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Ceria Nanoparticle Toxicity

Synthesis, Physicochemical Characterization, and Cytotoxicity Assessment of CeO₂ Nanoparticles with Different Morphologies

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Abstract: With the growing use of nanomaterials, it is essential to carefully determine whether they represent a risk for potential users. So far, validated stand-alone methods that allow a proper risk assessment are still rare. In the present study, the cytotoxicity of CeO₂ nanoparticles has been assessed. For this purpose, a variety of well-defined CeO₂ nanoparticles has been prepared by using either hydrothermal synthesis or flame spray pyrolysis (FSP), resulting in nanoparticles of different morphologies and sizes. The FSP technique is known to produce particles of a very small size (in the range of nanometers), which can easily become airborne. We employed a characterization procedure that makes use of physicochemical techniques, comprising N₂ physisorption, XRD, TEM, as well as ζ-potential and surface-charge measurements. The cytotoxicity of the nanoparticles was evaluated in vitro on two different human lung cell lines (A549 and MRC-5). The tests showed that, despite the differences in surface properties, size, and morphologies, neither of the CeO₂ samples gave rise to a cytotoxic response.

Introduction

Nanomaterials are defined as materials with an external dimension on the nanoscale (1–100 nm) or having an internal structure or surface structure on the nanoscale.[1] Compared to a bulk material, a nanomaterial may show remarkably different optical, electrical, mechanical, and chemical properties, that can be used in fields like solar energy conversion,[2] catalysis,[3] drug delivery,[4] and sensor technologies.[5] Due to the unique properties of nanomaterials, the world has seen a vast increase in their production and use in the last few decades. Although there is concern about the safety and toxicity effects of nanomaterials and lots of activities are devoted hereto, a uniform procedure that makes use of physicochemical techniques, comprising N₂ physisorption, XRD, TEM, as well as ζ-potential and surface-charge measurements. The cytotoxicity of the nanoparticles was assessed in vitro on two different human lung cell lines (A549 and MRC-5). The tests showed that, despite the differences in surface properties, size, and morphologies, neither of the CeO₂ samples gave rise to a cytotoxic response.

Cerium dioxide nanoparticles (CNPs) have attracted widespread attention, due to their unique chemical, mechanical, electrical, and optical properties, which are especially interesting when the size of the CNPs is on the nanoscale.[7] They find applications in heterogeneous catalysis,[8] chemical mechanical polishing,[9] UV-resistant coatings,[10,11] and nanomedicine.[12] One of the key aspects of CNPs is that they have unique regenerative properties, owing to their low reduction potential and the coexistence of both Ce³⁺/Ce⁴⁺ on their surfaces.[13–15] Defects in the crystal lattice, due to the presence of Ce³⁺, play an important role in tuning the redox activity of CNPs. The surface Ce³⁺/Ce⁴⁺ ratio is influenced by the microenvironment in which the CNPs are synthesized. Therefore, the adopted synthesis method plays an important role in determining the properties of the resultant CNPs, and the potential applications, as well as the biological activity and the toxicity of CNPs. CNPs have shown the ability to scavenge reactive oxygen species, which are harmful, and to reduce oxidative stress.[16,17] The ability to trap oxygen radicals has been linked to the abovementioned ease of switching from the Ce³⁺ to the Ce⁴⁺ oxidation state. Other authors, in contrast, claim that this property itself is the source of oxidative stress, and that CNPs induce genotoxicity through the production of oxygen radicals.[18] Reed et al.[19]
stated that when the size of CNPs decreases, the amount of Ce\textsuperscript{3+}, and thus, oxygen vacancies, on the surface increases.

