\text{Mg}(\text{OH})_2$ nanoflakes, are heavily used in pollutant adsorption and removal. Residues from these environmental remediations are potential hazardous materials. Safety evaluations of these materials are needed for environmental protection and human health. Although nanotoxicity has been widely investigated in recent years, research on the toxicity of nanoparticle/pollutant adducts has been rather inadequate. Here, we report the cellular perturbations and cytotoxicity of nano- $\text{Mg}(\text{OH})_2/\text{Cr}(\text{VI})$ adducts as a case study to elucidate how nanoparticle/pollutant adducts impact human cells. We found that $\text{Mg}(\text{OH})_2$ nanoflakes barely enter cells, while desorbed $\text{Cr}(\text{VI})$ anions enter cells, generate ROS, induce cell apoptosis, and cause cytotoxicity. This cytotoxicity is only a fraction of the cytotoxicity of free $\text{Cr}(\text{VI})$ because nano- $\text{Mg}(\text{OH})_2$ particles are able to retain more than half of their $\text{Cr}(\text{VI})$ anions.

INTRODUCTION

Air-borne or solution-suspended nanoparticles are health issues because of their toxicity and because of the toxic agents they carry.^{1,2} Nanoparticles have a large specific surface area. To maintain their stability, they tend to adsorb chemical or biological agents on their surfaces to reduce their surface energy. These adsorbents are likely toxic, carcinogenic, viral, or infectious substances. When these particles enter the human body, they pose considerable threats to human health. In addition to these incidental nanoparticle/pollutant adducts, millions of tons of such adducts are formed during environmental remediation, as nanotechnology is increasingly being applied in environmental engineering.^{3,4}

Chromium has been widely used in industry.⁵ The worldwide environmental release of chromium-containing waste is over 40.8 million tons annually.⁶ Chromium(VI) has been designated as a class 1 carcinogen.^{7–9} Chromium waste is generally landfilled after chemical reduction. However, $\text{Cr}(\text{III})$ is easily oxidized back to $\text{Cr}(\text{VI})$ in the environment.¹⁰ Furthermore, chromium-containing wastes are readily translocated through the air, water, and soil and endanger the entire ecosystem.¹¹ The removal of highly toxic $\text{Cr}(\text{VI})$ from industrial waste is very important. Several approaches, including electrochemical reduction,¹² ion-exchange,^{13,14} and biological remediation,¹⁵ have been developed for $\text{Cr}(\text{VI})$ removal from wastewater. With their small size, large surface area, and high

surface-absorptivity, nanomaterials have been increasingly used as adsorbents for pollutant removal.¹⁶ Nanoflakes or disk-shaped nanoparticles normally have a reduced internalization^{17,18} and are particularly meaningful for environmental remediation. Specifically, due to their low toxicity and lower cost, magnesium hydroxide nanoflakes (nano- $\text{Mg}(\text{OH})_2$) have been widely used in pollutant remediation, e.g., the removal of organic pollutants and heavy metal ions such as carcinogenic $\text{Cr}(\text{VI})$.^{3,19} This operation, however, creates a new class of species: nanoparticle/pollutant adducts. The annual generation of nano- $\text{Mg}(\text{OH})_2/\text{Cr}(\text{VI})$ by Chinese chloralkali and chlorate plants has reached 800 000 tons.^{20,21}

The adsorption of pollutants to nanoparticles alters the properties of both the pollutant and the nanoparticle. Therefore, the toxicity of these adducts to human and living systems may be quite different from that of the pollutant or nanoparticle alone. For example, the adsorption of natural organic matter to multiwalled carbon nanotubes increases the aqueous stability of hydrophobic nanotubes and toxicity of the adducts to daphnia.²² The tributyltin/nano- TiO_2 adduct is more toxic to abalone embryos than tributyltin alone.²³ Similar

Received: November 8, 2013

Revised: December 24, 2013

Accepted: January 6, 2014

Published: January 6, 2014

synergistic toxicities to aquatic organisms have also been found for As, Pb, Cu, and Cd in the presence of nano- Al_2O_3 , nano- Fe_2O_3 , and nano- TiO_2 .^{24–28} Recently, we have observed significant research efforts exerted toward the investigation of nanoparticle toxicity. However, very little attention has been paid to nanoparticle/pollutant adducts. In reality, bare nanoparticles are nonexistent. Nanoparticles always exist as adducts with molecules from the immediate microenvironment. Therefore, investigating the health impacts of such nanoparticle adducts is imperative.

