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 $\text{Ce}^{3+}/\text{Ce}^{4+}$ 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 $\text{Ce}^{3+}/\text{Ce}^{4+}$ 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 cells.

Keywords: STXM; structured illumination microscopy; cerium oxide nanoparticles; oxidation state

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 $\text{Ce}^{3+}/\text{Ce}^{4+}$ 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 $\text{Ce}^{3+}/\text{Ce}^{4+}$ ratio linked to SOD-like activity [29,41], and lower $\text{Ce}^{3+}/\text{Ce}^{4+}$ 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 $\text{Ce}^{3+}/\text{Ce}^{4+}$ 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 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 $\text{Ce}^{3+}/\text{Ce}^{4+}$ 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 $\text{Ce}^{3+}/\text{Ce}^{4+}$ 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 to 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 minutes 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 hr 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 $\mu\text{g/ml}$ 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 $\mu\text{g/mL}$ 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 Berkeley National Laboratory (Berkeley, CA). Images and spectra were collected on the 5.3.2.1, 5.3.2.2, and 11.0.2 end stations [50, 51]. Calibration was performed with CO_2 to the 292.74 eV feature at the beginning of each beam line shift. Typical slit widths were 30 μm for both entrance slits and 60 μm for the exit slit. The count rate for the detector was kept below 10^6 Hz to avoid non-linear responses [52]. The focal spot of the microscope was typically ~ 50 nm for cerium images and all images used for analysis were collected with 50 nm per pixel step size. STXM samples were prepared by sealing in the hydrated, fixed cells with epoxy. First a clean Si_3N_4 window was fixed in place on a standard STXM sample plate with epoxy (Hardman Double Bubble 'Red' epoxy). The window with cells was then placed on top of the clean window with the cells in between and slightly tacky epoxy was used to seal the edges. Samples were then rinsed with deionized water before insertion into the STXM chamber.

2.6. Super-resolution fluorescence microscopy: Structured illumination microscope (SIM) was performed on an inverted microscope (Elyra S.1, Zeiss). The sample was excited by a 561 nm

laser and emission was acquired using a 655 nm long pass filter. The SIM line spacing was provided by a 51 μm grating with 5 phase positions and 5 rotation angles. A 100x oil immersion objective and 1.6x tube lens were used for magnification. Images in the z axis were taken at 0.116 μm steps. Reconstruction was performed using the manufacturer's software (Zen, Zeiss) with default settings.

2.7. Image overlay: The slight variations in home position between SIM and STXM made it impossible to align the two image types with nm accuracy using a common reference home position or any other mechanical alignment approach. Therefore, overlay of the STXM and SIM images was accomplished using the Matlab image processing toolbox. Features that were consistent across the two image types were identified, including vesicles with clearly correlated shapes, and an affine transform was applied to bring the coincident points into alignment. ImageJ (National Institutes of Health, USA) and Volocity (PerkinElmer, Waltham, MA, USA) were used for visualization and the presence of CNP aggregates inside cells or lysosomes was determined manually.

2.8. STXM Data analysis: All spectral analyses were performed in Matlab using a combination of a custom user interface to select regions for analysis and extract spectra, and other custom scripts and functions for decomposition of spectra. This graphical user interface can be used to examine and extract information from image scans, x-ray absorption spectral stacks, and line scans to a variety of formats for use in other software, including Excel and Matlab. Single value decomposition analysis of the spectra was performed in Matlab using the Nelder-Meade downhill simplex search algorithm with the amplitude of each reference spectrum and baseline offset as the fitting parameters. While an energy calibration was performed at the beginning of each shift, the fitting routine included a slight correction in the energy axis of the search algorithm to compensate for differences in energy calibration at the beam line. To achieve the correction, the above fit was performed at a range of energy shifts and the best fit (sum of squares) was used for the overall fit result. This routine was used to determine the best energy shift to 1 meV accuracy with the value always less than 1 eV displacement. All statistical tests were performed using the Wilcoxon rank sum test, as appropriate for data sets that do not fit a normal distribution. While the histograms presented in this study ([Figures 3 and 5](#)) consist of

subpopulations with normal distributions, the populations, as a whole, do not obey the normal distribution.

3. Results

3.1. CNP Characterization

CNPs were synthesized in heated solvent, following the method described in Chanteau *et al* [48]. This approach generates smaller particles that are more stable in biological media and more uniform in shape, compared with other approaches [21]. This approach also generates CNPs with $\text{Ce}^{3+}/\text{Ce}^{4+}$ ratios between $\frac{1}{4}$ - $\frac{1}{2}$ (depending on the environment and age of the NPs), allowing for any shift in the ratio to occur in both directions. Primary particle average diameter was determined using transmission electron microscopy (TEM) to be 2.4 ± 0.6 nm (62 particles). CNP aggregate diameter was determined in RPMI growth medium using dynamic light scattering (DLS) to be 588 ± 84 nm, reflecting the well-documented tendency of NPs to aggregate in biological media [48,53–57]. Zeta potential was determined in the growth medium to be -14.1 ± 11.7 mV.