With the increasing use of CeO\textsubscript{2} in the nanomedicine field, for instance, in antimicrobial applications,\textsuperscript{20} there is a clear need for a more thorough characterization of the interaction between CeO\textsubscript{2} nanoparticles and human organs.\textsuperscript{21} The work of Gagnon et al.\textsuperscript{6} clearly underlines the confusion and lack of unambiguous conclusions that characterizes the topic. The test method should be able to reproduce, as closely as possible, the processes that take place during the exposure of an organism to nanomaterials. Studies on lung cells represent the inhalation route well, which is the most likely route for nanoparticles. Physicochemical methods can provide information on the size, shape, and surface properties of the CNPs, while biological tests offer evidence on absorption routes, nanoparticle–cell interactions, and most importantly, on toxicity. Lastly, the careful evaluation of the dissolution and dispersion of the CNPs in the biological medium is of great importance.

Lin et al.\textsuperscript{22} synthesized CNPs with a size of 20 nm and assessed their toxicity when a A549 human bronchoalveolar carcinoma-derived cell line was subjected to increasing doses of CNPs. The results show that CNPs generate oxidative stress on the cells and cause membrane leakage. The authors point out that not only does the concentration of the nanoparticles matter, but the overall cytotoxicity is also dependent on factors, such as the presence of dopants and impurities, nanoscale morphology, surface treatments, crystallinity, and agglomeration level. Xia et al.\textsuperscript{17} studied CeO\textsubscript{2} nanoparticles produced by flame spray pyrolysis and evaluated the cellular uptake and toxic effects on mice macrophages RAW 264.7 and human BEAS-2B lung epithelial cells. Their results show that CeO\textsubscript{2} nanoparticles are able to suppress the production of reactive oxygen species (ROS), proving to have a positive influence on the cells. Park et al.\textsuperscript{23} synthesized CNPs of sizes ranging from 15 to 45 nm by a supercritical synthesis method, and evaluated their toxicity to human BEAS-2B lung epithelial cells. The results show that the cells are subjected to severe negative effects, such as ROS increase, oxidative stress increase, and the induction of stress-related genes (oxygenase-1, catalase, glutathione S-transferase, and thioredoxin reductase). Moreover, it was demonstrated that the CNPs were able to penetrate into the cytoplasm of the cells. It was shown that the CNPs aggregate in the perinuclear region of the nucleus, which might be responsible for the high number of adverse reactions observed. A study conducted by Ji et al.\textsuperscript{24} underlined the importance of the careful distinction, not only of the chemical composition of a sample, but also of the particle aspect ratio, in the case of needle-shaped CNPs. The aspect ratio is defined as the length of a particle divided by its width, and it is essential for distinguishing fiber-like materials. Their study considers the synthesis of multiple CeO\textsubscript{2} nanorod systems and the subsequent evaluation of the effect of the sample on a THP-1 monomyelocytic leukemia cell line. The toxicity was evaluated by monitoring the activation of the NALP3 inflammasome due to lysosomal damage. Previous studies on asbestos fibers had established that if the NALP3 inflammasome was activated, it is likely to play a role in the generation of pulmonary inflammation and fibrosis.\textsuperscript{25} In their study, all nanorods with an aspect ratio of 1–16 proved to be nontoxic, while nanorods with aspect ratios higher than 52 caused cell death. Intermediate aspect ratio values triggered cell stress.

Flame spray pyrolysis is a technique that is nowadays widely used to produce high-purity oxides, such as SiO\textsubscript{2}, TiO\textsubscript{2}, Al\textsubscript{2}O\textsubscript{3}, FeO\textsubscript{3}, and CeO\textsubscript{2}.\textsuperscript{26} It starts from an organic solution of an organic precursor of the metal. The solution is sprayed into a CH\textsubscript{4}/O\textsubscript{2} flame and it is finely dispersed to tiny droplets. Due to the highly oxidizing atmosphere, the organic portion of the droplets is combusted and the growth of oxide nanoparticles starts. FSP-made particles are usually associated with relatively small size.\textsuperscript{27} Due to this feature, they are normally associated with the highest probability of becoming airborne. The information on nanosafety for CNPs prepared with FSP is rather scarce,\textsuperscript{28} and it is also not necessarily valid, as the specific FSP parameter settings may be different, and the properties of the particles might therefore be different as well.

