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Leading opinion

Shifts in oxidation states of cerium oxide nanoparticles detected inside intact hydrated cells and organelles

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ABSTRACT

Cerium oxide nanoparticles (CNPs) have been shown to induce diverse biological effects, ranging from toxic to beneficial. The beneficial effects have been attributed to the potential antioxidant activity of CNPs via certain redox reactions, depending on their oxidation state or Ce^{3+}/Ce^{4+} ratio. However, this ratio is strongly dependent on the environment and age of the nanoparticles and it is unclear whether and how the complex intracellular environment impacts this ratio and the possible redox reactions of CNPs. To identify any changes in the oxidation state of CNPs in the intracellular environment and better understand their intracellular reactions, we directly quantified the oxidation states of CNPs outside and inside intact hydrated cells and organelles using correlated scanning transmission x-ray and super resolution fluorescence microscopies. By analyzing hundreds of small CNP aggregates, we detected a shift to a higher Ce^{3+}/Ce^{4+} ratio in CNPs inside versus outside the cells, indicating a net reduction of CNPs in the intracellular environment. We further found a similar ratio in the cytoplasm and in the lysosomes, indicating that the net reduction occurs earlier in the internalization pathway. Together with oxidative stress and toxicity measurements, our observations identify a net reduction of CNPs in the intracellular environment, which is consistent with their involvement in potentially beneficial oxidation reactions, but also point to interactions that can negatively impact the health of the cells.

1. Introduction

Recent advances in the design of engineered nanoparticles (NPs) have opened a range of possible applications, including medical applications. One type of nanomaterials attracting interest are cerium oxide NPs (CNPs), where diverse effects have been reported in the literature, ranging from toxic [1–10] to beneficial [11–20]. These disparate effects have been linked, in part, to the NP synthesis methods and the resulting physicochemical properties of the NPs, including their oxidation state, described as the Ce^{3+}/Ce^{4+} ratio [21]. Harmful or no effects have been mostly reported for CNPs synthesized by high temperature and heating in solvent methods [1,2,4–9,22–27], whereas no or beneficial effects have been reported for CNPs synthesized by room temperature methods [28–38].

The beneficial effects of CNPs have been attributed to their potential antioxidant activity, possibly resulting from superoxide dismutase (SOD)-like [29,39,40] or catalase-like activities. These activities were directly observed only in solution [41–43] and were dependent on the oxidation state of the NPs, with high Ce^{3+}/Ce^{4+} ratio linked to SOD-like activity [29,41], and lower Ce^{3+}/Ce^{4+} ratio linked to catalase-like activity [39]. However, it is unclear whether and how CNPs are directly involved in such redox reactions in the complex and compartmentalized intracellular environment, especially because the Ce^{3+}/Ce^{4+} ratio is strongly dependent on the environment and age of the NPs [28,39,44].

When measured in solution, it has been shown that CNPs in the +3 oxidation state may act as a SOD, catalyzing the dismutation of superoxide (O_2^-) to hydrogen peroxide (H_2O_2), which is more easily catalyzed in living cells into harmless species, including water and molecular oxygen [28,39]. CNPs in solution have been

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also shown to have catalase-like activity, reacting with H_2O_2 to produce water, oxygen, and hydrogen ions [39,43]. This breakdown of H_2O_2 can produce other reactive oxygen species (ROS) intermediates, suggesting a Fenton/Haber Weiss (FHW) mechanism [42]. Both catalase and FHW mechanisms have the same overall reaction for a complete cycle, which produces harmless water and oxygen, but may also produce reactive intermediates that could be damaging when occurred in live cells. While each of these activities - SOD, catalase, and FHW - can cycle between oxidation and reduction reactions, oxidation is favored in low pH environments for all the above activities, while reduction is favored in high pH environments for both catalase and FHW activities. Considering the complex subcellular environment, it is likely that oxidation or reduction will be favored in different subcellular compartments.

To detect any changes that might occur in the oxidation state of CNPs as they enter cells and organelles and better understand their reactions in the intracellular environment, we combined the use of scanning transmission x-ray microscopy (STXM) and super resolution fluorescence structured illumination microscopy (SIM) to quantify the Ce^{3+}/Ce^{4+} ratio of the NPs outside and inside intact hydrated cells and organelles. Our studies were conducted in alveolar epithelial cells, which present an intended as well as unintended target for airborne NPs that enter the respiratory tract [45–47]. We show that CNPs undergo a net reduction with a shift to a higher Ce^{3+}/Ce^{4+} ratio in the intracellular environment. We also find a similar ratio in the cytoplasm and inside the lysosomes - the final accumulation, degradation or recycling compartment - with two more distinct NP subpopulations inside versus outside the lysosomes. These observations indicate that the net reduction occurs earlier in the internalization pathway of the NPs, with additional processes occurring uniquely in the lysosomes. Using toxicity assays, showing membrane damage with no cell death, and oxidative stress measurements showing an initial increase followed by a decrease in oxidative stress at certain NP concentrations, our observations indicate both harmful as well as beneficial effects of these CNPs. Together, we bring direct evidence for the reduction of CNPs in the intracellular environment, which is consistent with their involvement in potentially beneficial redox reactions, but we also show their potential for adverse effects on the cell.