In this investigation, we evaluated the effects of nano- $\text{Mg}(\text{OH})_2$ and nano- $\text{Mg}(\text{OH})_2/\text{Cr}(\text{VI})$ adducts on human cells by assessing their cellular uptake, oxidative stress induction, cell viability, and apoptosis. Because $\text{Cr}(\text{VI})$ is known to induce nephrotoxicity⁹ and hepatic failure,^{5–9} we selected the human hepatocellular carcinoma epithelial cell line HepG2 and embryonic kidney epithelial cell line HEK293 for our investigations. Our results demonstrate that $\text{Mg}(\text{OH})_2$ nanoflakes are not evidently internalized by cells and are not cytotoxic. However, $\text{Cr}(\text{VI})$ anions released from nanoadducts are able to enter cells through nonspecific anion channels and cause cell damage. In this case, nano- $\text{Mg}(\text{OH})_2/\text{Cr}(\text{VI})$ adduct formation significantly reduces the cellular uptake of $\text{Cr}(\text{VI})$ anions and its associated cytotoxicity.

MATERIALS AND METHODS

Nano- $\text{Mg}(\text{OH})_2$ and Nano- $\text{Mg}(\text{OH})_2/\text{Cr}(\text{VI})$. $\text{Mg}(\text{OH})_2$ nanoflakes were synthesized by coprecipitation of magnesium chloride hexahydrate and sodium hydroxide as described before.²⁹ Nano- $\text{Mg}(\text{OH})_2/\text{Cr}(\text{VI})$ was prepared by stirring nano- $\text{Mg}(\text{OH})_2$ in a $\text{K}_2\text{Cr}_2\text{O}_7$ solution (80 mg/L) for 4 h. The suspension was then washed and centrifuged at 5000 g 3 times.

Characterization of Nano- $\text{Mg}(\text{OH})_2$ and Nano- $\text{Mg}(\text{OH})_2/\text{Cr}(\text{VI})$. The morphology and size of the nanoparticles were analyzed using a transmission electron microscope (TEM, JEM-1011, JEOL, Japan). Their hydrodynamic size was analyzed using a dynamic light scattering instrument (DLS, Nano ZS 90, Malvern, UK). Samples were prepared in water or Dulbecco's modified eagle medium (DMEM). Samples were sonicated before DLS determination. The thickness of the nanoflakes was measured using an atomic force microscope (AFM, NanoScope IIIA, Veeco Instruments Inc., United States). Images were obtained in the tapping mode and then analyzed using the accompanying Veeco Nanoscope software. Crystal structures of the sample powders were also analyzed by X-ray diffraction (XRD) using a PANalytical X'Pert PRO diffractometer with $\text{Cu K}\alpha$ radiation. Data were collected with continuous scanning 2θ from 5° to 85° at 0.017° per step. To understand the surface charge properties of the nanoflakes, the zeta potentials of the suspensions were analyzed using a Zeta Sizer instrument (Nano ZS 90, Malvern, UK). The adsorption capacity of nano- $\text{Mg}(\text{OH})_2$ for $\text{Cr}(\text{VI})$ was determined by ICP-AES (Optima7000DV, PerkinElmer, Inc., United States). A sample of supernatant or nano- $\text{Mg}(\text{OH})_2/\text{Cr}(\text{VI})$ adducts was dissolved in HNO_3 and then diluted with double distilled water (DDW) (18.2 M Ω , Pall, Reservoir, United States). The dissolved or the adsorbed amount of $\text{Cr}(\text{VI})$ was calculated from the content of Cr as obtained from ICP-AES elemental analysis. Data were from the average of three independent measurements.

