Published in final edited form as:

Biomaterials. 2011 October ; 32(28): 6745–6753. doi:10.1016/j.biomaterials.2011.05.073.

A phosphate-dependent shift in redox state of cerium oxide nanoparticles and its effects on catalytic properties

Sanjay Singh^a, Talib Dosani^a, Ajay Karakoti^b, Amit Kumar^c, Sudipta Seal^c, and William T. Self^{a,*}

^a Burnett School of Biomedical Science, College of Medicine, University of Central Florida

^e Environmental Molecular Sciences Laboratory (EMSL), Pacific Northwest National Lab, Richland, WA 99352, USA

^c Mechanical, Materials and Aerospace Engineering, Nanoscience and Technology Center, Advanced Materials Processing and Analysis Center, University of Central Florida

Abstract

Cerium oxide nanoparticles (CeNPs) have shown promise as catalytic antioxidants in cell culture and animal models as both superoxide dismutase and catalase mimetics. The reactivity of the cerium (Ce) atoms at the surface of its oxide particle is critical to such therapeutic properties, yet little is known about the potential for a protein or small molecule corona to form on these materials *in vivo*. Moreover Ce atoms in these active sites have the potential to interact with small molecule anions, peptides, or sugars when administered in culture or animal models. Several nanomaterials have been shown to alter or aggregate under these conditions, rendering them less useful for biomedical applications. In this work we have studied the change in catalytic properties of CeNPs when exposed to various biologically relevant conditions *in vitro*. We have found that CeNPs are resistant to broad changes in pH and also not altered by incubation in cell culture medium. However to our surprise phosphate anions significantly altered the characteristics of these nanomaterials and shifted the catalytic behavior due to the binding of phosphate anions to cerium. Given the abundance of phosphate in biological systems in an inorganic form, it is likely that the action of CeNPs as a catalyst may be strongly influenced by the local concentration of phosphate in the cells and/or tissues in which it has been introduced.

INTRODUCTION

The potential for the use of nanomaterials in biomedical science is emerging with a variety of organic and inorganic materials. Among these rare earth oxides, especially cerium oxide nanoparticles (CeNPs), have garnered attention as catalytic antioxidants in biological model systems. In terms of catalysis, CeNPs were first shown to exhibit superoxide dismutase activity (SOD), or conversion of superoxide anion to hydrogen peroxide [1, 2]. This catalytic activity was less efficient in particles with a higher level of Ce in the 4+ oxidation state, whether through synthesis or by modification through oxidation with peroxides [1]. More recently we have shown that CeNPs also exhibit catalase mimetic activity, i.e. they

© 2011 Elsevier Ltd. All rights reserved.

*Corresponding author: wself@mail.ucf.edu, 4000 Central Florida Blvd., Bldg. 20 Room 124, Orlando, Florida 32816-2364, Phone (407)823-4262, Fax (407)823-0956.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

convert hydrogen peroxide to water and molecular oxygen [3]. This activity was pronounced in CeNPs that had lower levels of Ce in the 3+ oxidation state, opposite to the observations made for SOD mimetic activity. Taken together these studies show that the redox state of Ce atoms at the surface of the nanoparticle can modulate the reactivity of these nanomaterials to biologically relevant reactive oxygen species (ROS). It is yet to be established which of either catalytic behaviors are indeed the molecular mechanism behind the antioxidant properties of these nanomaterials in animal models of disease [1, 4–7].

CeNPs have been studied as bare oxide nanoparticles and functionalized materials with surface groups such as dextran and polyethylene glycol [1, 5, 7–10]. Functionalization and subsequent dispersion of materials into aqueous solutions is a critical aspect of the synthesis of biomaterials. The behavior of CeNPs, after dispersion into cell culture media, buffers, serum and other chemical treatments has not yet been studied in detail. One report suggests that changes in pH alter the reactivity of CeNPs [11], even though this work focused on *in vitro* applications. For materials other than CeNPs, studies have shown that surface changes can occur to particles such as adsorption of a protein corona [12] and aggregation [13]. Dispersion of nanoparticles in biologically relevant media can cause their irreversible aggregation; dissolution in form of ions and/or change in oxidation state, and any of these changes can be undesirable for stable administration as a catalyst in biological systems.

The core catalytic property of CeNPs has been proposed to be due to the regeneration of +3 oxidation state of Ce at the surface of the nanoparticles [8, 9]. Due to their small size, nanoparticles exhibit high surface to volume ratio which would lead them to have a high capacity to absorb molecules such as amino acids, proteins, sugars or salts present with in biological media. It is possible that interaction with these biological molecules might passivate the surface and thereby alter the stability and/or catalytic activity, which could also lead to these particles becoming toxic agents or reducing their efficacy as a catalyst [14–16]. In order to better understand how interactions with buffers, media and serum might affect CeNPs we have carried out this study to determine changes in the surface chemistry, superoxide dismutase and catalase mimetic activity of CeNPs upon exposure to biologically relevant media, buffers and ions.