Results and Discussion

The goal of our study is to assess the feasibility of a characterization and test method that can serve as a reference for researchers who need to evaluate the toxicity of CNPs or other nanoparticulate materials. In the present study, we performed the synthesis of model CNPs with different shapes and sizes using both hydrothermal and flame spray pyrolysis (FSP) methods, as described in the Exp. Sect. The products of the hydrothermal synthesis method differ by morphology and size. The rod-shaped CNPs are named after their aspect ratio (Rods9 and Rods15). Hydrothermal synthesis also yielded CNPs with a cubic morphology. Flame spray pyrolysis provided remarkably small CNPs, with a polyhedral shape. We used a combination of physicochemical characterization techniques and biological in vitro testing to obtain substantial information on the particle size, morphology, and surface properties of the CNPs, and their effects on biological systems. The CNPs were characterized in powder form by powder X-ray diffraction (XRD) and N\textsubscript{2} physisorption. For the hydrodynamics, ζ-potential, surface-charge, TEM, and biological testing, it was necessary to prepare dispersions of CNPs in appropriate media.

We investigated the effect of CeO\textsubscript{2} particles of different morphologies, immersed in cell-culture media on human lung cells. This method is suitable for nanotoxicity screening and is recommended, for example, by the EU project NanoValid.\textsuperscript{29} Inhaled particles smaller than 100 nm are very likely to reach the alveoli.\textsuperscript{30} Therefore, alveolar epithelial cells A549 have been used.\textsuperscript{31–34} Since A549 cells are derived from adenocarcinoma and might not accurately represent normal airway epithelium in all aspects, we also investigated the non-immortalized cell type MRC-5, which represents normal lung fibroblasts as the most abundant cell type of connective tissue, which together with lung epithelium, basement membrane, and capillary endothelium, act as structural barriers against the uptake of inhaled particles.
Particle Size, Surface Area, and Crystallinity

The particle-size analysis performed on the TEM images (Figure 1) showed that the shorter nanorods (Rods9) have a width of \((10 \pm 2)\) nm and a length of \((90 \pm 30)\) nm, while the longer nanorods (Rods15) have a width of \((11 \pm 2)\) nm and a length of \((160 \pm 50)\) nm. The nanocubes have an average size of \((17 \pm 4)\) nm, while the FSP-made nanoparticles have an average size of \((5 \pm 2)\) nm. The FSP-made nanoparticles have a polyhedral morphology, and a small portion of them displays an octahedral shape. Notably, for all four samples, a small fraction of particles shows a nonspecific morphology and a variable size smaller than 2 nm.

All samples are heavily agglomerated, even after the ultrasonication procedure, as can be seen in Figure 1. Due to the agglomerated state of the samples, only particles that could be recognized without doubt were included in the particle-size calculation.

The N\(_2\) physisorption results are summarized in Table 1. The FSP-made particles exhibit the highest specific surface area, while the nanocubes show the lowest value. The specific surface area values are comparable with those obtained in previous studies.[7] The surface area displayed by the FSP-made nanoparticles is much higher than that normally obtained for CeO\(_2\) materials, and this is in line with the smaller particle size. None of the CNPs showed relevant microporosity.

All charge-density measurements were performed at basic pH, in the range 8–9, by adding NH\(_3\)/NH\(_4^+\) buffer, to be able to generate the maximum number of negative surface groups, verified by a large negative streaming potential at the start of the charge titration. The titration measurements gave a charge density in the range of \(-0.06\) to \(-0.05\) mEq/g for all CNPs, with fair reproducibility. The raw charge-density data, in mEq/g, were converted into surface-specific data by dividing the charge value by the surface area of the sample. The results reported in Table 1 show a relatively high surface-charge density for the cubes and a low value for the FSP CNPs, while the rod-shaped CNPs have similar intermediate values. This then points to differences in crystal faceting, in terms of the facets involved and the extent to which they are exposed during the different synthetic routes.