Cell Culture. The human hepatocellular carcinoma epithelial cell line HepG2 (ATCC) and embryonic kidney epithelial cell line HEK293 (ATCC) were cultured in

Dulbecco's Modified Eagle's Medium (GIBCO, Life Technologies, United States) with 10% fetal bovine serum (HyClone, Thermo Fisher Scientific Inc., United States), 100 U/mL penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin solution (HyClone, Thermo Fisher Scientific Inc., United States) at 37°C in a 5% CO_2 incubator.

Cellular Uptake. Cells were incubated with 200 $\mu\text{g}/\text{mL}$ nano- $\text{Mg}(\text{OH})_2$ or 206.2 $\mu\text{g}/\text{mL}$ nano- $\text{Mg}(\text{OH})_2/\text{Cr}(\text{VI})$ for 2 h. After washing, cell pellets were fixed for 1 h in 2% osmium tetroxide with 3% potassium ferricyanide. Thin (70 nm) sections were cut on a Leica UC6 ultramicrotome for TEM observation. Quantification of cellular uptake was performed by ICP-MS analysis after incubation with nanoparticles. The cells were washed, sonicated, and digested overnight in Aqua Regia at 37°C . The cellular uptake of Mg and Cr was determined by ICP-MS (Agilent 7500 series, Agilent Technologies, United States), and a series of Mg/Cr standard solutions (0, 5, 10, 20, 50, 100, and 200 $\mu\text{g}/\text{mL}$) with ^{236}Y as an internal standard for calibration. To quantify the cellular uptake of free $\text{Cr}(\text{VI})$, the anion exchanger inhibitor 4,4'-diisothiocyano-2,2'-stilbenedisulfonic acid (DIDS) (Sigma Corporation of America) was used to block free $\text{Cr}(\text{VI})$ ($\text{Cr}_2\text{O}_7^{2-}$) permeation. A nontoxic inhibition concentration of DIDS (0.1 mM) was selected using the WST-1 assay (Supporting Information Figure 2). Cells were preincubated with 0.1 mM DIDS for 30 min, and cell uptake experiments were carried out as described.

Cell Viability. Cells (5000 cells/well) were seeded into a 96-well plate 24 h before incubation with nanoparticles, adducts, and $\text{Cr}(\text{VI})$ for 24 or 48 h. The exposure concentrations of nano- $\text{Mg}(\text{OH})_2$ were 25, 100, and 400 $\mu\text{g}/\text{mL}$. According to the determined adsorption capacity of $\text{Cr}(\text{VI})$ on $\text{Mg}(\text{OH})_2$, the concentrations of adducts were 25.8, 103.1, and 412.4 $\mu\text{g}/\text{mL}$, while concentrations of $\text{Cr}(\text{VI})$ were 0.8, 3.1, and 12.4 $\mu\text{g}/\text{mL}$. Cell mitochondria dehydrogenase activity, which is proportional to cell viability, was determined using the WST-1 assay (Beyotime Institute of Biotechnology, China). Cell morphology after being treated with 100 $\mu\text{g}/\text{mL}$ of nano- $\text{Mg}(\text{OH})_2$, the corresponding adducts, and $\text{Cr}(\text{VI})$ were also monitored using an optical microscope (Olympus IX71, Olympus Corporation, Japan).

Reactive Oxygen Species (ROS) Generation and Cell Apoptosis. The production of cellular ROS was determined by flow cytometry (Guava Millipore, Merck KGaA, Germany) using dichlorofluorescein diacetate as a fluorescent marker. The mean fluorescence intensity of a given amount of cells was obtained using GuavaSoft, which is the analysis software for Guava Millipore Flow Cytometer by BD Biosciences (San Jose, California, U.S.A.). Cells given the same treatments were also washed, trypsinized, and resuspended in PBS for apoptosis analysis. The Nexin-V reagent (Guava, Millipore, Merck KGaA, Germany) was used for cell staining, followed by flow cytometry analysis.

RESULTS AND DISCUSSION

Nanoparticle/pollutant adducts constitute a newly emerging environmental hazard. To evaluate their cytotoxicity, a careful characterization of nanoparticles and their adducts as well as their solubilization, desorption behavior, and aggregation tendency is crucial.

