Colloids and Surfaces B: Biointerfaces 147 (2016) 45–53

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

Green synthesized cerium oxide nanoparticle: A prospective drug against oxidative harm

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ARTICLE INFO

Article history:
Received 7 May 2016
Received in revised form 15 July 2016
Accepted 19 July 2016
Available online 25 July 2016

Keywords:
Green synthesis
Cerium oxide nanoparticles
Aloe-vera extract
Anti-oxidant activity
Mouse neural cells

ABSTRACT

Cerium oxide nanoparticle (CeONP) of size 2–3 nm was synthesized by a new, simple and green method at ambient temperature, using cerium nitrate as prime precursor and Aloe vera leaf extract as both oxidizing and stabilizing agent. Of the two oxidation states (+3) and (+4) of cerium, it was dominantly present in (+3) state in CeONP and cyclic conversion of Ce(III)O → Ce(IV)O → Ce(III)O by reaction with H_2O_2 implied uninterrupted antioxidant property of CeONP. Moreover, the higher oxygen defect in the crystal lattice produced particles with higher antioxidant activity. CeONP was found to neutralize the deleterious effects of H_2O_2 viz., cell death, generation of intracellular reactive oxygen species and loss of connectivity in mouse neural cells. Therefore, CeONP might have potential use in future as an anti-oxidant drug.

1. Introduction

Oxidative stress has been implicated as a cause of different diseases like arthritis, cardiac disorder, diabetes, Alzheimer's and Parkinson's diseases, macular and retinal degeneration etc. [1,2]. Cellular oxidative damage is mediated by 'reactive oxygen species' (ROS), which include free radicals such as superoxide anion ($O_2^{\bullet -}$), hydroxyl radical (•OH), singlet oxygen ($^1O_2$) and non-free radical species like hydrogen peroxide (H_2O_2). Usually, antioxidants protect cells from oxidative stress, by neutralizing ROS. Most of the antioxidants are exhausted to neutralize ROS; however, cerium oxide (CeO) has an exclusive uninterrupted antioxidant activity, because cerium can cycle between the cerous (Ce^{3+}) and ceric (Ce^{4+}) oxidation states. CeO(III) is fast oxidized to CeO(IV) by ROS and by that CeO(III) successfully neutralizes ROS. Again CeO(IV) is slowly reduced by ROS to slip back to CeO(III), leaving oxygen vacancies in the CeO crystal lattice that becomes further reactive towards ROS [3,4]. Thus, cycling of CeO between +3 and +4 states can uninterruptedly break down ROS.

Over the past few years there has been increasing research to explore the therapeutic capacity of nanoscale cerium oxide [5–9]. Thermal history of synthesis of CeO_2 nanoparticles is suggested to be a factor that strongly influences the biological impact of nano-ceria. Synthesis methods, involving high temperature exposure, produce CeONP of pro-oxidative inflammatory response; on the other hand, methods requiring low heat or room temperature form particles of anti-oxidative response [10,11]. Moreover, various chemical processes of synthesis produce nanoparticles with undesirable functionality. We, therefore, ventured to synthesize biocompatible CeONP of potential antioxidant activity.

In this communication we report about a simple, green method of preparation of CeONP at room temperature, using cerium nitrate [$Ce(NO_3)_3$] as the prime precursor and Aloe vera leaf extract as the stabilizing agent. Important physico-chemical characteristics of the synthesized CeONP have also been reported here. The organic layer of Aloe vera on the CeONP surface made it prone to interact with biological cell surface and thereby facilitated its internalization into the cell. Our CeONP has high pharmacological potential to neutralize the H_2O_2-induced oxidative stress in mouse neuroblastoma cells (N2A).

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http://dx.doi.org/10.1016/j.colsurfb.2016.07.045
0927-7765/

2. Materials and methods

2.1. Preparation of aloe vera leaf extract

Fresh leaf pulp (30 g) of Aloe vera was boiled in 100 ml distilled water for 10 min. The aqueous extract was then filtered through Whatman filter paper-40 (GE Healthcare, UK). The filtrate was centrifuged at 15000 RPM for 15 min to remove any un-dissolved substance; the supernatant was collected and stored at −20 °C.

2.2. Synthesis of CeONP

For preparation of 10 ml CeONP suspension, 1 ml cerium nitrate (10 mM), 1 ml aqueous Aloe vera leaf extract and 7 ml Milli-Q water were first mixed at room temperature (28 °C); 1 ml of sodium hydroxide (30 mM) was then added drop-wise to the mixture. The suspension was stirred for 48 h, when it became transparent. It was finally sonicated at 75% amplitude with 59/10 s 'on and off' pulses respectively for 10 min, to obtain well-dispersed nano-ceria particles.

2.3. Characterization of CeONP

Light absorption property of CeONP suspension was checked by a spectrophotometer (Shimadzu, UV-1800) in the wavelength region 220–800 nm, using a mixture of cerium nitrate and Aloe vera extract (in the same proportions as they were present in CeONP) in the reference cuvette.

Band gap energy E_g between valence and conduction bands of the nano-ceria particles was measured spectrophotometrically, using Tauc's equation $\alpha(h\nu) = A(h\nu\text{-}E_g)^{m/2}$ [12], where 'α' was absorption co-efficient of the NP suspension, $h\nu$ (= hc/λ) was energy of incident light of wavelength λ, 'A' was a constant and 'm' depended on nature of transition (m = 1 for direct and m = 0.5 for indirect transition of electrons) [13]. Since the transition is direct for ceria NPs [13], therefore putting m = 1, Tauc's equation became $(\alpha h\nu)^2 = A^2 (h\nu - E_g)$. From the plot of $(\alpha h\nu)^2$ vs. $h\nu$, the point of intersection of the slope of the plot on X-axis denoted the value of E_g.