The XRD patterns of CNPs synthesized by both hydrothermal and FSP methods are shown in Figure 2. The peak broadening due to the small size of the CeO\(_2\) crystallites is easily visible, especially in the case of the FSP-made samples. All samples show the CeO\(_2\) diffraction peaks, which are consistent with the cubic fluorite structure and space group \(Fm\bar{3}m\) reported in the literature (ICCD card 43–1002).[14,35] The calculation of particle size using the Scherrer method is not meaningful in our case, since the equation approximates all particles as having a spherical shape. A table comparing Scherrer-based particle sizes with TEM-based size determination can be found in the Supporting Information (Table S1).

| Sample | BET area \([\text{m}^2/\text{g}]\) | Charge density \([\text{mEq/m}^2]\) |
|--------|-------------------------------|----------------------------------|
| Rods9  | 87                            | \(-6.3 \times 10^{-4}\)           |
| Rods15 | 78                            | \(-7.1 \times 10^{-4}\)           |
| Cubes  | 28                            | \(-2.0 \times 10^{-3}\)           |
| FSP    | 153                           | \(-3.6 \times 10^{-4}\)           |

Figure 1. TEM images and particle-size distribution of the CeO\(_2\): (A) nanorods with an aspect ratio of 9; (B) nanorods with an aspect ratio of 15; (C) nanocubes; and (D) FSP-made particles. For both types of nanorods, only the size distribution of the width is reported.
Hydrodynamic Size, Dispersion, $\zeta$ Potential, and Isoelectric Point

The purpose of the tests described so far was the characterization of the bare CNPs. Subsequently, further studies were performed on the CNPs in media that are closer to those commonly employed in in vitro nanotoxicity studies.[36]

The dynamic light scattering and $\zeta$-potential results are summarized in Table 2. Herein, $Z_{av}$ is a measure of the hydrodynamic diameter, and the $\zeta$ potential describes the surface charge of the CNP aggregates. Moreover, the polydispersity index (PDI) and the $\zeta$ range represent the corresponding distribution widths. The pH of the dispersed CNPs in 1 mM KCl, here called the "natural pH", differs considerably (between 4.5 and 6.4 in Table 2), as does the accompanying $\zeta$ potential, which we attribute to differences in the synthetic procedure. The lowest natural pH value is displayed by the FSP-made particles, corresponding to a large positive $\zeta$ potential. The FSP-made CNPs also possess the highest value of the $\zeta$-potential range, with a wide distribution. The isoelectric point, to be considered as a specific material (surface) property, is defined as the pH at which nanomaterials have no net charge ($\zeta = 0$ mV). At lower pH values, the particles are positively charged ($\zeta > 0$ mV), while above the IEP, they bear a negative charge ($\zeta < 0$ mV). The isoelectric point of the nanorods can be estimated as being close to 6, while the cubes have an isoelectric-point value lower than 5.8.

Table 2. Results of the DLS and $\zeta$-potential measurements.

| CNP type | $Z_{av}$ [nm] | PDI   | Natural pH | $\zeta$ [mV] | $\zeta$ range [mV] |
|----------|---------------|-------|------------|--------------|-------------------|
| Rods9    | 2231          | 0.295 | 6.43       | -16.6        | 3.6               |
| Rods15   | 2997          | 0.145 | 5.96       | +8.16        | 2.7               |
| Cubes    | 1759          | 0.197 | 5.82       | -9.88        | 3.2               |
| FSP      | 557           | 0.477 | 4.51       | +34.4        | 10                |