Properties of Nano- $\text{Mg}(\text{OH})_2$ and Nano- $\text{Mg}(\text{OH})_2/\text{Cr}(\text{VI})$ Systems. The morphology, size distribution, aqueous dispersity, crystal structure, and adsorption capacity of nano- $\text{Mg}(\text{OH})_2$ and nano- $\text{Mg}(\text{OH})_2/\text{Cr}(\text{VI})$ were characterized

Figure 1. Characterization of nano-Mg(OH)₂ and nano-Mg(OH)₂/Cr(VI) adducts by TEM and AFM. The scale bar in A is for all TEM panels.

Table 1. Characterization of Nano-Mg(OH)₂ and Nano-Mg(OH)₂/Cr(VI)^a

	Nano-Mg(OH) ₂		Nano-Mg(OH) ₂ /Cr(VI)	
XRD
Primary size/ TEM and AFM (nm)	62-71			
Hydrodynamic size/ DLS (nm)	859.8±79.1 (In water)	313.6±25.7 (In culture media)	733.0±142.4 (In water)	340.1±47.6 (In culture media)
Thickness/ AFM (nm)	8.5±1.2			
Zeta potential (mV)	33.5±0.5 (In water)	-22.5±0.4 (In culture media)	4.5±0.6 (In water)	-21.7±0.9 (In culture media)
Adsorption/ K ₂ Cr ₂ O ₇ μg/mg Mg(OH) ₂	NA		15.2	15.2
Cr(VI) desorption after 24 hrs	NA		25.8%	35.6%

^aAFM, XRD, and TEM diameter and thickness (AFM) are not related to “water” or “culture medium”. They can be listed at the top of table. The rest are listed below those four properties and only at the top list “in water” and “in culture medium”, not at every item.

(Figure 1 and Table 1). TEM (Figure 1) showed hexagonal shape of nano-Mg(OH)₂ and the adducts with a diameter of ~70 nm. AFM (Figure 1) showed that the thickness of these nanoflakes was about 8–9 nm. The XRD pattern of nano-Mg(OH)₂ particles (Table 1) also reveals that diffraction peaks can be well-indexed as hexagonal nanoflakes with a calculated crystallite size of 10.2 ± 0.4 nm in the [001] direction using the

Scherrer equation. This value was very close to the value of 8–9 nm measured by AFM. The hydrodynamic diameter of the nanoflakes in water was 700–800 nm as determined by dynamic light scattering. The increased size was most likely due to the formation of a hydration shell around the nanoflakes, their disk-like shape, and partial aggregation of the nanoflakes.^{30,31} Because the nanoparticles were suspended in cell

Figure 2. Cellular uptake of nano-Mg(OH)₂ and nano-Mg(OH)₂/Cr(VI). (A) TEM observation of cells treated with nanoflakes (100 μg/mL) for 24 h. (B and C) Quantitative determination of the internalized Mg and Cr(VI) by ICP-MS. Total uptake% = The internalized Mg²⁷/ total dosed Mg²⁷ × 100%. All the uptake data were normalized to the cell numbers. (D) Cell uptake after the anion channels are blocked. In B–D, left panels are for HEK293 and right panels are for HepG2. (0.01 < **p* value < 0.05, 0.001 < ***p* value < 0.01). The scale bar in A is for all TEM panels.

culture medium for the cell-based assays, we also analyzed their hydrodynamic diameter in this medium. Protein adsorption on nanoparticles' surface^{32,33} can break large aggregates of particles into smaller aggregates because protein coatings effectively reduce the surface energy of nanoparticles. We observed that serum proteins facilitated nanoparticle dispersion and reduced their hydrodynamic size to ~300 nm. Protein adsorption should increase the size of each individual particle.^{33,34} Yet, the average dynamic size of the nanoflakes was reduced from 800 to 300 nm, suggesting that protein binding actually broke up most of the aggregates. The zeta-potential value of nano-Mg(OH)₂ was approximately +30 mV in water. The positive

charge of the nanoflakes promoted the adsorption of Cr(VI) anions. After adsorption, the zeta potential of adducts became negative (−22 mV). According to the electric double layer diffusion theory, zeta potential primarily depends on the charge difference of the positive colloidal nucleus and the surrounding negative ions. The Cr₂O₇^{2−} anions neutralized the colloidal nucleus and reduced the positive potential of the whole colloidal particles. The diffraction peaks for adducts in the XRD spectra matched the hexagonal crystal and the dimension of nano-Mg(OH)₂. Therefore, the Cr(VI) adsorption did not change the crystal lattice of nano-Mg(OH)₂. This demonstrated that the chemical nature of the nano-Mg(OH)₂ crystal and