3.2. Quantitative analysis of oxidation states of CNPs outside and inside intact hydrated cells identifies a shift to a higher Ce^{3+} content in the intracellular environment

Cells exposed to the NPs were imaged using STXM to generate elemental maps and scans. A typical STXM scan was acquired at 50 nm pixel resolution. A cerium map was generated by subtracting the values in images acquired at energy below the cerium edge (875 eV), where cerium absorption is minimal, from values in images acquired at energy above the cerium edge (883.8 eV), where cerium absorption is high relative to other elements. An example of the resulting cerium-specific map is shown in [Figure 1](#), where the edge of the cell and the nucleus are outlined by the dashed line. CNP aggregates are shown in a gray scale, with white representing maximal cerium absorbance.

Individual small aggregates from these maps were chosen for oxidation state analysis using a line scan across each aggregate. The spectra that were generated were compared to reference spectra for Ce^{3+} (CePO_4) and Ce^{4+} (commercial CeO_2 NPs) using singular value decomposition (SVD) [58]. This approach finds the optimal combination of two or more spectra that

approximate an unknown or sample spectrum, and enables the quantification of Ce^{3+} and Ce^{4+} content in each aggregate, as demonstrated in Figure 2. The fractions of Ce^{3+} (purple) and Ce^{4+} (orange) in the measured spectra (blue) were calculated from the relative ratios of the reference spectra for Ce^{3+} (CePO_4) and Ce^{4+} (fully oxidized CeO_2) in the integrated fitted spectra (red). Ce^{3+} has lower energy absorptions at 882, 883, 899, and 900 eV, while Ce^{4+} absorption has prominent peaks at 885, 890, 902, and 907 eV. The median R^2 value for the decomposition fits in our study was 0.92, indicating a good fit for most of the sample spectra. It is important to note that x-ray photons pass through the entire particle, including the core, which is expected to have a higher Ce^{4+} concentration than the surface [59]. Thus, the fraction of Ce^{3+} in our measurements of individual aggregates is expected to reach values that are far below 100%.

A total of 292 aggregates outside the cells and 382 aggregates inside the cells were analyzed using SVD. The data is summarized in Figure 3, where a clear shift to a higher Ce^{3+} content is observed for particles found inside (gray bars, solid fitted line) versus outside (white bars, dashed fitted line) the cells. The distributions were fitted using multiple normal distributions corresponding to the centers of the NP subpopulations, with peak values and R^2 values to evaluate goodness of fits listed in Table 1. Two main subpopulations were observed for both the particles inside (Figure 3, solid fitted line) and outside (Figure 3, dashed fitted line) the cells. The peak values of the left (0.158 ± 0.025) and right (0.251 ± 0.033) NP subpopulations inside the cells were significantly higher than the peak values of the left (0.076 ± 0.035) and right (0.176 ± 0.032) NP subpopulations outside the cells, respectively ($p < 0.01$ for both left and right comparisons). The average Ce^{3+} value of the two NP subpopulations outside the cells (0.202 ± 0.155) was also significantly lower than the average value of the two subpopulations inside the cells (0.218 ± 0.087) ($p < 0.001$).

3.3. Quantitative analysis of the oxidation states of CNPs outside and inside the lysosomes indicates that the shift to a higher Ce^{3+} content starts early in the internalization pathway

To identify the subcellular compartment in which the shift in the oxidation state of the NPs occurs, fluorescence immune-staining was applied to all samples using an antibody against LAMP-1, a lysosome-specific protein. Early and late endocytic vesicles merge with lysosomes, which are the final accumulation, degradation or recycling compartment [60]. Following STXM, Super resolution fluorescence SIM was used to image the cells, and the two image types were

overlaid. [Figure 4](#) shows an example of overlaid images of small areas inside the cytoplasm, where the lysosomes are shown in red and the CNPs are shown in green. Examples of small CNP aggregates that are encased within lysosomes are indicated by the arrows.

A careful examination of the oxidation state of 108 aggregates that were found inside the lysosomes ([Figure 5, white bars, dashed fitted line](#)) showed a similar trend in the distribution of Ce^{3+} content as the distribution found for 273 aggregates inside the cells but outside the lysosomes ([Figure 5, gray bars, solid fitted line](#)), with no significant difference in the peak values of the two subpopulations in each distribution ([Table I](#)). This observation indicates that the shift to a higher Ce^{3+} content starts early in the internalization pathway, before reaching the lysosomes. However, two more discrete subpopulations were observed in the lysosomes ([Figure 5, white bars, dashed fitted line](#)) compared with the more distributed subpopulations observed outside the lysosomes ([Figure 5, gray bars, solid fitted line](#)), indicating additional processes unique to the lysosomes.

3.4. Toxicity assessments and oxidative stress measurements detect both harmful and beneficial effects of CNP

Using the lactate dehydrogenase (LDH) assay to quantify membrane integrity ([Figure 6A](#)), we found a significant membrane damage starting at 5 $\mu\text{g/ml}$. However, using the MTS proliferation assay to quantify cell viability ([Figure 6A](#)), no cell death was detected at all tested NP concentrations (1 - 100 $\mu\text{g/ml}$) at 24 hour post exposure.