MATERIALS AND METHODS

Characterization of CeNPs

UV-visible spectra were obtained using a Hewlett-Packard diode array UV-visible 8453 spectrophotometer in a 1.0 cm path length quartz cuvette. High-resolution transmission electron microscope (HR-TEM) images were obtained using a Philips (Tecnai Series) TEM operating at 300 keV and samples were prepared on 400 mesh copper grid coated with a thin layer of carbon. The surface chemistry of CeNPs dispersed in different biological buffers was studied using X-ray photoelectron spectroscopy (XPS) using a 5400 PHI ESCA (XPS) spectrometer. The base pressure during XPS measurement was 10^{-9} Torr and Mg-K α X-ray radiation (1253.6 eV) at a power of 300 watts was used. C-1S peak at 284.8 eV was used as a reference to compensate for any peak shift due to charging. Zeta potential measurements were carried out using dynamic light scattering measurements from Zeta Sizer Nano (Malvern Instruments) which uses a laser with wavelength at 633 nm.

Analysis of SOD mimetic activity

SOD mimetic activity was measured essentially as described by Korsvik et al [2]. In short, a competition for reduction of ferricytochrome C was utilized to assess the superoxide scavenging activity of CeNPs incubated in different biological buffers, media and different pH conditions. Superoxide was generated by hypoxanthine/xanthine oxidase system, and an

excess of catalase was added to remove hydrogen peroxide. Reactions were carried out in 96 well plates with a 100 μ L final volume using a Spectramax 190 UV-visible spectrophotometer (Molecular Devices, Sunnyvale, CA).

Catalase mimetic activity

Assays to follow the concentration of hydrogen peroxide were performed essentially as described [3]. Briefly, changes in the concentration of H_2O_2 (Acros Organics) was followed by its absorbance at 240 nm using UV-visible spectrophotometry. A total 100 μ L sample volume was used for kinetic measurement containing H_2O_2 and CeNPs suspensions and 1 mM DTPA (diethylene triamine pentaacetic acid), in order to avoid any potential interference by adventitious metals. This reaction mixture was buffered using tris (50 mM, pH 7.0).

Preparation of culture medium and buffers and subsequent analysis of CeNP catalytic activities

Phosphate buffer was prepared by dissolving monosodium phosphate (13.8 g/L) and its conjugate base, disodium phosphate (14.1 g/L), in one liter of water to give a 0.1 M solution, and the pH was adjusted by titration with 1 M HCl until a pH value of 7.4 was achieved. Dulbecco's modification of Eagle's medium (DMEM) was obtained from Mediatech, Manassas, VA. When added, fetal bovine serum (Mediatech, Manassas, VA.) was present at a 10% vol/vol concentration in DMEM. To study the effect of altering the pH environment on CeNPs, the pH was adjusted accordingly (range from 3 to 9) using HCl or NaOH. For analysis of stability of CeNPs in culture medium, water dispersed CeNPs (100 μ M) were suspended in 1 mL of DMEM, with or without FBS.

XPS analysis

For XPS measurements, CeNPs (1 mM) were incubated with phosphate buffer (50 mM) for 72 hrs and particles were isolated by centrifugation (10,000 rpm, 30 min) and used for sample preparation. XPS sample was prepared on cleaned silicon wafer by drop coating of so obtained resultant solution and allowed for air drying.

Zeta potential and particle size measurements

Water dispersed CeNPs (100 μ M) were suspended in different concentration of phosphate (10 μ M, 50 μ M, 100 μ M, 500 μ M, etc.) and incubated for 24 h followed by zeta potential and particle size measurements using Zeta-sizer (Nano-ZS) from Malvern Instruments.

RESULTS

pH Stability of SOD mimetic activity of CeNPs

Nanoparticles can undergo alteration in their surface properties when presented in a system with altering pH. Although the cytosolic pH of most cells remains near neutral, the subcellular organelles can be acidic or basic over a broad range of pH (4.00 to 9.00). Further, the likely contact of nanoparticles in human body may occur orally [17] and this route of delivery would expose nanoparticles to the environment with wide range of pH values. In the stomach, nanoparticles would be exposed to low pH ($\sim$ 2.0)[18] [19], followed by basic pH ($\sim$ 9.0) exposure in intestine.

We incubated CeNPs in aqueous acidic or basic solutions and followed the changes in absorption using UV-visible spectroscopy (Figure 1A). We found no significant changes to the oxidation state of Ce at the particle surface based on UV-visible measurements. Likewise when we analyzed the superoxide scavenging properties of these particles were

found no significant changes (Figure 1B). At pH values higher than 9.0, dispersed particles apparently aggregated and precipitated (data now shown) therefore we did not follow the changes in catalytic property of CeNPs at higher pH values. These results demonstrate that CeNPs produced by aqueous phase synthesis have a good stability in varying pH environments. It has been shown that after cellular internalization nanoparticles accumulate in lysosomal compartment and are highly likely to carry a positive surface charge (due to acidic pH of lysosomes) which can lead to release of particles from endo-lysosome [20]. However, particles having negative charge in lysosomes tend to retain in endosomes (21) for longer duration. A study on CeNPs reported that negatively charged particles localized in lysosomal compartment and exhibited oxidase like activity, caused radical formation, thus cell death [21]. Our *in vitro* experiments (figure – 1B) show that this harsh acidic environment is not likely to alter the SOD mimetic activity CeNPs, since the SOD mimetic activity has remained intact over a variety of pH changes.

