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Role of phosphate on stability and catalase mimetic activity of cerium oxide nanoparticles

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ABSTRACT

Cerium oxide nanoparticles (CeNPs) have been recently shown to scavenge reactive oxygen and nitrogen species (ROS and RNS) in different experimental model systems. CeNPs (3+) and CeNPs (4+) have been shown to exhibit superoxide dismutase (SOD) and catalase mimetic activity, respectively. Due to their nanoscale dimension, CeNPs are expected to interact with the components of biologically relevant buffers and medium, which could alter their catalytic properties. We have demonstrated earlier that CeNPs (3+) interact with phosphate and lose the SOD activity. However, very little is known about the interaction of CeNPs (4+) with the phosphate and other anions, predominantly present in biological buffers and their effects on the catalase mimetic-activity of these nanoparticles. In this study, we report that catalase mimetic-activity of CeNPs (4+) is resistant to the phosphate anions, pH changes and composition of cell culture media. Given the abundance of phosphate anions in the biological system, it is likely that internalized CeNPs would be influenced by cytoplasmic and nucleoplasmic concentration of phosphate.

1. Introduction

The use of organic and inorganic nanomaterials as therapeutic and diagnostic agent, including imaging, targeting and systematic drug delivery, has grown tremendously worldwide during past two decades. Among these, CeNPs have attracted much attention as a catalyst in the biological model system due to their unique antioxidant properties. CeNPs have found several applications such as treatment of oxidative stress related diseases [1], neurodegenerative disorders [2,3] and even in commercial fields as oxygen ion conductor and as anode of solid oxide fuel cells [4,5]. "Ce" atoms in CeNPs undergo redox reaction and can exist in the ratio of 3+ and 4+ oxidation states, which can be the possible mechanism behind the free-radical scavenging activity [6]. SOD and catalase mimetic activities exhibited by CeNPs are due to the high 3+ and 4+ oxidation states of surface "Ce" atoms present on their surface, respectively [7–9]. Mechanism behind the switching of CeNPs between the two oxidation states are proposed to be due to the oxygen vacancy/defect in their crystal structure [10]. Loss of oxygen or its electron is responsible for the alternation between the two oxidation states during redox reaction [11]. Owing to the high surface to volume ratio, nanomaterials suspended in biological media or buffer tend to react with the surrounding biomolecules which may ultimately lead to the adsorption of particular amino acids, sugars, salts and other molecules [12]. It is well reported that nanoparticles undergo the formation of "protein corona," and understood that only specific amino acids are preferably adsorbed on the nanoparticle surface and certain amino acids are in dynamic form and endure adsorption and desorption from the surface of nanoparticles [13]. More recently, Singh et al. [14] have reported that CeNPs (3+) lose SOD activity when exposed to high concentration of phosphate anions but not to carbonate and sulfate ions. This further supports the selectivity of molecules too, which can adsorbed on the surface of nanoparticles. Therefore, it is also possible that certain molecules adsorb on the surface and alter the catalytic activity of nanoparticles. Further, as described by DLVO theory, in colloids the stability of nanoparticles are due to electrostatic repulsion and van der Waals attraction forces operating between the charged particles and controlled by ionic strength of the fluid [15]. The disruption of this force by the addition of ions could cause aggregation of the particles which may again lead to the alteration of intrinsic properties of nanomaterials. It has also been reported that the covalent conjugation of carboxyfluorescein to CeNPs passivates the surface and decreases the SOD mimetic-activity [16]. Such alteration to nanoparticles could impart toxicity when exposed to mammalian cells/tissues and environment. Therefore, in order to understand the stability and interaction of CeNPs (4+) with phosphate buffer and consequent alteration in surface chemistry, oxidation state and

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catalase mimetic-activity, due to dispersion in different pH conditions and composition of cell culture media, a systematic study was carried out.

2. Materials and methods

2.1. Materials

Xanthine oxidase, catalase, cytochrome C and ammonium cerium nitrate were purchased from Sigma Aldrich (St. Louis, MO, USA). Hydrogen peroxide (H_2O_2) and DTPA (diethylenetriaminepentaacetic acid) was obtained from S D Fine Chem. Limited (Mumbai, India). Hypoxanthine, potassium carbonate (K_2CO_3), magnesium sulphate ($MgSO_4$), calcium sulphate ($CaSO_4$) and Dulbecco's Minimum essential media (DMEM: F12) were purchased from Hi-Media Pvt. Ltd. (Mumbai, India).

2.2. CeNPs synthesis

CeNPs (4+) were prepared by dissolving ammonium cerium nitrate in Milli Q water, followed by drop wise addition of ammonium hydroxide until the white precipitate got to disappear from the solution. Finally, a light pale yellow solution of CeNPs in 4+ oxidation state was formed.

2.3. Characterization of CeNPs

UV–visible spectra was acquired using BioSpectrometer (Eppendorf AG 22331, Hamburg) using quartz cuvette having 1.0 cm path length. Zeta potential measurements were carried out using dynamic light scattering measurements from Zeta Sizer Nano (Malvern Instruments) which uses a laser with wavelength of 633 nm.