To determine whether CNPs can modulate the level of oxidative stress in the intracellular environment, dichlorofluorescein (DCF) was used to quantify ROS at 1, 3 and 24 hr post exposure to CNPs at 0, 1, 10 and 50 $\mu\text{g/ml}$. Due to its indiscriminate response to free radicals, DCF is useful in quantifying overall oxidative stress. Here we used flow cytometry to screen 18,000 cells per treatment group and the mean values are plotted in [Figure 7](#). At 1 hr post exposure, a significant increase in oxidative stress was observed in response to the three tested CNP concentrations relative to control (0 $\mu\text{g/ml}$) ($p < 0.0001$). However, a significant decrease in oxidative stress was detected at the 3 hr post exposure in response to CNPs at 1 and 10 $\mu\text{g/ml}$ ($p < 0.0001$). The decrease was maintained at the 24 hr time point only in response to CNPs at 10

$\mu\text{g/ml}$ ($p < 0.0001$). CNPs at $50 \mu\text{g/ml}$ induced oxidative stress responses at all time-points. These observations indicate both beneficial as well as harmful effects of CNPs in these cells.

4. Discussion

The main new finding to emerge from this work is the shift that occurs in the oxidation state of CNPs to a higher Ce^{3+} content as they enter the intracellular environment, with two, more distinct oxidation state subpopulations forming in the lysosomes. To our knowledge, this study is the first to quantify the oxidation state of NPs inside organelles in intact hydrated cells. The shift to a higher Ce^{3+} content indicates a net reduction of the NPs as they enter the cellular environment. Although a complete redox reaction cycle could end with no shift in the oxidation state of the NPs, the occurrence of such a shift is consistent with the involvement of the NPs in potentially beneficial oxidation reactions in the intracellular environment.

STXM allowed us to determine the oxidation state of individual CNP aggregates outside and inside intact hydrated cells and organelles with high spatial resolution and chemical selectivity. STXM [50] enables high resolution (10-50 nm) imaging and speciation analysis in dried or hydrated cells [61,62,64], as well as the quantification of oxidation states of particles in biofilms or non-biological media [63,65,66]. The minimal damage to biological systems caused by STXM allows subsequent imaging of the cells using other methods. We took advantage of this property and followed the STXM imaging with super resolution fluorescence imaging using SIM, which enables 120-130 nm lateral resolution. Together with immunocytochemistry, it was possible to further determine the oxidation state of the NPs specifically in lysosomes – the final degradation or recycling organelle, and determine the subcellular environment in which the shift occurred. The high spatial resolution of SIM allowed for accurate overlap with STXM images, acquired with 50 nm resolution, and unambiguous identification of CNPs that were encased within the lysosomes.

Based on measurements conducted in solution, three chemical reactions have been proposed as possible reactions that CNP might be involved in. These include SOD-like reaction (Scheme 1) [29,38,40], catalase-like reaction (Scheme 2) [28,38], and Fenton Haber/Weiss (FHW) reaction[42], which is similar to the overall catalase reaction. If the oxidation and reduction take place at equal rates, no overall change in the oxidation state of the NPs is expected. However,

our observations indicate that the reduction of the NPs is favored over their oxidation in the intracellular environment, which is consistent with their involvement in these reaction schemes, where they might oxidize harmful free radicals and convert them to less harmful or non-harmful species. Following the SOD-like activity ([Scheme 1](#)), CNPs might catalyze the conversion of the harmful O_2^- to H_2O_2 , which is more easily catalyzed in living cells into harmless water and molecular oxygen [28,39]. Following the catalase-like activity ([Scheme 2](#)), CNPs might react with H_2O_2 to produce harmless water, oxygen, and hydrogen ions [39,43]. Sorted by their Ce^{3+} content, two main CNP subpopulations were detected, both outside and inside the cells ([Figure 3](#)). The two subpopulations might reflect the original heterogeneities in size, crystallinity, oxygen vacancies and other properties of the synthesized particles, all of which govern the oxidation state [38,59,67,68].

The two subpopulations outside the cell shifted to higher Ce^{3+} content values inside the cells and their distribution became considerably narrower in the lysosomes, possibly reflecting the more defined chemical environment within these organelles. NPs enter the cellular environment mostly encased within endocytic vesicles [69–72] and in rare cases can also directly cross the cell membrane [73]. In both cases, the NPs enter an environment where the pH is around 7.4. Endocytic vesicles carrying NPs often merge with lysosomes [69,72], which are the final degradation or recycling compartment, where the pH is around 4.5. The two more distinct subpopulations observed inside versus outside the lysosomes ([Figure 5](#)) could be explained in the context of the two, SOD- and catalase-like activities ([Scheme 1 and 2](#)). It is likely that the redox cycling occurs more freely in early endosomes or in the cytosol where the pH is nearly neutral, causing the oxidation state of the CNPs to span over a continuum of states, whereas in lysosomes, where the pH is acidic, the mechanism to return to Ce^{3+} is limited, possibly locking the CNPs in two more distinct subpopulations.