Further, we determined any changes in the surface oxidation chemistry or catalytic activity that could occur during incubation in a commonly used culture medium. CeNPs suspended in DMEM (with or without serum) at concentration of 100 μ M (cerium concentration) formed a stable suspension as no precipitate was observed even after 72 hrs of incubation at 37° C. The SOD mimetic activity of CeNPs was not altered by this treatment (Figure 2A and B). We supplemented DMEM with 10% serum in our study, whereas, only 1% of serum has been shown to be sufficient to achieve high dispersion of nanoparticles[22]. Ji et al have shown that presence of serum in cell culture media makes nanoparticles highly dispersed [22]. Proteins or peptides present in serum can be adsorbed on the nanoparticle surface and provide better dispersion by making them more hydrophilic. The retention of catalytic activity (figure 2A and 2B) in culture medium demonstrates that any resulting protein corona that exists over CeNPs do not alter their catalytic function *in vitro*. Recently, Karakoti et al have shown that a thick coating of PEG (polyethylene glycol) did not affect the SOD mimetic activity [8] when compared to the bare CeNPs. However, a study on peroxidase activity of magnetite nanoparticles show a decrease in catalytic activity when compared with high molecular weight polymers such as dextran or PEG coating on nanoparticles [23].

Exposure of CeNPs to phosphate shifts redox state

Phosphate buffer is a typical biological buffering system used to administer biological agents (drugs, peptides, antibodies) to cells in culture or animals. Typical formulations of phosphate buffer contain phosphate anions at concentrations from 50–100 mM. Given the widespread use of phosphate buffers in biomedical science and medicine, we tested the stability and activity of CeNPs after incubation with phosphate buffer (50 mM). To our surprise we observed changes in the UV-visible absorption spectra (Figure 3B) of CeNPs after mixing with phosphate buffer. UV-visible spectra of water dispersed CeNPs showed the presence of absorbance maxima at ~250 nm (Figure 3B) which could be assigned to the +3 oxidation state of Ce in CeNPs. This absorbance peak disappears after incubation with phosphate buffer and a new absorbance peak centering at 275 nm (Figure 3B) appears, which has been ascribed to cerium phosphate [24]. Following the change in surface chemistry, we measured SOD mimetic activity (Figure 3A). To our surprise we observed complete loss of SOD activity of CeNPs after incubation with phosphate buffer

The decrease in absorbance intensity at 250 nm corresponds directly to the loss of SOD mimetic activity in phosphate treated CeNPs. Recent work has shown that Ce ions can be used as the better capture agent of inorganic phosphate released during the enzymatic hydrolysis of phosphate-containing substrates by a variety of phosphatases enzymes[25], suggesting the strong association of cerium ions at the surface of the particle with phosphate anions.

To probe this interaction further, we determined the concentration-dependence of the interaction of phosphate with CeNPs. Here we limited our experiment to 100 μM concentrations of phosphate as we found similar spectral patterns at higher phosphate concentrations (data not shown). Indeed a concentration-dependent change in SOD mimetic activity and UV-visible spectra respectively, were obtained from CeNPs dispersed in different concentrations of phosphate (Figure 4A and B). No significant change in UV-visible spectral pattern or SOD mimetic activity was observed at phosphate concentrations less than 50 μM (Figure 4A and B). However at higher concentrations (100 μM and above) a complete inhibition of SOD activity was seen and concomitantly UV-visible spectra from CeNPs incubated in phosphate buffer showed a clear absorbance peak shift from 250 nm to 275 nm (Figure 4A), again correlating well with catalytic activity. This observation suggests that CeNPs (100 μM) incubated in 100 μM phosphate concentration (equimolar) forms cerium phosphate like species which leads to the loss of SOD mimetic activity of CeNPs.

Catalase mimetic activity of CeNPs exposed to phosphate

Recently, we have shown that CeNPs with higher $\text{Ce}^{4+}/\text{Ce}^{3+}$ ratio can exhibit catalase like activity [3]. Therefore, we further tested any potential catalase like activity in phosphate-treated CeNPs suspension. Interestingly, we found that resulting suspensions of phosphate-treated CeNPs exhibited catalase like activity. This activity was also found to be dose-dependent as increasing concentrations (10 μM , 50 μM or 100 μM) of PO_4 anions incubated with CeNPs (100 μM) led to the rapid degradation of H_2O_2 (Figure 4C) when followed using UV-visible spectrophotometry at 240 nm (Fig. 5B). Our results clearly show that equimolar (100 μM) concentrations of phosphate anions and CeNPs leads to the complete disappearance of SOD activity and concomitant increase in catalase mimetic activity.