Presence of cerium in different oxidation states Ce(III) and Ce(IV) and their relative amounts in CeONP were determined by X-ray photoelectron spectroscopy (XPS) of vacuum dried NP powder, in a Omicron Multiprobe (Omicron NanoTechnology GmbH., UK) spectrometer fitted with an EA125 hemispherical analyzer. An X-ray source of monochromated Al Kα radiation, operated at 150W, was used and the analyzer pass energy was kept fixed at 40 eV for all scans.

Size of the NPs was determined by high resolution transmission electron microscope (HR-TEM); a drop of CeONP suspension was dried on a carbon-coated copper grid in a vacuum desiccator and was finally analyzed by an HR-TEM (JEOL, JEM-2010), operated at 200 kV.

Crystallinity of vacuum-dried CeONP powder was characterized by an x-ray diffractometer (Bruker, D8) with Cu-Kα radiation (λ = 1.5406 Å); scanning was done from 20 to 80° of 2θ with 0.02^0/min.

Surface oxygen defect on nano-ceria crystal lattice was studied through Surface Enhanced Raman Spectroscopy (SERS) of vacuum dried NPs, in a Raman Spectrometer (HORIBA, Jobin Yvon) using 633 nm laser beam.

Zeta potential of nano-ceria was measured by dynamic light scattering (DLS) instrument (Malvern, Nano-ZS).

Chemical bonds in CeONP were investigated by Fourier transformed infra-red (FTIR) spectroscopy. 1 mg lyophilized NP powder was mixed with 250 mg KBr to prepare a homogeneous KBr pellet as FTIR sample. The spectroscopy was done in a FTIR machine (Perkin Elmer L 120-000A) in transmission mode, with wave number range 450–4000 cm^{-1} and resolution 4 cm^{-1}.

Antioxidant activity of CeONP was determined by the DPPH (2diphenyl1picrylhydrazyl) assay [14]. DPPH was composed of stable free radical molecules and its purple colored solution had a strong absorption peak at 517 nm. When its radical property was scavenged upon absorption of hydrogen from an antioxidant, the color turned from purple to yellow with decrease of intensity of $(abs)_{517nm}$. The antioxidant activity was calculated as:

$$\%\ \text{of radical scavenging} = \frac{[(\text{DPPH})\,517\text{nm} - (\text{DPPH} + \text{antioxidant})\,517\text{nm}]}{[(\text{DPPH})\,517\text{nm}]} \times 100$$

To determine in vitro antioxidant capacity of CeONP, 300 μl of freshly prepared DPPH solution (0.2 mM) in methanol was added to different concentrations of the NP. The final volume of the reaction mix was made 1 ml by adding Milli-Q water. After 30 min of incubation in dark at room temperature, absorbance of each sample was measured at 517 nm.

2.4. Antioxidant activity of CeONP against H_2O_2-induced oxidative stress in mouse neural cells

2.4.1. Cell viability assay

Toxic effect of H_2O_2 on the viability of mouse neuroblastoma cells N2A was determined by 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) reduction assay [15], taking healthy grown cells in 96-well plate at a concentration of about 6×10^3 cells/well. In principle, mitochondrial dehydrogenases of metabolically active cells cleaved the tetrazolium ring of MTT, yielding purple colored insoluble formazan crystals, which were subsequently solubilized in acidified isopropanol; the absorbance of the resulting purple solution was measured at 595 nm. The degree of cytotoxicity got reflected directly from the decrease in absorbance, because less tetrazolium cleavage took place with an increase of cell killing. In this assay, healthy attached cells in wells were exposed to H_2O_2 for 24 h, the exhausted medium was drained out followed by mild washing with phosphate buffer saline (PBS—NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 10 mM, KH_2PO_4 1.8 mM, pH 7.4). Cells were then incubated with 20 μl MTT (1 mg/ml) at 37 °C for 4 h, after which the MTT was removed and 100 μl acidic isopropanol was added and mixed thoroughly on a plate shaker. Absorbance was measured at 595 nm with a micro-plate reader.

2.4.2. Measurement of intracellular ROS production

The extent of ROS production in H_2O_2-exposed cells was estimated by using the dye 2′, 7′-dichlorodihydrofluorescein diacetate ($DCFH_2$-DA) [16]. After diffusion into the cell, the dye was deacetylated by cellular esterases to a non-fluorescent compound, which was later oxidized by ROS into 2′,7′-dichlorofluorescein (DCF) [17]. Cells (3×10^5/2 ml) were treated with 40 μM H_2O_2 for 2 h and were subsequently washed twice with PBS. $DCFH_2$-DA (10 μM) was then added to the cells and incubated in the dark for 30 min at 37 °C. Cells were washed twice with PBS and analyzed by a flow cytometer (FACS Calibur; Becton Dickinson, USA) through the FL1-H channel. The data were plotted as histogram using 'Flowing Software 2.5.1′ (University of Turku).

2.4.3. Study of cell morphology by scanning electron microscope (SEM)

Cells were cultured on glass cover slip, fixed with 2.5% glutaraldehyde in PBS at 37 °C for 15 min, followed by washing with PBS. The cell pellet was then treated with 2.5% osmium tetroxide, washed again, dehydrated by rinsing with increasing concentrations of aqueous ethanol (25, 50, 70, 90 and 100% for 5 min at each step) solution and finally washed with fresh 100% ethanol