Figure 3. Cytotoxicity of nano-Mg(OH)₂ and nano-Mg(OH)₂/Cr(VI). (A) Cell viability assessment by quantifying mitochondria dehydrogenase activity. (B) Cytotoxicity after blocking anion channels. (C) Morphology of treated cells at 24 h (equivalent to 10.5 µM Cr(VI)).

Cr(VI) anions were not altered and that Cr(VI) anions were only physically adsorbed onto the surface of the nanoflakes. The adsorption of Cr(VI) or Cr₂O₇²⁻ reduced the positive surface charge of nano-Mg(OH)₂ slightly in water but did not change the size, surface charge, or aqueous dispersion properties of nano-Mg(OH)₂ in cell culture medium. This was likely due to serum proteins heavily bound to nanoparticles^{32,33} and dominated the modification on surface electrical properties.

Because Cr(VI) anions are only physically adsorbed onto nano-Mg(OH)₂, an adsorption/desorption equilibrium must occur in solution. By quantitatively determining the amount of Cr(VI) by ICP-MS, the adsorption capacity of the nanoflakes was found to be 15.2 mg K₂Cr₂O₇/g nano-Mg(OH)₂. This value was used to determine the concentration of the control Cr(VI) in the cell-based assays. By using HEPES buffer solution

in cell culture media, the pH was maintained at 7.0. The balance of the adsorption/desorption equilibrium indicated that, in the nano-Mg(OH)₂/Cr(VI) system, Cr(VI) anions existed as free and adsorbed forms. In a simulated adsorption/desorption experiment, approximately 25–35% of the Cr(VI) anions were in free-anion form after incubation for 24 h in water and cell culture medium (Supporting Information, Figure S1).

Adsorption to Nano-Mg(OH)₂ Outside of Cells Reduced the Cellular Uptake of Cr(VI) Anions. Two-dimensional polymeric nanodisks mostly bound to cell surfaces with little internalization.¹⁸ Nano-Mg(OH)₂ nanoflakes were rarely detected inside *E. coli* and primarily bound to the bacterial surface.²⁹ Because of the repellent interaction between electronegative cell membranes, negatively charged nanoparticles are internalized less than positively charged nano-

particles.^{35–38} $\text{Mg}(\text{OH})_2$ nanoflakes were nearly two-dimensional nanoparticles and were negatively charged after $\text{Cr}(\text{VI})$ adsorption. On the basis of these considerations, we hypothesized that nano- $\text{Mg}(\text{OH})_2$ nanoflakes might not be internalized well. As a result, the cell uptake and cytotoxicity of $\text{Cr}(\text{VI})$ anions might be reduced. To test this hypothesis, we first investigated the cellular uptake of nano- $\text{Mg}(\text{OH})_2$ and its $\text{Cr}(\text{VI})$ adducts by TEM and ICP-MS. The nanoflakes were rarely observed inside cells after incubation for 24 h (Figure 2A). Consistent with the TEM observations, cellular Mg was less than 0.7 pg/cell (Supporting Information, Figure S2A) or 0.2% of the total applied nano- $\text{Mg}(\text{OH})_2$ dose, as determined by ICP-MS (Figure 2B). $\text{Cr}(\text{VI})$ adsorption did not increase the cellular uptake of $\text{Mg}(\text{OH})_2$ nanoflakes. These findings indicated that the cellular uptake of $\text{Cr}(\text{VI})$ through nano- $\text{Mg}(\text{OH})_2$ carriers was rare.