In addition we monitored the changes in photoluminescence (PL) properties of cerium oxide nanoparticles as a function of exposure to phosphate. Ce in +3 oxidation state has a weak fluorescence that is dependent on the composition of the cerium compound. As depicted in figure 5 CeNPs display a broad PL peak in blue UV region centered at ~ 345 nm [26] while cerium phosphate depicts a peak at ~ 335 nm due to the $^2\text{D}(5\text{d}^1)-^2\text{F}_{5/2}(4\text{f}^1)$ transition [27]. The presence of PL peak also suggests the predominance of Ce^{3+} in both these compounds as Ce^{4+} does not show any PL properties. It can also be observed that the PL peak shows a blue shift as well as decrease in intensity upon mixing with increasing concentration of phosphate. The trend in PL clearly suggests that phosphate anions react chemically with CeNPs and form a product (at least on the surface) that closely resembles the properties of cerium phosphate. This surface interaction may be responsible for arresting the SOD mimetic activity of CeNPs upon exposure to phosphate.

Exposure of CeNPs to sulfate and carbonate

Since other anions, similar to phosphate, could in theory bind to CeNPs and alter charge, chemistry or aggregation, we incubated CeNPs with other biologically important anions such as carbonates and sulfates, which are found in biological tissues such as in human plasma. Figure 6A shows the UV-visible absorbance pattern of CeNPs after dispersing in sulfates (calcium and magnesium) or carbonates (calcium and magnesium). Exposure of CeNPs to sulfates and carbonates did not change the absorbance spectrum of CeNPs. Likewise sulfate and carbonate treated CeNPs suspension exhibited a similar SOD mimetic activity as compared to aqueous CeNPs (Figure 6C). Further, sulfate and carbonate anion treatment did not trigger catalase mimetic activity as was seen when particles were incubated with phosphate (Figure 6B). Taken together these results suggest that a specific reaction or complex is formed upon addition of phosphate anions to CeNPs which could also affect catalytic activity, and thus could impact the mechanism by which these particles impart their protective effects both in vitro and in vivo [28].

Crystalline structure of CeNPs exposed to phosphate

High resolution TEM images (Figure ESI-1B) revealed no significant changes in the average crystal size of CeNPs even after incubation for 72 hrs in the presence of phosphate. Well separated particles present in form of clusters, made up of individual particles of 3–5 nm, similar to the water dispersed CeNPs crystals (Figure ESI-1A), suggested that phosphate ions did not cause any noteworthy aggregation or alteration in the crystal structure. The SAED patterns (Inset, Figure C) from the nanoparticles matched with the fluorite lattice of cerium oxide. High resolution TEM images show the presence of amorphous particles along with the crystalline CeNPs which clearly suggests the presence of two different types of particles in solution. The observation of fluorite lattice from SAED pattern is in contrast to the observations from UV-Visible spectroscopy however, the presence of amorphous particles suggests that the CeNPs that reacted with phosphate may be present in amorphous state. This data also suggests that the transformed nanoparticles may be stable in aqueous medium since the presence of high concentration of phosphate and may transform back to CeNPs upon drying or under the influence of electron beam.

To further investigate the identity of chemical species formed due to the interaction between CeNPs and phosphate (absorbance peak at 275 nm) we analyzed CNP-phosphate complex using XPS, a surface sensitive technique. Figure 7 shows the “P” 2p (7A and C) and “Ce” 3d (7B and D) core level spectra obtained from phosphate buffer alone (Figure 7C and D) and CeNPs treated with phosphate buffer (Figure 7A and B) respectively. The “P” 2p core level spectrum from phosphate buffer shows a clear binding energy peak at 133.5 eV that matches with the literature reported value for disodium hydrogen phosphate [29]. However, this peak shifts to 133.0 eV upon interaction with CeNPs, thus a 0.5 eV shift towards lower binding energy, which could be assigned to the interaction of phosphate group with cerium ions. This value is also 0.3 eV less than the literature reported value of 133.3 eV for P-2p core level in CePO_4 [30] suggesting the formation of mixed composite phase than pure cerium phosphate. Furthermore, the “Ce” 3d core level spectrum was also recorded from phosphate buffer (Figure 7D) and CeNPs treated with phosphate buffer (Figure 7B). As expected, no signal from “Ce” 3d was observed from phosphate buffer alone however, strong Ce 3d signals ($3d_{3/2}$ at 903.9 eV, $3d_{5/2}$ at 885.2 eV) were observed, which are characteristic for cerium atoms in the +3 oxidation state [31]. The absence of characteristic 916.8 eV peak for cerium oxide confirms that cerium was predominantly present in 3+ oxidation state and that the reaction with phosphate did not change the oxidation state of cerium as expected from the low SOD mimetic and high catalase mimetic activity of CeNPs after interaction with phosphate. XPS analysis suggests that the interaction of CeNPs with phosphate may have caused the formation of cerium phosphate at the particle surface, in which cerium is mainly present in +3 oxidation state.