2. Materials and methods

2.1. Nanoparticle synthesis

CNPs were synthesized by thermal hydrolysis procedure, adopted from Chanteau et al., 2009 [48]. Briefly, ammonium cerium (IV) nitrate precursor in DI water (pH ~ 0.6–0.7) was thermally hydrolyzed at 75 °C to yield colloidal dispersion CNPs. The size of the nanoparticles was controlled by drop wise addition of stoichiometric amount of base hydroxide ions with stirring at 450 RPM. The synthesized CNP suspension was centrifuged at 9000 RPM for 15 min followed by isolation of top gradient solution to remove any large agglomerates. The synthesized nanoparticle suspension (top gradient) was dialyzed against DI water in regenerated cellulose tubing, MWCO 6000–8000 for 48–72 h to remove any excess free ions in the particles solution.

2.2. Cell culture, NP exposure and immuno-staining

Mouse alveolar type II epithelial cell line (C10) was used in this study. Cells were grown on fibronectin-coated silicon nitride windows (TEM size, 100 nm membrane, Silson Ltd). Coating was done by immersing the windows in a 10 μg/ml human fibronectin solution in deionized water for 24 h incubation, followed by a brief rinse with deionized water and drying. Cells were grown in RPMI growth medium, supplemented with 10% fetal bovine serum and 0.1% penicillin/streptomycin, for 48 h before exposure to CNPs suspension for correlated STXM and SIM imaging, a 50 μg/ml CNP suspension in growth medium was used. Following 24 h incubation, the NPs were washed away using PBS and the cells were fixed using 2% paraformaldehyde for 15 min incubation. Cells were then washed with PBS and permeabilized using 0.5% saponin in PBS for 15 min incubation. Blocking buffer was added (0.2% saponin, 1% bovine serum albumin, in PBS) to cells for 30 min incubation, followed by incubation with the primary antibody (rabbit anti LAMP1, ab24170, Abcam) at 1:2000 dilution in blocking buffer for 1 h incubation. Primary antibody was washed and the secondary antibody, biotinylated goat anti-rabbit (ab64256, Abcam), was added at 2.5 μg/mL in blocking buffer for 1 h incubation. The secondary antibody was washed and streptavidin conjugated quantum dots (705 nm emission, Q10161MP, Life Technologies) were added at a concentration of 20 nM in blocking buffer for 1 h incubation. Cells were then washed with PBS and the silicon nitride windows were mounted on a STXM sample plate for imaging.

2.3. Toxicity assessment

Cell viability and membrane integrity were determined using the MTS and LDH assays, respectively (CellTiter 96 and CytoTox 96, Promega). Measurements were performed on three separate days in four 96 well plates, with a total of 12 wells per NP concentration. For each experiment, 3000 cells were seeded in each well for 48 h incubation. CNPs were then added at the indicated concentrations for 24 h incubation, and the assays were conducted following the manufacturer's protocols. Cell viability and LDH release were calculated by dividing the absorbance value for each well by the average no-particle absorbance. These normalized values were then averaged for each NP concentration and a standard deviation was determined. Significance was calculated by unpaired, unequal variance, two-tailed Student's t-test (also known as Welch's t-test).

2.4. Oxidative stress measurement

ROS generation was measured at 1, 3, and 24 h post exposure through the oxidation of the cell-permeant compound 5-(and-6)-chloromethyl-2′,7′- dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA or DCF). CNP concentrations of 0, 1, 10, and 50 μg/ml were used. 25 μM CMH2DCFDA was added to the growth media and incubated for 30 min at 37 °C under light protection. The medium was removed, and the cells were trypsinized until fully detached and resuspended in 1 mL RPMI growth media, followed by centrifugation (IEC Centa MP4R) at 1000 rpm for 2 min. The cells were resuspended in 0.5 mL growth media with propidium iodide (PI) at 1 μg/mL to distinguish live from dead cells. DCF and PI fluorescence were measured by flow cytometry using the Influx (BD Biosciences, Seattle, WA). Forward and side scatter were used to gate out cellular debris, and a secondary gate, based on PI emission at 585/29 nm when excited with a 561-nm laser, was used to detect and exclude the dead cell population from the DCF analysis. DCF fluorescence was measured at 520/15 nm when excited with a 488-nm laser. Gating and mean calculations from 18,000 cells per treatment group were done using Flow Jo software (Tree Star, Ashland, OR). Significance was determined using Monte-Carlo simulation of synthetic data sets technique [49] in Matlab (Mathworks, Natick, MA) to determine standard deviation of the center of the fluorescence intensity histograms.

2.5. Scanning transmission x-ray microscopy

STXM was performed at the Advanced Light Source at Lawrence