Our toxicity assessments showed membrane damage with no cell death ([Figure 6](#)). It is possible that the induced membrane damage was at the level that could be controlled or managed by the cell to avoid cell death. Our oxidative stress measurements showed an initial increase in ROS production in response to the NPs at all tested concentrations, followed by a decrease in ROS at 3 and 24 hr post exposure to certain NP concentrations. These observations point to initial processes that were harmful to the cell, but later reactions that reduced the presence of harmful

free radicals, possibly by the SOD- or catalase-like activities. This possibility is supported by the shift that we observed in the oxidation state of the NPs in the cellular environment. Studies using CNPs that were synthesized by heating in solvent methods, such as the method used to generate the NPs in our study, have mostly reported either no effects [23–27], or harmful effects *in vitro* [1,2,5], including DNA damage and oxidative stress, and *in vivo* [3,8,9], including lung inflammatory and injury in the rat and mouse models. Our observations are consistent with these reports, showing membrane damage but no cell death even at high doses and concentration- and time-dependent decrease and increase in oxidative stress, adding to the overall understanding that CNPs synthesized by such methods should be avoided. In contrast, CNPs synthesized in room temperature methods have mostly shown either no or beneficial effects *in vitro* and *in vivo*, including protection from inflammation, oxidative stress, radiation damage, retinal degeneration or pathological development, among other beneficial effects [30–36]. Although not pursued in this study, it is likely that CNPs synthesized in room temperature also undergo shifts in their oxidation state in the complex intracellular environment, consistent with their involvement in potentially beneficial redox reactions.

5. Conclusion

Our studies provide, for the first time, direct measurements of oxidation state dynamics of NPs inside intact hydrated cells and organelles. Using correlated STXM and SIM, two techniques that support imaging with nm spatial resolution, we identified a shift to a higher $\text{Ce}^{3+}/\text{Ce}^{4+}$ ratio in CNPs inside versus outside the cells, indicating that an overall reduction of the NPs occurs in the intracellular environment. This observation is consistent with the involvement of the NPs in potentially beneficial redox reactions in this complex environment, where harmful reactive oxygen species are oxidized to a less harmful or non-harmful species, possible through SOD-like or catalase-like activities. We further found a similar $\text{Ce}^{3+}/\text{Ce}^{4+}$ ratio in the cytoplasm and inside the lysosomes. This observation indicates that the reduction of the NPs and their potential beneficial activity starts early in their internalization pathway before reaching the lysosomes. Two more distinct NP subpopulations were found inside versus outside the lysosomes, which could be driven by the acidic or more defined environment in these organelles. Our observations of a time- and concentration-dependent decrease in ROS support the involvement of the NPs in beneficial redox reactions, while the observed increase in ROS indicates their involvement also

in harmful processes. Although our toxicity assessment showed no cell death, significant membrane damage was detected. These observations indicate that, in addition to potentially beneficial oxidation reactions, other interactions that compromise the health of the cell can also take place between CNPs and the cellular environment.

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References

- [1] Auffan M, Rose J, Orsiere T, De Meo M, Thill A, Zeyons O, et al. CeO₂ nanoparticles induce DNA damage towards human dermal fibroblasts in vitro. *Nanotoxicology* 2009;3:161–71. doi:10.1080/17435390902788086.
- [2] Eom H-J, Choi J. Oxidative stress of CeO₂ nanoparticles via p38-Nrf-2 signaling pathway in human bronchial epithelial cell, Beas-2B. *Toxicol Lett* 2009;187:77–83. doi:10.1016/j.toxlet.2009.01.028.
- [3] Lin W, Huang Y, Zhou X-D, Ma Y. Toxicity of Cerium Oxide Nanoparticles in Human Lung Cancer Cells. *Int J Toxicol* 2006;25:451–7. doi:10.1080/10915810600959543.
- [4] Ma JY, Zhao H, Mercer RR, Barger M, Rao M, Meighan T, et al. Cerium oxide nanoparticle-induced pulmonary inflammation and alveolar macrophage functional change in rats. *Nanotoxicology* 2011;5:312–25. doi:10.3109/17435390.2010.519835.
- [5] Park E-J, Choi J, Park Y-K, Park K. Oxidative stress induced by cerium oxide nanoparticles in cultured BEAS-2B cells. *Toxicology* 2008;245:90–100. doi:10.1016/j.tox.2007.12.022.
- [6] Yokel RA, Florence RL, Unrine JM, Tseng MT, Graham UM, Wu P, et al. Biodistribution and oxidative stress effects of a systemically-introduced commercial ceria engineered nanomaterial. *Nanotoxicology* 2009;3:234–48. doi:10.1080/17435390902974496.