Because the adsorbed $\text{Cr}(\text{VI})$ anions might be desorbed from nanoparticles and free $\text{Cr}(\text{VI})$ anions might enter a cell through nonspecific anion channels in the cell membrane, we then analyzed the cellular uptake of free $\text{Cr}(\text{VI})$ (Figure 2C). The uptake rates of $\text{K}_2\text{Cr}_2\text{O}_7$ in HEK293 and HepG2 cells were determined to be 7.69×10^{-5} and 1.22×10^{-4} pmol/min·cell, respectively. Free $\text{Cr}(\text{VI})$ anions are known to enter cells through anion channels with an uptake rate of 5.15×10^{-4} pmol/min·cell after treatment with 20 mM $\text{Cr}(\text{VI})$.^{39,40} The cellular uptake of Cr in the nano- $\text{Mg}(\text{OH})_2/\text{Cr}(\text{VI})$ group was significantly reduced compared to free $\text{Cr}(\text{VI})$ at all time points (Figure 2C). After a 24-h incubation, the intracellular Cr uptake of the nano- $\text{Mg}(\text{OH})_2/\text{Cr}(\text{VI})$ group was approximately 45% of free $\text{Cr}(\text{VI})$ in HEK293 and 50% of that in HepG2 cells. Because nano- $\text{Mg}(\text{OH})_2$ rarely enter cells, $\text{Cr}(\text{VI})$ anions must have entered the cells after they were desorbed from the nanoflakes. Incubation of nano- $\text{Mg}(\text{OH})_2/\text{Cr}(\text{VI})$ adducts with cell culture medium for 24 h released 35% of $\text{Cr}(\text{VI})$ anions. This value was slightly different from the cellular uptake of Cr (45–50%) compared to free $\text{Cr}(\text{VI})$, likely due to enhanced $\text{Cr}(\text{VI})$ release when a portion of the extracellular $\text{Cr}(\text{VI})$ anions were internalized.

$\text{Cr}(\text{VI})$ anions readily penetrate plasma membranes through the nonspecific anion exchange carrier “band-3” proteins.⁴¹ To determine whether $\text{Cr}(\text{VI})$ entered cells through these channels, we used the anion channel blocker 4,4'-diisothiocyanato-2,2'-stilbenedisulfonic acid (DIDS) to block free $\text{Cr}(\text{VI})$ permeation through channels. DIDS covalently binds to “band-3” proteins and blocks over 80% of the anion channels in live cells.⁴² We determined the $\text{Cr}(\text{VI})$ uptake in the presence and absence of nano- $\text{Mg}(\text{OH})_2$ before and after the anion channels were blocked (Figure 2D). Cellular uptake of free $\text{Cr}(\text{VI})$ anions was almost completely blocked by the channel inhibitor. Furthermore, uptake of $\text{Cr}(\text{VI})$ from nano- $\text{Mg}(\text{OH})_2/\text{Cr}(\text{VI})$ was also significantly blocked (Figure 2D), demonstrating that $\text{Cr}(\text{VI})$ anions entered cells through anion channels after they were desorbed.

The interactions between nanoparticle/toxicant adducts and cells are complex. Adducts can enter cells and cause synergistic toxicity, while desorbed toxicants can also enter cells and generate further cellular damage. In the case of the nano- $\text{Mg}(\text{OH})_2/\text{Cr}(\text{VI})$ adduct, the situation is simplified because nanoflakes do not enter cells. The only possibility is that desorbed $\text{Cr}(\text{VI})$ enters cells and causes cytotoxicity, and the above-mentioned results demonstrating the blocking of $\text{Cr}(\text{VI})$ internalization by channel blockers support this hypothesis.