Surface charge of CeNPs exposed to phosphate

In aqueous colloidal suspension charged particles are stabilized by exquisite balance between electrostatic repulsion and van der Waals attractive forces amongst charged particles and which depends on the ionic strength of the fluid. Water dispersed CeNPs are shown to have a positive zeta potential (+32.0 mV) at concentration of 0.86 mg/mL (5 mM). Addition of phosphate anions to the CeNPs suspension could decrease the electrostatic repulsion between particles due to charge neutralization and decrease in the strength of the double layer leading to the aggregation of CeNPs. To test this we determined zeta potential of CeNPs suspended in phosphate buffer. CeNPs (100 μM) dispersed in high phosphate concentration (50 mM) immediately aggregated, therefore we were not able to measure zeta potential or image by TEM. We followed the change in zeta potential value and variation in hydrodynamic size of CeNPs under increasing concentrations of phosphate at concentrations at or below the level of CeNPs (Figure 8A and B). Freshly prepared, water dispersed, stable

colloidal suspension of CeNPs exhibited an average size of ~11 nm, which again correlates our observation under TEM. As we began to expose CeNPs to increasing concentrations of phosphate (10 μ M, 50 μ M and 100 μ M), increase in resultant particle size (~24 nm, ~220 nm and ~290 nm) was observed (Figure 8A). However, when CeNPs (100 μ M) dispersed in high concentration of phosphate (500 μ M), particles tend to agglomerate and showed average aggregate size of ~ 5500 nm indicating heavy agglomeration of particles.

Water dispersed stable suspension of CeNPs displayed positive zeta potential (+ 41.5 mV), which decreases due to the addition of increased phosphate concentration (Figure 8B). Interestingly, despite the decrease in zeta potential value or an increase in the hydrodynamic size, particles did not precipitate out rather displayed a stable colloidal suspension up to a concentration of 100 μ M of phosphate. This observation suggests the adsorption of phosphate anions has occurred. Stable colloidal suspension of water dispersed CeNPs are due to the electrostatic repulsion between positive surface charges. Addition of negatively charged phosphate ions to these particles leads to a decrease in zeta potential due to the electrostatic attraction between phosphate anions and positive charge at CeNPs surface, resulting in neutralization of charge. The loss in SOD mimetic activity could be due to adsorption of phosphate anions that can chemically alter the surface composition of CeNPs.

CeNP-phosphate reaction with hydrogen peroxide

Phosphorus has been shown as poison to the catalytic activity of cerium and other oxides in diesel and automotive exhaust catalyst components [32, 33]. It should be clearly mentioned here that the catalytic capability of CeNPs involves redox cycling between Ce in the +3 and +4 oxidation states, however, formation of cerium phosphate is expected to trap the ceria in the +3 oxidation state, and thus blocks the free inter-conversion between +3 and +4 oxidation states. Similar mechanisms have been proposed for the observed oxygen storage capacity (OSC) of ceria-based materials [32]. These reports further support our observation from XPS data which also shows the presence of ceria in its +3 oxidation state (Figure 7B) after treatment with phosphate buffer.

It has been proposed that the radical scavenging properties of CeNPs are due at least in part to the mixed valence state (+3 and +4) present on nanoparticle surface [6]. Therefore, to determine the regenerative capability of CeNPs treated with phosphate buffer, we performed a reaction using high concentrations of H₂O₂, a strong oxidizer. CeNPs, having high Ce⁺³/Ce⁺⁴ ratio, have been reported to show rapid (within 5 min) color change (from colorless to yellow) upon addition of H₂O₂, which is probably due to the oxidation of Ce⁺³ to Ce⁺⁴ state and thus increase in Ce⁺⁴/Ce⁺³ ratio on CeNPs surface[6, 9]. This yellow colored solution becomes colorless after ~ 15 days due to the reduction of Ce⁺⁴ back to Ce⁺³ oxidation state. Interestingly, when phosphate buffer treated CeNPs were exposed to H₂O₂, no spectral change was observed (Figure 9, bottle 3). However a colorless CeNPs solution (Figure 9, bottle 1) was clearly oxidized (yellow emission) within 5 min after H₂O₂ addition (Figure 9, bottle 2). UV-visible spectra were also analyzed to confirm that although no change was observed in CNP-phosphate dispersions treated with peroxide (Figure 9), CeNPs alone did show a red shift in absorbance after H₂O₂ treatment.