- [7] Zhang H, He X, Zhang Z, Zhang P, Li Y, Ma Y, et al. Nano-CeO₂ exhibits adverse effects at environmental relevant concentrations. *Environ Sci Technol* 2011;45:3725–30. doi:10.1021/es103309n.
- [8] Cho W-S, Duffin R, Poland CA, Howie SEM, MacNee W, Bradley M, et al. Metal oxide nanoparticles induce unique inflammatory footprints in the lung: important implications for nanoparticle testing. *Environ Health Perspect* 2010;118:1699–706. doi:10.1289/ehp.1002201.
- [9] Park E-J, Cho W-S, Jeong J, Yi J, Choi K, Kim Y, et al. Induction of Inflammatory Responses in Mice Treated with Cerium Oxide Nanoparticles by Intratracheal Instillation. *J Health Sci* 2010;56:387–96.
- [10] Hussain S, Al-Nsour F, Rice AB, Marshburn J, Yingling B, Ji Z, et al. Cerium Dioxide Nanoparticles Induce Apoptosis and Autophagy in Human Peripheral Blood Monocytes. *ACS Nano* 2012;6:5820–9. doi:10.1021/nn302235u.
- [11] Chigurupati S, Mughal MR, Okun E, Das S, Kumar A, McCaffery M, et al. Effects of cerium oxide nanoparticles on the growth of keratinocytes, fibroblasts and vascular endothelial cells in cutaneous wound healing. *Biomaterials* 2013;34:2194–201. doi:10.1016/j.biomaterials.2012.11.061.
- [12] Sack M, Alili L, Karaman E, Das S, Gupta A, Seal S, et al. Combination of conventional chemotherapeutics with redox-active cerium oxide nanoparticles - a novel aspect in cancer therapy. *Mol Cancer Ther* 2014;molcanther.0950.2013. doi:10.1158/1535-7163.MCT-13-0950.
- [13] Celardo I, De Nicola M, Mandoli C, Pedersen JZ, Traversa E, Ghibelli L. Ce³⁺ Ions Determine Redox-Dependent Anti-apoptotic Effect of Cerium Oxide Nanoparticles. *ACS Nano* 2011;5:4537–49. doi:10.1021/nn200126a.
- [14] Babu S, Cho J-H, Dowding JM, Heckert E, Komanski C, Das S, et al. Multicolored redox active upconverter cerium oxide nanoparticle for bio-imaging and therapeutics. *Chem Commun* 2010;46:6915. doi:10.1039/c0cc01832e.
- [15] Lord MS, Jung M, Teoh WY, Gunawan C, Vassie JA, Amal R, et al. Cellular uptake and reactive oxygen species modulation of cerium oxide nanoparticles in human monocyte cell line U937. *Biomaterials* 2012;33:7915–24. doi:10.1016/j.biomaterials.2012.07.024.
- [16] Cai X, Seal S, McGinnis JF. Sustained inhibition of neovascularization in vldlr^{-/-} mice following intravitreal injection of cerium oxide nanoparticles and the role of the ASK1-P38/JNK-NF- κ B pathway. *Biomaterials* 2014;35:249–58.
- [17] Alili L, Sack M, Karakoti AS, Teuber S, Puschmann K, Hirst SM, et al. Combined cytotoxic and anti-invasive properties of redox-active nanoparticles in tumor-stroma interactions. *Biomaterials* 2011;32:2918–29. doi:10.1016/j.biomaterials.2010.12.056.
- [18] Kumar A, Das S, Munusamy P, Self W, Baer DR, Sayle DC, et al. Behavior of nanoceria in biologically-relevant environments. *Environ Sci Nano* 2014. doi:10.1039/C4EN00052H.
- [19] Kong L, Cai X, Zhou X, Wong LL, Karakoti AS, Seal S, et al. Nanoceria extend photoreceptor cell lifespan in tubby mice by modulation of apoptosis/survival signaling pathways. *Neurobiol Dis* 2011;42:514–23. doi:10.1016/j.nbd.2011.03.004.
- [20] Wason MS, Zhao J. Cerium oxide nanoparticles: potential applications for cancer and other diseases. *Am J Transl Res* 2013;5:126–31.
- [21] Karakoti AS, Munusamy P, Hostetler K, Kodali V, Kuchibhatla S, Orr G, et al. Preparation and characterization challenges to understanding environmental and biological impacts of ceria nanoparticles. *Surf Interface Anal* 2012;44:882–9. doi:10.1002/sia.5006.