Notably, one reported isoelectric point of CeO$_2$ is found at the much higher value of 6.75.[37] The large values of $Z_{av}$ show that the CNPs exist in aggregates of substantial sizes, and as the PDI values of all samples are all higher than 0.1, a broad hydrodynamic distribution can be expected. The results indicate that different synthetic procedures and conditions sensitively influence the surface composition and properties of the CNPs with different isoelectric points and charge-density values. The natural pH of the CNP dispersions differs considerably, which actually justifies the use of a buffer to test the particles under comparable conditions, where they will all be negatively charged and far from their IEP for optimum charge stability. Consequently, for further biological tests, all CNPs were tested at equal pH by applying a buffer at a pH of 7.4 (Modified Eagle’s Medium, MEM, supplemented with Glutamax™ and 10 % fetal bovine serum). Notably, in the following cytotoxicity tests, dispersion parameters can influence the cell–CNP interactions.[38]

Cytotoxicity

Before the biological tests, all CNPs were dispersed in a bovine serum albumin (BSA)-containing solution, using a standardized protocol, as described in the Experimental Section, to achieve a better dispersion in the biological media and to prevent uncontrolled aggregation upon encounter with serum proteins in the cell-culture medium. To avoid immediate agglomeration of the different CNPs upon dissolution in the cell-culture medium, a standardized protocol utilizing precoating with BSA was employed. With this protocol, dispersions that were stable for at least 1 h in the cell-culture medium were obtained for all particle types (data not shown). It is well known that NPs dispersed in biological media will quickly be covered by a dynamic protein corona, the nature of which will determine the cellular response to the NPs.[39] The BSA-precoated CNPs were analyzed by TEM. All samples appear to be homogeneously coated by the BSA (Figure 3).
The particles underwent cytotoxicity testing as described in the Exp. Sect. The results of the cytotoxicity tests are reported in Figure 4. CNPs were well tolerated by both A549 cells and MRC-5 cells, irrespective of morphology, despite the wide dose range applied. The highest doses we applied can be considered as particle overload doses, based on available calculated data for realistic and extreme in vivo exposures. Indeed, optical microscopic observation of the cells shows a large extent of particle deposits on the cellular surfaces (cf. Supporting Information, Figures S1–S5). Nevertheless, no adverse effect on cellular metabolism could be detected for any of the cell lines (nor did the hydrothermally prepared samples, regardless of aspect ratios, and surface properties, including a sample prepared by flame spray pyrolysis. The FSP-made sample showed a remarkably small particle size, which is often considered to be linked to toxicity. Unexpectedly, this sample did not show any negative interference with A549 or MRC-5 lung cells, and neither did the hydrothermally prepared samples, regardless of morphology and aspect ratio. Moreover, since the sample-denominated Rods15 and Cubes were synthesized by following a widely used procedure, we believe that the lack of toxicity of these particles is an important result for the safety of researchers working with these materials. This further implicates that they are likely to be relatively well tolerated in vivo, although further testing is needed to allow for a thorough risk assessment. The combination of physicochemical characterization techniques and biological in vitro tests applied to an extensive set of samples led to a comprehensive cytotoxicity assessment of the selected set of CNPs.

Experimental Section

Chemicals: Ce(NO₃)₃·6H₂O (99.5 % purity) was purchased from Alfa Aesar. CeCl₃·7H₂O (99.9 % purity), NaOH (reagent grade, ≥ 98 %), ethanol, Ce(CH₃COO)₂·H₂O (99.9 % purity), glacial acetic acid (99.7 % purity) were purchased from Sigma Aldrich. Polyelectrolyte poly-di-allyl-N,N-dimethyl-ammoniumchloride (poly-DADMAC) solution (0.001 N) was purchased from BTG. All compounds were used without further purification. Bovine serum albumin (BSA) was purchased from Sigma Aldrich. All cell-culture reagents (see below) were from ThermoScientific Hyclone.