DISCUSSION

CeNPs represent a unique catalyst that is being developed for many applications. The results presented in this work demonstrate that phosphate anions can alter the catalytic nature of these materials in a fundamental way, and suggest that our interpretation of how these materials behave in biological systems may need revision. We and others have seen that these materials can prevent damage due to increases in reactive oxygen and potentially reactive nitrogen species [28]. It is well established that both superoxide and hydrogen

peroxide are two critical ROS that help to determine the balance between oxidants and reducing potential and catalytic antioxidants. Since the spontaneous decomposition of superoxide occurs with such efficiency, many have been overzealous about the overexpression of the enzyme superoxide dismutase in model systems [34]. Given the altered catalytic properties we have found when phosphate is present with CeNPs, the question can be raised as to whether the level of phosphate present *in vivo* will alter the catalytic nature of these materials. Since inorganic phosphate is likely to be abundant (micromolar to millimolar) in cells and tissues, it is highly likely that CeNPs in the biological milieu will exhibit more reactivity with peroxides based on the results presented in this work.

The increase in catalase mimetic activity of phosphate-treated CeNPs and its subsequent decrease in the SOD mimetic activity is interesting as the XPS analysis have shown that these phosphate treated particles have predominantly Ce³⁺ oxidation state. Thus far we have relied on the specific redox chemistry of CeNPs in which we have attributed the Ce³⁺ oxidation state to be high in SOD mimetic activity and Ce⁴⁺ to possess higher catalase mimetic activity. The current investigation shows that mere presence of 3+ and 4+ oxidation state of cerium does not alone explain the catalytic activity and the redox potential of the final compound is also important in deciding its reactivity with ROS. Specific to the decomposition of hydrogen peroxide it can be realized immediately that the catalase activity observed in phosphate treated CeNPs with higher levels of cerium in the 3+ oxidation state will follow a different mechanism than the catalase activity shown by CeNPs which predominantly have cerium in the 4+ oxidation state. Hydrogen peroxide can in principle decompose in two different ways

By accepting electrons or acting as an oxidizing agent

By donating electrons or acting as a reducing agent

CeNPs with high concentration of cerium in the 4+ oxidation state can only accept electrons and decompose hydrogen peroxide by reaction 2 while phosphate treated CeNPs (or cerium phosphate) with cerium in the 3+ oxidation state may catalytically decompose hydrogen peroxide by donating electrons through reaction 1. The redox potential of the final compounds may be driving these reactions at neutral pH conditions and measurement of the same in actual aqueous conditions will be a focus of future investigations. It must be noted that redox potential may only provide the thermodynamic driving potential for a reaction to happen and it does not take into account the rate at which these reaction may occur at the surface of nanoparticles.

The stability of aqueous dispersions of nanomaterials is critical for application, and also for long term storage of these materials. We have been careful since our initial discoveries of the catalytic nature of CeNPs to test our preparations over an extended period of time. We have found that the most stable form of CeNPs is that of simple aqueous suspension, and as such most all of our studied use this material to understand the basic nature of these nanoparticles [1–3, 8, 28]. The stability of these particles is quite remarkable in the presence of extremes in pH and also in their ability to maintain a dispersed nature in the presence of

proteins and small molecules. We have also shown that conjugated forms of CeNPs are taken up rapidly and efficiently by cells in culture [35]. Depending on the level of phosphate present in the environment, it is possible that CeNPs can alter their behavior in the presence of excess phosphate and then become better superoxide scavengers when phosphate is limiting. Moreover the ability to reverse the ‘poisoning’ of the SOD mimetic activity *in vivo* can be a valid question in future studies, in which the basic changes that we have observed *in vitro* can be extended to careful *in vivo* studies.

CONCLUSIONS

The chemical stability, dispersion characteristics and SOD mimetic catalytic activity of CeNPs are resistant to changes in pH. However the presence of phosphate alters the surface chemistry and catalytic activity of CeNPs at micromolar concentrations. During this process the electrostatic repulsion between positively charged CeNPs is likely shielded by anionic phosphate ions, thus, once a substantial drop in surface charge has been achieved, aggregation occurs. Cerium phosphate formation blocks the redox cycling between $\text{Ce}^{+3}/\text{Ce}^{+4}$, which is essential for antioxidant/catalytic activity of CeNPs. Since CeNPs are a material of interest for biomedical applications our findings suggest that these interactions with phosphate should be taken into account when designing experiments for cell culture or animal studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

These studies were supported by NIH grant (1R01AG031529-01) to WS and SS and by NSF grant (NIRT 0708172 CBET) to SS and WS.

References

1. 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. [PubMed: 18395249]
2. Korsvik C, Patil S, Seal S, Self WT. Superoxide dismutase mimetic properties exhibited by vacancy engineered ceria nanoparticles. *Chem Comm*. 2007:1056–8. [PubMed: 17325804]
3. Pirmohamed T, Dowding JM, Singh S, Wasserman B, Heckert E, Karakoti AS, et al. Nanoceria exhibit redox state-dependent catalase mimetic activity. *Chem Comm*. 2010; 46:2736–8. [PubMed: 20369166]
4. Hirst SM, Karakoti AS, Tyler RD, Sriranganathan N, Seal S, Reilly CM. Anti-inflammatory properties of cerium oxide nanoparticles. *Small*. 2009; 5:2848–56. [PubMed: 19802857]
5. Niu J, Azfer A, Rogers LM, Wang X, Kolattukudy PE. Cardioprotective effects of cerium oxide nanoparticles in a transgenic murine model of cardiomyopathy. *Cardio Res*. 2007; 73:549–59.
6. 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. [PubMed: 17222903]
7. 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. [PubMed: 16351218]
8. Karakoti AS, Singh S, Kumar A, Malinska M, Kuchibhatla SVNT, Wozniak K, et al. Pegylated nanoceria as radical scavenger with tunable redox chemistry. *J Amer Chem Soc*. 2009; 131:14144–5. [PubMed: 19769392]
9. Perez JM, Asati A, Nath S, Kaftanis C. Synthesis of biocompatible dextran-coated nanoceria with pH-dependent antioxidant properties. *Small*. 2008; 4:552–6. [PubMed: 18433077]