- [22] D'Angelo B, Santucci S, Benedetti E, Di Loreto S, Phani RA, Falone S, et al. Cerium Oxide Nanoparticles Trigger Neuronal Survival in a Human Alzheimer Disease Model By Modulating BDNF Pathway. *Curr Nanosci* 2009;5:167–76.
- [23] Hardas SS, Butterfield DA, Sultana R, Tseng MT, Dan M, Florence RL, et al. Brain distribution and toxicological evaluation of a systemically delivered engineered nanoscale ceria. *Toxicol Sci Off J Soc Toxicol* 2010;116:562–76. doi:10.1093/toxsci/kfq137.
- [24] Pierscionek B, Keenan J, Yasseen A, Colhoun L, Li Y, Schachar R, et al. CeO₂ Nanoparticles have No Detrimental Effect on Eye Lens Proteins. *Curr Anal Chem* 2010;6:172–6. doi:10.2174/157341110790945517.
- [25] Gojova A, Lee J-T, Jung HS, Guo B, Barakat AI, Kennedy IM. Effect of cerium oxide nanoparticles on inflammation in vascular endothelial cells. *Inhal Toxicol* 2009;21 Suppl 1:123–30. doi:10.1080/08958370902942582.
- [26] Park B, Donaldson K, Duffin R, Tran L, Kelly F, Mudway I, et al. Hazard and risk assessment of a nanoparticulate cerium oxide-based diesel fuel additive - a case study. *Inhal Toxicol* 2008;20:547–66. doi:10.1080/08958370801915309.
- [27] Safi M, Sarrouj H, Sandre O, Mignet N, Berret J-F. Interactions between sub-10-nm iron and cerium oxide nanoparticles and 3T3 fibroblasts: the role of the coating and aggregation state. *Nanotechnology* 2010;21:145103. doi:10.1088/0957-4484/21/14/145103.
- [28] Karakoti AS, Singh S, Kumar A, Malinska M, Kuchibhatla SVNT, Wozniak K, et al. PEGylated nanoceria as radical scavenger with tunable redox chemistry. *J Am Chem Soc* 2009;131:14144–5. doi:10.1021/ja9051087.
- [29] Heckert EG, Karakoti AS, Seal S, Self WT. The role of cerium redox state in the SOD mimetic activity of nanoceria. *Biomaterials* 2008;29:2705–9. doi:10.1016/j.biomaterials.2008.03.014.
- [30] Hirst SM, Karakoti AS, Tyler RD, Sriranganathan N, Seal S, Reilly CM. Anti-inflammatory Properties of Cerium Oxide Nanoparticles. *Small* 2009;5:2848–56. doi:10.1002/sml.200901048.
- [31] Das M, Patil S, Bhargava N, Kang J-F, Riedel LM, Seal S, et al. Auto-catalytic ceria nanoparticles offer neuroprotection to adult rat spinal cord neurons. *Biomaterials* 2007;28:1918–25. doi:10.1016/j.biomaterials.2006.11.036.
- [32] Schubert D, Dargusch R, Raitano J, Chan S-W. Cerium and yttrium oxide nanoparticles are neuroprotective. *Biochem Biophys Res Commun* 2006;342:86–91. doi:10.1016/j.bbrc.2006.01.129.
- [33] Niu J, Wang K, Kolattukudy PE. Cerium oxide nanoparticles inhibit oxidative stress and nuclear factor- κ B activation in H9c2 cardiomyocytes exposed to cigarette smoke extract. *J Pharmacol Exp Ther* 2011;338:53–61. doi:10.1124/jpet.111.179978.
- [34] Zhou X, Wong LL, Karakoti AS, Seal S, McGinnis JF. Nanoceria inhibit the development and promote the regression of pathologic retinal neovascularization in the Vldlr knockout mouse. *PloS One* 2011;6:e16733. doi:10.1371/journal.pone.0016733.
- [35] Colon J, Herrera L, Smith J, Patil S, Komanski C, Kupelian P, et al. Protection from radiation-induced pneumonitis using cerium oxide nanoparticles. *Nanomedicine Nanotechnol Biol Med* 2009;5:225–31. doi:10.1016/j.nano.2008.10.003.
- [36] Chen J, Patil S, Seal S, McGinnis JF. Rare earth nanoparticles prevent retinal degeneration induced by intracellular peroxides. *Nat Nanotechnol* 2006;1:142–50. doi:10.1038/nnano.2006.91.

- [37] Karakoti AS, Tsigkou O, Yue S, Lee PD, Stevens MM, Jones JR, et al. Rare earth oxides as nanoadditives in 3-D nanocomposite scaffolds for bone regeneration. *J Mater Chem* 2010;20:8912–9. doi:10.1039/C0JM01072C.
- [38] Tarnuzzer RW, Colon J, Patil S, Seal S. Vacancy engineered ceria nanostructures for protection from radiation-induced cellular damage. *Nano Lett* 2005;5:2573–7. doi:10.1021/nl052024f.
- [39] Singh S, Dosani T, Karakoti AS, Kumar A, Seal S, Self WT. A phosphate-dependent shift in redox state of cerium oxide nanoparticles and its effects on catalytic properties. *Biomaterials* 2011;32:6745–53. doi:10.1016/j.biomaterials.2011.05.073.
- [40] Korsvik C, Patil S, Seal S, Self WT. Superoxide dismutase mimetic properties exhibited by vacancy engineered ceria nanoparticles. *Chem Commun Camb Engl* 2007:1056–8. doi:10.1039/b615134e.
- [41] Lee SS, Song W, Cho M, Puppala HL, Nguyen P, Zhu H, et al. Antioxidant Properties of Cerium Oxide Nanocrystals as a Function of Nanocrystal Diameter and Surface Coating. *ACS Nano* 2013. doi:10.1021/nn4026806.
- [42] Heckert EG, Seal S, Self WT. Fenton-Like Reaction Catalyzed by the Rare Earth Inner Transition Metal Cerium. *Environ Sci Technol* 2008;42:5014–9. doi:10.1021/es8001508.
- [43] Pirmohamed T, Dowding JM, Singh S, Wasserman B, Heckert E, Karakoti AS, et al. Nanoceria exhibit redox state-dependent catalase mimetic activity. *Chem Commun* 2010;46:2736–8. doi:10.1039/B922024K.
- [44] Karakoti AS, Kuchibhatla SVNT, Babu KS, Seal S. Direct Synthesis of Nanoceria in Aqueous Polyhydroxyl Solutions. *J Phys Chem C* 2007;111:17232–40. doi:10.1021/jp076164k.
- [45] Donaldson K, Borm PJA, Oberdorster G, Pinkerton KE, Stone V, Tran CL. Concordance Between In Vitro and In Vivo Dosimetry in the Proinflammatory Effects of Low-Toxicity, Low-Solubility Particles: The Key Role of the Proximal Alveolar Region 2008.
- [46] Oberdorster G, Oberdorster E, Oberdorster J. Nanotoxicology: An Emerging Discipline Evolving from Studies of Ultrafine Particles. *Environ Health Perspect* 2005;113:823–39. doi:10.1289/ehp.7339.
- [47] Mercer RR, Hubbs AF, Scabilloni JF, Wang L, Battelli LA, Schwegler-Berry D, et al. Distribution and persistence of pleural penetrations by multi-walled carbon nanotubes. *Part Fibre Toxicol* 2010;7:28. doi:10.1186/1743-8977-7-28.
- [48] Chanteau B, Fresnais J, Berret J-F. Electrosteric Enhanced Stability of Functional Sub-10 nm Cerium and Iron Oxide Particles in Cell Culture Medium. *Langmuir* 2009;25:9064–70. doi:10.1021/la900833v.
- [49] Press WH, Teukolsky SA, Vetterling WT, Flannery BP. *Numerical Recipes: The Art of Scientific Computing*. (Cambridge University Press, 2007).
- [50] Bluhm H, Andersson K, Araki T, Benzerara K, Brown GE, Dynes JJ, et al. Soft X-ray microscopy and spectroscopy at the molecular environmental science beamline at the Advanced Light Source. *J Electron Spectrosc Relat Phenom* 2006;150:86–104. doi:10.1016/j.elspec.2005.07.005.
- [51] Kilcoyne D, Ade H, Attwood D, Hitchcock A, McKean P, Mitchell G, et al. A new Scanning Transmission X-ray Microscope at the ALS for operation up to 2500eV. *AIP Conf. Proc.*, vol. 1234, AIP Publishing; 2010, p. 465–8. doi:10.1063/1.3463241.