Hydrothermal synthesis was performed following the procedure detailed in reference. Briefly, a Ce(NO₃)₃·6H₂O precursor was used as the precursor to obtain nanorods with an aspect ratio of 15 (Rods15), as well as nanocubes, and CeCl₃·7H₂O was used to obtain nanorods with an aspect ratio of 9 (Rods9). In a typical synthetic procedure, the precursor was dissolved in deionized water to achieve 5 mL of a 5 mM solution. The solution was poured into a NaOH solution (35 mL, 17.1 M) in deionized water at room temperature and stirred for 1 h. Deionized water was added to the resulting slurry until a volume of 100 mL was reached, and the mixture was poured into the Teflon liner of a 125 mL stainless steel autoclave. The hydrothermal treatment temperature chosen was 100 °C for both types of rods (static autoclave), and 180 °C for the nanocubes (tumbling autoclave). The hydrothermal treatment was prolonged for 24 h. The product was then washed with deionized water and ethanol and dried at 80 °C for 2 h in a vacuum oven. Subsequently, it was calcined at 500 °C for 4 h under a 2080 O₂/He flow.

Flame spray pyrolysis was performed with a Tethis NPS10 setup. The cerium precursor was Ce(CH₃COO)₂, which was dissolved in glacial acetic acid. The concentration of the solution was 0.15 M. To obtain a homogeneous solution, stirring was necessary for 1 h at 80 °C. We employed 30 mL of the starting 0.15 M solution and
injectd it in the nozzle at a speed of 5 mL/min. The flame was fed with a 1.5 SLPM flow of methane and a 3.0 SLPM flow of oxygen, as well as a dispersion flow of oxygen of 5.0 SLPM. We were able to collect ca. 0.25 g of powder on a glass fiber filter after the procedure.

XRD patterns were recorded with a Bruker D2 Phaser powder diffractometer system using Cu-Kα radiation, with a time per step of 0.5 min and a step size of 0.02° in the range 10°–90° 2θ. N2 physisorption was performed at –196 °C with a Tristar II 3020 instrument. Typically, 200 mg of each sample were transferred into a glass sample holder. The samples were previously heated to 120 °C under a N2 flow overnight to allow for desorption of water and impurities. The Brunauer–Emmet–Teller (BET) method was used to calculate the surface area.

Ultrasonication was used to achieve reproducible dispersion and to be able to conduct further experiments that require a homogeneous system. For this purpose, a 1.8 mg/mL solution was made in deionized water for each nanoparticle type. We made use of a Sonics VibraCell VCX 130 Ultrasonicator using the following settings: 130 W power, 40 % amplitude, total sonication time of 16 min, with a pulsing time of 10 seconds and a resting time of 10 seconds. Nanorods required a longer time to achieve satisfactory dispersion; therefore, a time of 24 min was used. Notably, in the cases of Rods9 and Rods15, in spite of the described vibrasonic treatment, some material deposited on the containers over time (overnight), indicating limited dispersion and colloidal stability.

TEM measurements were performed with a FEI Tecnai 20 electron microscope at an electron acceleration voltage of 200 kV with a LaB6 filament. A few milligrams of sample were ultrasonicated as required and imaged using a transmission electron microscope at an electron acceleration voltage of 200 kV with a LaB6 filament. A few milligrams of sample were ultrasonicated as required and imaged using a transmission electron microscope. TEM measurements were used to achieve reproducible dispersion and to be able to conduct further experiments that require a homogeneous system. For this purpose, a 1.8 mg/mL solution was made in deionized water for each nanoparticle type. We made use of a Sonics Vibra Cell VCX 130 Ultrasonicator using the following settings: 130 W power, 40 % amplitude, total sonication time of 16 min, with a pulsing time of 10 seconds and a resting time of 10 seconds. Nanorods required a longer time to achieve satisfactory dispersion; therefore, a time of 24 min was used. Notably, in the cases of Rods9 and Rods15, in spite of the described vibrasonic treatment, some material deposited on the containers over time (overnight), indicating limited dispersion and colloidal stability.