Adsorption to Nano- $\text{Mg}(\text{OH})_2$ Reduces the Cytotoxicity of $\text{Cr}(\text{VI})$. Because nano- $\text{Mg}(\text{OH})_2$ did not enter cells, the cytotoxicity of nano- $\text{Mg}(\text{OH})_2/\text{Cr}(\text{VI})$ might be attributable to free $\text{Cr}(\text{VI})$ anions. Adsorption of $\text{Cr}(\text{VI})$ to nano- $\text{Mg}(\text{OH})_2$ reduced the amount of $\text{Cr}(\text{VI})$ that was readily taken up by cells. We therefore expected that nano- $\text{Mg}(\text{OH})_2/\text{Cr}(\text{VI})$ might lead to less cytotoxicity when compared to free $\text{Cr}(\text{VI})$. To test this, we determined the viability of cells by analyzing the activity of mitochondria dehydrogenase, which is proportional to the number of live cells. Cells treated with nano- $\text{Mg}(\text{OH})_2/\text{Cr}(\text{VI})$ and $\text{K}_2\text{Cr}_2\text{O}_7$ exhibited time- and dose-dependent cell growth inhibition (Figure 3A). To corroborate this finding, microscopic images showed cell shrinkage, round shapes, and the loss of attachment after treatment with nano- $\text{Mg}(\text{OH})_2/\text{Cr}(\text{VI})$ or $\text{Cr}(\text{VI})$ (Figure 3C). Nano- $\text{Mg}(\text{OH})_2/\text{Cr}(\text{VI})$ rescued these cells by increasing cell viability by 2-fold. Because $\text{Cr}(\text{VI})$ anions were partially desorbed from nanoflakes in cell culture media, we speculated that the cytotoxicity of nano- $\text{Mg}(\text{OH})_2/\text{Cr}(\text{VI})$ was mainly caused by free $\text{Cr}(\text{VI})$. We examined this scenario by blocking anion channels using DIDS (Figure 3B). Cell viability was significantly increased when anion channels were blocked. For the HEK293 cell line, cell viability increased to approximately 90%, while HepG2 cells showed nearly 100% viability. Thus, the cytotoxicity of nano- $\text{Mg}(\text{OH})_2/\text{Cr}(\text{VI})$ was attributed to free $\text{Cr}(\text{VI})$ anions that were desorbed from nanoflakes. This result was consistent with the above-mentioned cellular uptake results.

Adsorption to Nano- $\text{Mg}(\text{OH})_2$ Remediated $\text{Cr}(\text{VI})$ -Induced Oxidative Stress and Apoptosis. The mode of action for the carcinogenicity of $\text{Cr}(\text{VI})$ has been proposed before.⁴³ $\text{Cr}(\text{VI})$ anions readily diffuse into cells through nonspecific anion channels⁴¹ and are reduced to the “reactive intermediates” $\text{Cr}(\text{V})$ and $\text{Cr}(\text{III})$ by antioxidative enzymes inside the cells.^{44,45} During this process, molecular oxygen is reduced to superoxide ($\text{O}_2^{\bullet-}$), H_2O_2 , and hydroxyl radicals ($\text{OH}^{\bullet}$) through a Fenton-like reaction.⁴⁶ The generation of oxidative stress causes considerable cell dysfunction, including DNA damage,^{47,48} cell cycle arrest,⁴⁹ and apoptosis.^{50–52} We therefore investigated the induction of oxidative stress and cell apoptosis after various treatments. Nano- $\text{Mg}(\text{OH})_2$ alone did not significantly increase cellular ROS; however, the exposure of both cell lines to $\text{K}_2\text{Cr}_2\text{O}_7$ significantly elevated the cellular ROS levels (Figure 4A), which was consistent with a previous observation in HepG2 cells.⁸ In contrast to free $\text{Cr}(\text{VI})$, the nano- $\text{Mg}(\text{OH})_2/\text{Cr}(\text{VI})$ group generated ROS levels that were 40% of that induced by $\text{Cr}(\text{VI})$ in HEK293 and 51% of that by $\text{Cr}(\text{VI})$ in HepG2 cells at 48 h (Figure 4B).