- [52] Collins BA, Ade H. Quantitative compositional analysis of organic thin films using transmission NEXAFS spectroscopy in an X-ray microscope. *J Electron Spectrosc Relat Phenom* 2012;185:119–28. doi:10.1016/j.elspec.2012.05.002.
- [53] Allouni ZE, Cimpan MR, Høl PJ, Skodvin T, Gjerdet NR. Agglomeration and sedimentation of TiO₂ nanoparticles in cell culture medium. *Colloids Surf B Biointerfaces* 2009;68:83–7. doi:10.1016/j.colsurfb.2008.09.014.
- [54] Díaz B, Sánchez-Espinel C, Arruebo M, Faro J, de Miguel E, Magadán S, et al. Assessing Methods for Blood Cell Cytotoxic Responses to Inorganic Nanoparticles and Nanoparticle Aggregates. *Small* 2008;4:2025–34. doi:10.1002/smll.200800199.
- [55] Boldt K, Bruns OT, Gaponik N, Eychmüller A. Comparative Examination of the Stability of Semiconductor Quantum Dots in Various Biochemical Buffers. *J Phys Chem B* 2006;110:1959–63. doi:10.1021/jp056371p.
- [56] Petri-Fink A, Steitz B, Finka A, Salaklang J, Hofmann H. Effect of cell media on polymer coated superparamagnetic iron oxide nanoparticles (SPIONs): Colloidal stability, cytotoxicity, and cellular uptake studies. *Eur J Pharm Biopharm* 2008;68:129–37. doi:10.1016/j.ejpb.2007.02.024.
- [57] Liu Y, Shipton MK, Ryan J, Kaufman ED, Franzen S, Feldheim DL. Synthesis, Stability, and Cellular Internalization of Gold Nanoparticles Containing Mixed Peptide–Poly(ethylene glycol) Monolayers. *Anal Chem* 2007;79:2221–9. doi:10.1021/ac061578f.
- [58] Press WH, Teukolsky SA, Vetterling WT, Flannery BP. *Numerical Recipes: The Art of Scientific Computing*. 3rd ed. Cambridge: Cambridge University Press; 2007.
- [59] Zhang F, Wang P, Koberstein J, Khalid S, Chan S-W. Cerium oxidation state in ceria nanoparticles studied with X-ray photoelectron spectroscopy and absorption near edge spectroscopy. *Surf Sci* 2004;563:74–82. doi:10.1016/j.susc.2004.05.138.
- [60] Appelqvist H, Wäster P, Kågedal K, Öllinger K. The lysosome: from waste bag to potential therapeutic target. *J Mol Cell Biol* 2013;5:214–26. doi:10.1093/jmcb/mjt022.
- [61] Lawrence JR, Swerhone GDW, Leppard GG, Araki T, Zhang X, West MM, et al. Scanning Transmission X-Ray, Laser Scanning, and Transmission Electron Microscopy Mapping of the Exopolymeric Matrix of Microbial Biofilms. *Appl Environ Microbiol* 2003;69:5543–54. doi:10.1128/AEM.69.9.5543-5554.2003.
- [62] Methe O, Spring H, Guttman P, Schneider G, Rudolph D, Trendelenburg MF, et al. Transmission X-ray microscopy of intact hydrated PtK2 cells during the cell cycle. *J Microsc* 1997;188:125–35.
- [63] Lawrence JR, Dynes JJ, Korber DR, Swerhone GDW, Leppard GG, Hitchcock AP. Monitoring the fate of copper nanoparticles in river biofilms using scanning transmission X-ray microscopy (STXM). *Chem Geol* n.d. doi:10.1016/j.chemgeo.2011.07.013.
- [64] Stasio GD, Rajesh D, Ford JM, Daniels MJ, Erhardt RJ, Frazer BH, et al. Motexafin-Gadolinium Taken Up In vitro by at Least 90% of Glioblastoma Cell Nuclei. *Clin Cancer Res* 2006;12:206–13. doi:10.1158/1078-0432.CCR-05-0743.
- [65] Takahama S, Gilardoni S, Russell LM. Single-particle oxidation state and morphology of atmospheric iron aerosols. *J Geophys Res Atmospheres* 2008;113:n/a – n/a. doi:10.1029/2008JD009810.
- [66] Ressler T, Wong J, Roos J, Smith IL. Quantitative Speciation of Mn-Bearing Particulates Emitted from Autos Burning (Methylcyclopentadienyl)manganese Tricarbonyl-Added