10. Rzigalinski BA, Meehan K, Davis RM, Xu Y, Miles WC, Cohen CA. Radical nanomedicine. *Nanomedicine*. 2006; 1:399–412. [PubMed: 17716143]
11. Asati A, Santra S, Kaittanis C, Nath S, Perez JM. Oxidase-like activity of polymer-coated cerium oxide nanoparticles. *Angew Chem*. 2009; 48:2308–12. [PubMed: 19130532]
12. Cedervall T, Lynch I, Lindman S, Berggard T, Thulin E, Nilsson H, et al. Understanding the nanoparticle protein corona using methods to quantify exchange rates and affinities of proteins for nanoparticles. *Proc Natl Acad Sci*. 2007; 104:2050–5. [PubMed: 17267609]
13. Buettner KM, Rinciog CI, Mylon SE. Aggregation kinetics of cerium oxide nanoparticles in monovalent and divalent electrolytes. *Colloids Surfaces A: Physicochem Engin Aspects*. 2010; 366:74–9.
14. Brunner TJ, Wick P, Manser P, Spohn P, Grass RN, Limbach LK, et al. In vitro cytotoxicity of oxide nanoparticles: A comparison to asbestos, silica, and the effect of particle solubility. *Environ Sci Technol*. 2006; 40:4374–81. [PubMed: 16903273]
15. Lin W, Huang Y-w, Zhou X-D, Ma Y. Toxicity of cerium oxide nanoparticles in human lung cancer cells. *Intern J Toxicol*. 2006; 25:451–7.
16. Thill A, Zeyons OL, Spalla O, Chauvat F, Rose Jm, Auffan MI, et al. Cytotoxicity of CeO₂ nanoparticles for *Escherichia coli*. Physico-chemical insight of the cytotoxicity mechanism. *Environ Sci Technol*. 2006; 40:6151–6. [PubMed: 17051814]
17. Gatti AM, Tossini D, Gambarelli A, Montanari S, Capitani F. Investigation of the presence of inorganic micro- and nanosized contaminants in bread and biscuits by environmental scanning electron microscopy. *Crit Rev Food Sci Nutr*. 2009; 49:275–82. [PubMed: 19093270]
18. Rodriguez RR, Basta NT, Casteel SW, Pace LW. An in vitro gastrointestinal method to estimate bioavailable arsenic in contaminated soils and solid media. *Environ Sci Technol*. 1999; 33:642–9.
19. Beak DG, Basta NT, Scheckel KG, Traina SJ. Bioaccessibility of arsenic(v) bound to ferrihydrite using a simulated gastrointestinal system. *Environ Sci Technol*. 2006; 40:1364–70. [PubMed: 16572798]
20. Panyam J, Zhou W-Z, Prabha S, Sahoo SK, Labhasetwar V. Rapid endo-lysosomal escape of poly(dl-lactide-co-glycolide) nanoparticles: Implications for drug and gene delivery. *FASEB J*. 2002; 16:1217–26. [PubMed: 12153989]
21. Asati A, Santra S, Kaittanis C, Perez JM. Surface-charge-dependent cell localization and cytotoxicity of cerium oxide nanoparticles. *ACS Nano*. 2010; 4:5321–31. [PubMed: 20690607]
22. Ji Z, Jin X, George S, Xia T, Meng H, Wang X, et al. Dispersion and stability optimization of TiO₂ nanoparticles in cell culture media. *Environ Sci Technol*. 2010; 44:7309–14. [PubMed: 20536146]
23. Gao L, Zhuang J, Nie L, Zhang J, Zhang Y, Gu N, et al. Intrinsic peroxidase-like activity of ferromagnetic nanoparticles. *Nat Nano*. 2007; 2:577–83.
24. Xing Y, Li M, Davis SA, Mann S. Synthesis and characterization of cerium phosphate nanowires in microemulsion reaction media. *J Phys Chem B*. 2005; 110:1111–3. [PubMed: 16471651]
25. Robinson J, Karnovsky M. Ultrastructural localization of several phosphatases with cerium. *J Histochem Cytochem*. 1983; 31:1197–208. [PubMed: 6309949]
26. Gu H, Soucek MD. Preparation and characterization of monodisperse cerium oxide nanoparticles in hydrocarbon solvents. *Chem Mater*. 2007; 19:1103–10.
27. Guany M, Sun J, Shang T, Zhou Q, Han J, Ji A. A facile synthesis of cerium phosphate nanofiber by solution-solid method. *J Mater Sci Technol*. 2010; 26:45–8.
28. Karakoti A, Singh S, Dowding JM, Seal S, Self WT. Redox-active radical scavenging nanomaterials. *Chem Soc Rev*. 2010; 39:4422–32. [PubMed: 20717560]
29. Fluck E, Weber Z. P 2p binding energies in phosphorus (iii) compounds, phosphonium salts and oxiacids of phosphorus. *Naturforsch B*. 1974; 29:603.
30. Pemba-Mabiala JM, Lenzi M, Lenzi J, Lebugle A. XPS study of mixed cerium–terbium rhophosphate catalysts. *Surf Interface Anal*. 1990; 15:663–7.
31. Deshpande S, Patil S, Kuchibhatla SVNT, Seal S. Size dependency variation in lattice parameter and valency states in nanocrystalline cerium oxide. *Appl Phys Lett*. 2005; 87:133113.