Dynamic light-scattering (DLS) characterization was performed with a Malvern Nano ZS (HeNe-laser, 633 nm) on a stock dispersion (1000 μL) of the CNP in Milli-Q water (1.8 mg/mL) diluted into 10 mL of filtered (0.45 μm) KCl solution (1 mM) in Milli-Q water. At a similar sample dilution, the pH and the ζ potential (applying Smoluchowski’s law) was determined with the Malvern Nano ZS, using the M3 PALS technique (phase angle light scattering). The pH meter had been calibrated just prior to the measurement. All the measurements were performed in triplicate.

Before the cytotoxicity tests, the particles were dispersed according to a modified version of the NanoGenotox protocol, where particles after the ultrasonication treatment, as described above, were subsequently suspended in Milli-Q water containing bovine serum albumin (0.5 % w/v). The BSA protein coated the nanoparticles and enabled further dispersion in biological media containing salts, vitamins, and many other different types of proteins, without immediate aggregation, which was likely to be the result without the precoating procedure. For this purpose, a CNP dispersion (1.8 mg/mL) containing BSA (0.5 % w/v) was made. The freshly made dispersion was then spread onto a carbon-coated copper grid and TEM images were recorded, as described above, in order to evaluate the effectiveness of the BSA coating procedure.

A549 cells and MRC-5 cells were cultured in Modified Eagle’s Medium (MEM) supplemented with Glutamax™ and 10 % fetal bovine serum (FBS). The pH of the buffer was 7.4 under 5 % CO2. Cells were maintained at 37 °C and 5 % CO2 and were trypsinized every 3–4 d, and were split at a 1:3–1:6 ratio. For toxicity evaluation, the cell culture medium was supplemented with antibiotics (1 % penicillin/streptomycin). Nanocytotoxicity was assessed by evaluating the metabolizing capacity of cells by conversion of a tetrazolium compound [(3-[4,5-dimethylthiazol-2-yl]-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS(a))] to a colored soluble formazan (CellTiter 96® AQueous One Solution Reagent, Promega). The amount of produced formazan was assessed by spectrophotometric evaluation at 490 nm. To calculate the viability percentage, the absorbance values at 490 nm were normalized to the absorbance of untreated cells cultured in hexaplicate in each plate. The mean of the six wells with untreated cells was used for normalization.

The BSA-containing stock of CNP dispersions in Milli-Q water (1.8 mg/mL) were further diluted in biological media for the biological tests. For the assessment, cells were seeded at 10000 cells per well in the inner wells of a 96-well plate. After 24 h, cells were exposed to nanoparticles at doses of 100 μg/mL, 50 μg/mL, 25 μg/mL, 10 μg/mL, and 1 μg/mL, with each concentration being assessed in triplicate (separate dilutions from the stock solution). On each 96 well plate, one type of CNPs was assessed. Controls consisting of cells not exposed to nanoparticles, nanoparticles in the medium with no cells present, the medium with neither nanoparticles nor cells, and positive-control CdSO4 (doses 150 μM down to 9.4 μM, which are equivalent to 31.3 and 2.0 μg/mL, respectively) were included on each plate. After 24 h exposure with nanoparticles, the cells were photographed at 10× magnification before they were washed once with cell-culture medium. Thereafter, the MTS reagent was added and cells were returned to the incubator for 1 h. Care was taken to avoid formation of bubbles upon addition of the MTS reagent to wells. After the incubation period, supernatants were transferred to new 96 well plates and formazan absorbance was measured at 490 nm with a Synergy 2 plate reader (BioTek Inc., VT, U.S.). Three independent biological runs were performed. Potential interference of NPs with the assay was controlled by the inclusion of CNPs added to the medium alone in two columns on each plate. These blanks underwent the same procedure as the exposed cells throughout the assay. The averaged absorbance values from these CNP blanks were subtracted from absorbance values for corresponding cells incubated with the same concentration of the CNPs or the chemical positive control, respectively.

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