Although the molecular mechanism of toxicity due to $\text{Cr}(\text{VI})$ has not been completely clarified, the consensus is that $\text{Cr}(\text{VI})$ suppresses cellular receptors that are involved in the immune response and activates cell cycle-related and proapoptosis genes.⁵³ The reduction of $\text{Cr}(\text{VI})$ to $\text{Cr}(\text{III})$ induces cellular oxidative stress and leads to cell cycle arrest, apoptosis, and neoplastic transformation.⁵⁴ We found that $\text{Cr}(\text{VI})$ induced significant cell apoptosis, but the adsorption of $\text{Cr}(\text{VI})$ onto nano- $\text{Mg}(\text{OH})_2$ reduced the number of apoptotic cells (Figure 4C). The apoptotic cell population was reduced by 40% in the nano- $\text{Mg}(\text{OH})_2/\text{Cr}(\text{VI})$ group when compared to the $\text{Cr}(\text{VI})$ group (Figure 4C). Therefore, the presence of $\text{Mg}(\text{OH})_2$ nanoflakes reduced the ROS and cell apoptosis induced by $\text{Cr}(\text{VI})$. The above results show that nano- $\text{Mg}(\text{OH})_2$ particles are not toxic and that cellular ROS generation and apoptosis induction result from highly toxic $\text{Cr}(\text{VI})$ anions. Because of

Figure 4. Induction of cellular ROS and cell apoptosis. (A) Determination of ROS level by flow cytometry and (B) quantification of flow cytometry data after normalization. (C) Cell apoptosis analysis by flow cytometry after cells were stained with Guava Nexin (0.01 < *p value < 0.05, 0.001 < **p value < 0.01).

the strong adsorption between nano-Mg(OH)₂ flakes and Cr(VI) anions, the adducts exhibit significantly reduced cytotoxicity (Table 2).

Ambient and solubilized nanoparticles always exist as adducts with environmental molecules; however, the adsorbed species are often environmental pollutants. In addition to investigating the toxicity of nanoparticles, understanding the toxicity of nanoparticle/pollutant adducts has become imperative. Nanoparticle/pollutant adducts are normally in equilibrium with adsorbed and desorbed pollutant species. In the case of nano-

Mg(OH)₂/Cr(VI) adducts (Figure 5), nearly half of the Cr(VI) anions could be desorbed from nanoparticles. Mg-

Figure 5. Cartoon illustrations showing interactions of free Cr(VI) and nano-Mg(OH)₂/Cr(VI) with cells.

(OH)₂ nanoflakes do not enter cells, most likely because of their nanoflake shape. However, desorbed Cr(VI) anions enter cells through nonspecific anion channels. These internalized Cr(VI) anions increase cellular ROS, induce cell apoptosis, and cause cell death. Because nanoflakes strongly retain about half of their Cr(VI) anions, the cytotoxicity of the adducts is significantly reduced when compared to Cr(VI) anions. Our findings have important implications for the environmental use of nanotechnology. Because two-dimensional nanoparticles are not readily to enter cells without significant aggregation, we believe that various nanomaterials that are engineered into two-dimensional shape are highly suitable for environmental remediation. Because Mg(OH)₂ nanoflakes themselves have little cytotoxicity and have superb adsorption capacity, they will continuously be used in pollutant removal applications. However, although the resulting nanoadducts have significantly reduced cytotoxicity, they are still toxic and need to be well-contained. It is highly necessary to further process these adducts to eliminate or recover nanomaterials and the desorbed pollutants.⁴

■ ASSOCIATED CONTENT

Supporting Information

Figures S1 and S2. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Table 2. Comparison of Cellular Effects Induced by Nano-Mg(OH)₂/Cr(VI) and Free Cr(VI)^a

comparison item	HEK293			HepG2		
	nano-Mg(OH) ₂ /Cr(VI)	free Cr(VI)	remediation (%)	nano-Mg(OH) ₂ /Cr(VI)	free Cr(VI)	remediation (%)
free Cr(VI) in solution	35%	100%	65%	35%	100%	65%
cellular uptake of Cr(VI)	3.5 pg/cell	7.4 pg/cell	53%	2.2 pg/cell	4.4 pg/cell	50.0%
ROS	1.2	1.8	33%	1.1	2.0	45%
apoptosis	7.4%	11.5%	36%	7.8%	12.8%	39%
cell viability	66%	26%	40%	61%	28%	33%

^aCell assays were carried out using 10.5 μM Cr(VI) or equivalent amount of Nano-Mg(OH)₂ and its adduct. Incubation time is 24 h.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was supported by the National Basic Research Program of China (2010CB933504) and National Natural Science Foundation of China (21077068 and 21137002).

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