- Gasolines Using XANES Spectroscopy. *Environ Sci Technol* 2000;34:950–8. doi:10.1021/es990787x.
- [67] Wu L, Wiesmann HJ, Moodenbaugh AR, Klie RF, Zhu Y, Welch DO, et al. Oxidation state and lattice expansion of CeO_{2-x} nanoparticles as a function of particle size. *Phys Rev B* 2004;69:125415. doi:10.1103/PhysRevB.69.125415.
- [68] Cafun J-D, Kvashnina KO, Casals E, Puentes VF, Glatzel P. Absence of Ce³⁺ Sites in Chemically Active Colloidal Ceria Nanoparticles. *ACS Nano* 2013;7:10726–32. doi:10.1021/nn403542p.
- [69] Orr G, Panther DJ, Phillips JL, Tarasevich BJ, Dohnalkova A, Hu D, et al. Submicrometer and Nanoscale Inorganic Particles Exploit the Actin Machinery To Be Propelled along Microvilli-like Structures into Alveolar Cells. *ACS Nano* 2007;1:463–75. doi:10.1021/nn700149r.
- [70] Orr G, Panther DJ, Cassens KJ, Phillips JL, Tarasevich BJ, Pounds JG. Syndecan-1 mediates the coupling of positively charged submicrometer amorphous silica particles with actin filaments across the alveolar epithelial cell membrane. *Toxicol Appl Pharmacol* 2009;236:210–20. doi:10.1016/j.taap.2009.01.022.
- [71] Orr GA, Chrisler WB, Cassens KJ, Tan R, Tarasevich BJ, Markillie LM, et al. Cellular recognition and trafficking of amorphous silica nanoparticles by macrophage scavenger receptor A. *Nanotoxicology* 2011;5:296–311. doi:10.3109/17435390.2010.513836.
- [72] Mihai C, Chrisler WB, Xie Y, Hu D, Szymanski CJ, Tolic A, et al. Intracellular accumulation dynamics and fate of zinc ions in alveolar epithelial cells exposed to airborne ZnO nanoparticles at the air–liquid interface. *Nanotoxicology* 2013:1–14. doi:10.3109/17435390.2013.859319.
- [73] Verma A, Uzun O, Hu Y, Han H-S, Watson N, et al. Surface-structure-regulated cell-membrane penetration by monolayer-protected nanoparticles. *Nat Mater* 2013;12:376–376. doi:10.1038/nmat3593.

FIGURE LEGENDS

Figure 1. STXM map of a cell showing the presence of all cerium. A scan taken at energy below the cerium edge (875 eV) was subtracted from a scan taken at energy above the cerium edge (883.8 eV) to create cerium-specific map. The edge of the cell and the nucleus (dashed lines) were traced from maps generated at energies above the cerium edge (883.8 eV). The gray scale-bar is in cerium absorbance units, with maximal absorbance in white.

Figure 2. Single value decomposition analysis for quantifying the Ce³⁺/Ce⁴⁺ ratio from CNP spectra. The measured spectrum (blue) was decomposed into the Ce³⁺spectrum (purple) and the Ce⁴⁺spectrum (orange), to form an approximate fit (red) to the measured spectrum. The residual with a negative offset is shown in green.

Figure 3. Histogram showing the distribution of Ce^{3+} content in individual CNP aggregates found inside the cell (gray bars, solid fitted line) versus aggregates found outside the cell (white bars, dashed fitted line). A shift to a higher Ce^{3+} content occurs in particles found inside the cells.

Figure 4. Overlaid cerium maps, acquired using STXM, and fluorescence images, acquired using SIM, of small areas inside the cytoplasm, showing small cerium aggregates (green), encased inside lysosomes (red) (arrows).

Figure 5. Histogram showing the distribution of Ce^{3+} content in individual CNP aggregates found inside lysosomes (white bars, dashed fitted line) versus aggregated found outside the lysosomes (gray bars, solid fitted line).

Figure 6. Quantitative analysis of membrane integrity using the LDH assay (A), and cell viability using the MTS assay (B). * indicates $p < 0.05$, and ** indicates $p < 0.01$.

Figure 7. Mean DCF fluorescence intensity values, measured by flow cytometry, calculated from 18,000 cells per time point and CNP dose.

SCHEMES

Scheme 1. Superoxide dismutase-like reaction cycle

Scheme 2. Catalase-like reaction cycle

TABLES

	Left NP subpopulation	Right NP subpopulation	Combined R ²
NPs outside cells	0.076 ± 0.035	0.176 ± 0.032	0.89
NPs inside cells	0.158 ± 0.025	0.251 ± 0.033	0.94
NPs outside lysosomes	0.162 ± 0.033	0.249 ± 0.036	0.92
NPs inside lysosomes	0.155 ± 0.017	0.259 ± 0.025	0.90

Table I: Peak value ± standard deviation for the fitting of the subpopulations in the distributions of Ce³⁺ content per aggregate

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7