2.4. Analysis of SOD mimetic activity

SOD mimetic activity of CeNPs was measured by the method reported by Korsvik et al. [8]. In brief; the competition for reduction of ferricytochrome C by superoxide radicals was measured by increasing absorbance at 550 nm using UV–visible spectrophotometer (Biotek, Synergy HT spectrophotometer). Superoxide radicals were generated by incubating 5 mM hypoxanthine with xanthine oxidase in a reaction system. Reactions were carried out in a 96 well plate with total volume of 100 μl for 20 min and reaction was buffered with 10 mM Tris pH 7.5. Catalase was added to avoid any interference by the hydrogen peroxide (H_2O_2) in the system.

2.5. Analysis of catalase mimetic activity

Catalase mimetic activity of nanoceria was measured by the decrease in the absorbance of H_2O_2 at 240 nm, by UV–visible spectrophotometer. 1 mM DTPA (diethylenetriaminepentaacetic acid) was used in the reaction to avoid any possible interference by the metals. Reaction kinetics was performed in total volume of 1 ml and buffered with 50 mM Tris, pH 7.5 [9].

2.6. Preparation of culture medium and buffers and analysis of CeNPs catalytic activity

Phosphate buffer was prepared by dissolving monosodium phosphate (13.8 g/L) and its conjugate base, disodium phosphate (14.1 g/L), in 1 L of water to obtain a 0.1 M solution, and further the pH was adjusted by titrating it with 1 M HCl until a pH value of 7.0 was achieved. Different pH range (2.0–10.0) was prepared by using 1 N HCl and 1 N NaOH in Milli Q water. Different salt solutions of potassium carbonate (K_2CO_3), magnesium sulphate ($MgSO_4$) and calcium sulphate ($CaSO_4$) were prepared in a concentration of 200 μM in water. DMEM: F12 culture was obtained from Hi-media. Complete media was prepared by adding Fetal Bovine Serum (FBS) at 10% (v/v) in incomplete media. To check the stability and activity of CeNPs (4+) in the phosphate buffer, at different pH range, in different salts and in complete and incomplete media, 200 μM of CeNPs were suspended in the solutions followed by stability and activity measurement.

2.7. Zeta potential and particle size measurements

CeNPs were suspended in the different concentrations of phosphate (10, 50, 100 μM and 10 mM), different pH solutions (2, 4, 6, 8, 10), salt solutions (K_2CO_3, $MgSO_4$ and $CaSO_4$) and complete and incomplete cell culture medium, followed by size and charge measurement by Zeta Sizer Nano (Malvern Instruments) using a laser of wavelength at 633 nm.

2.8. FTIR analysis

CeNPs (4+) (1 mM) were suspended in phosphate buffer (1 mM), complete (with FBS) and incomplete (without FBS) media and incubated for 24 h followed by FTIR analysis by FTIR-8400S, Shimadzu.

2.9. Photoluminescence analysis

CeNPs (4+)/(3+) (1 mM) were suspended in 1 mM phosphate buffer and incubated for 24 h followed by Photoluminescence spectra measurement by RF-5301, Shimadzu pc Spectrofluorophotometer.

3. Results and discussion

Nanoparticles can undergo change in their surface chemistry and thus behavior after incubation in solutions of different pH. The cellular cytoplasm has neutral pH, but sub-cellular organelles can have a wide range of pH. It has been reported by Labhasetwar and co-workers [17] that after cellular uptake nanomaterials accumulate in lysosomes, and it is likely that the acidic pH imparts positive surface charge (due to the acidic pH of lysosomes) which causes the release of particles from lysosomes. In contrast, nanomaterials carrying negative charge in lysosomes do not get released and tend to be retained in endosomes [18]. Similar observation was reported by Asati et al. [19] where negatively charged CeNPs were found localized in lysosomes and retain their oxidase-like activity. In the human body too nanoparticles can undergo exposure to a wide range of pH variation, like stomach has an acidic pH of ~2.0 and intestine has alkaline pH of ~9.0 [20]. CeNPs (4+) with high surface $Ce^{[4+/3+]}$ ratio has shown biological catalase enzyme like activity [9]; therefore, if these particles are envisaged to be delivered orally in the human body, the determination of stability assessment at different pH values is necessary. CeNPs (4+) were incubated at selected pH ranging from pH 2 to 10 followed by determination of absorbance using UV–visible spectroscopy. No substantial alteration in the absorbance pattern of CeNPs (4+) was observed indicating no change to the oxidation state to the surface of CeNPs (Fig. 1A). Subsequently, when analyzed, slight decrease was observed in the catalase mimetic activity of these particles (Fig. 1B). The CeNPs (4+) dispersed in water show diameter of ~10 nm, which did not get altered by the pH variation of the solution except the particles dispersed at pH 10, which showed the increase in particle size (Fig. 2A). This could be ascribed to the aggregation of CeNPs (4+) at alkaline pH. Likewise, no significant change in particle zeta potential was observed when exposed to varying pH solutions (Fig. 2B). These results demonstrate that CeNPs (4+), prepared by aqueous phase synthesis, are stable in different pH