32. Rokosz MJ, Chen AE, Lowe-Ma CK, Kuchеров AV, Benson D, Paputa Peck MC, et al. Characterization of phosphorus-poisoned automotive exhaust catalysts. *Appl Catal B*. 2001; 33:205–15.
33. Xu L, Guo G, Uy D, O'Neill AE, Weber WH, Rokosz MJ, et al. Cerium phosphate in automotive exhaust catalyst poisoning. *Appl Catal B*. 2004; 50:113–25.
34. Liochev SI, Fridovich I. The effects of superoxide dismutase on H_2O_2 formation. *Free Rad Bio Med*. 2007; 42:1465–9. [PubMed: 17448892]
35. Singh S, Kumar A, Karakoti A, Seal S, Self WT. Unveiling the mechanism of uptake and sub-cellular distribution of cerium oxide nanoparticles. *Mol Biosyst*. 2010; 6:1813–20. [PubMed: 20697616]

Figure 1. Superoxide dismutase activity of CeNPs is not altered by acidic or basic environment CeNPs were dispersed in solution with different pH (3 to 9) and incubated for 24 hrs followed analysis by using UV-visible spectroscopy (A) and SOD mimetic activity measurement (B) from resultant suspensions. SOD mimetic activity was followed by the reduction of ferricytochrome by superoxide spectrophotometrically (at 550 nm) for 20 min using hypoxanthine/xanthine oxidase to generate superoxide radicals[2].

Figure 2. SOD mimetic activity of CeNPs is not altered by incubation in culture medium (with or without serum)

CeNPs were dispersed in DMEM (without serum, Figure A) and DMEM (with serum, Figure B) and incubated for 24 hrs followed by SOD mimetic activity measurements from the respective suspensions.

Figure 3. Incubation of CeNPs in phosphate alters the surface charge of CeNPs

CeNPs were incubated in 50 mM phosphate (PO_4) for 24 hrs and subsequently analyzed for SOD mimetic activity (A) and UV-visible spectroscopy (B) measurements from the resultant suspensions.

Figure 4. Dose-dependent shift in surface oxidation state of CeNPs by treatment with phosphate anions (PO_4)

CeNPs (100 μM) were dispersed in a range of concentrations of phosphate (10 μM , 50 μM and 100 μM) and incubated for 24 hrs before UV-visible spectroscopy (A), SOD mimetic (B) and catalase mimetic (C) activity measurements from the resulting suspensions. A kinetic measurement of catalase like activity was analyzed by following H_2O_2 degradation at 240 nm.

Figure 5. PL spectra of CeNPs treated with phosphate

The changes in the PL spectra of CeNPs upon addition of various concentration of phosphate depicting a shift in the peak position (10 nm) and peak intensity upon treatment with phosphate.

Figure 6. Sulphate and carbonate anions are unable to alter redox state of cerium in CeNPs
 CeNPs (100 μM) were incubated with sulphate or carbonate anions (100 μM) and incubated for 24 hrs before UV-visible spectroscopy measurements (A), catalase (B) and SOD (C) mimetic activity was determined.

Figure 7. High resolution XPS spectra show that incubation of CeNPs with phosphate leads to the formation of cerium phosphate

CeNPs (1 mM) were incubated with PO_4 (50 mM) for 72 hrs and the precipitate was analyzed using XPS. XPS spectra were recorded for “P” 2p and “Ce” 3d from phosphate alone (c and d) and phosphate treated CeNPs (a and b).

Figure 8. Formation of cerium phosphate arrests “Ce” in +3 state thereby blocks the oxidation by hydrogen peroxide

CeNPs (1 mM) and phosphat treated CeNPs (1 mM) were treated with H_2O_2 (0.2 M) and incubated for 24 hrs followed by UV-visible spectra measurements.

Figure 9. Incubation of CeNPs with phosphate buffer leads to the increase in particle size (A) and decrease in zeta potential (B)