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Auto-catalytic Ceria Nanoparticles Offer Neuroprotection to Adult Rat Spinal Cord Neurons

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Abstract

This paper describes the evaluation of the auto-catalytic anti-oxidant behavior and biocompatibility of Cerium oxide nanoparticles for applications in spinal cord repair and other diseases of the CNS. The application of a single dose of nano-Ceria at a nano-molar concentration is biocompatible, regenerative and provides a significant neuroprotective effect on adult rat spinal cord neurons. Retention of neuronal function is demonstrated from electrophysiological recordings and the possibility of its application to prevent ischemic insult is suggested from an oxidative injury assay. A mechanism is proposed to explain the auto-catalytic properties of these nanoparticles.

Keywords

Adult spinal cord neurons; Auto-catalytic; Cerium oxide; Nanoparticle; Neuroprotection; Nerve regeneration

Introduction

In this study we have demonstrated that auto-catalytic nano-Ceria particles will enhance survival of adult spinal cord neurons in a unique, defined *in vitro* system. Ultra fine, non-agglomerated Cerium oxide nanoparticles (2–5 nm) were synthesized by a microemulsion process [1] for their suitability for neuroprotective applications in spinal cord injury and degenerative diseases of the central nervous system (CNS). To test the general biocompatibility and neuroprotection capability of the synthesized nanoparticles, we evaluated the activity of nano-Ceria in a novel serum-free cell culture model of adult rat spinal cord [2]. Administration of a single dose of nano-Ceria (10 nM) at the time of cell plating promotes significantly higher neuronal survival as compared to control cultures. Nanoparticle treated neurons indicated normal electrical activity as compared to the control culture to demonstrate functional biocompatibility. Nano-Ceria treated cells also had significantly higher cell survival upon hydrogen peroxide-induced oxidative injury in the adult spinal cord model system. Based on our cell culture assays, UV-visible spectroscopic studies, a hydrogen peroxide-induced oxidative injury assay and our proposed working hypothesis, we conclude that the

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supplementation of adult neuronal cultures with auto-catalytic Ceria nanoparticles has a significant effect in neuronal survival and retention of function.

Cerium oxide is a rare earth oxide that is found in the lanthanide series of the periodic table. It is used in various applications such as: histochemistry [3,4] electrolytes for solid oxide fuel cells [5], ultraviolet absorbents [6], oxygen sensors [7,8], and automotive catalytic converters [9]. Nanocrystalline Cerium oxide exhibits a blue shift in the ultra violet absorption spectrum [6], the shifting and broadening of Raman allowed modes [10] and lattice expansion as compared to bulk cerium oxide [6,11]. It was these attributes of this material that were an early indicator that it has unique properties.

These results demonstrate that the use of nano-Ceria could prove beneficial for the *in vivo* repair of spinal cord neurons based on our experiments evaluating the nano-ceria in a more realistic *in vitro* model of spinal cord utilizing **adult** CNS cells. It is also anticipated that they could be good candidates for drug delivery and imaging applications. Based on the surface chemical properties of Ceria nanoparticles [12] we propose a hypothesis to explain the neuroprotective role of this material.

Experimental Section

Nanoparticle Synthesis

Cerium oxide nanoparticles were prepared by a microemulsion method. The nanosized micelles act as reactors for particle formation. The microemulsion system consisted of the surfactant, sodium bis(2-ethylhexyl) sulphosuccinate (AOT), toluene and water. All the chemicals were purchased from Aldrich Chemicals Company, Inc. Details of the synthesis are published elsewhere [1]. The particles obtained in toluene were re-dispersed in water by evaporating the toluene prior to use in the cell culture studies.

Nanoparticles Characterization

The particle morphology was studied using HRTEM. The surface chemistry of the nano-Ceria particles was studied using XPS. The Ceria nanoparticles were deposited on the carbon coated copper grid for HRTEM analysis by the dip coating method. The HRTEM images of the as prepared particles were obtained with a Philips (Tecnai Series) transmission electron microscope operating at 300 keV. The XPS data was obtained using a 5400 PHI ESCA (XPS) spectrometer. The base pressure during XPS analysis was 10^{-9} Torr and Mg-K α X-ray radiation (1253.6eV) at a power of 200 watts was used. The binding energy of Au (4f $_{7/2}$) at 84.0 ± 0.1 eV was used to calibrate the binding energy scale of the spectrometer. Any charging shift produced in the spectrum was corrected by referencing to the C (1s) position (284.6 eV) [13]. XPS spectra smoothening and baseline subtraction was carried out using PeakFit (Version 4) software.

Cell Culture

Surface modification of the coverslips for cell culture—The DETA (United Chemical Technologies Inc. T2910KG) films were formed by the reaction of the cleaned glass surface with a 0.1% (v/v) mixture of the organosilane in freshly distilled toluene (Fisher T2904). The DETA coated coverslips were heated to just below the boiling point of toluene, rinsed with toluene, reheated to just below the boiling temperature, and then oven dried. The detailed procedure is described elsewhere [14,15]

Surface characterization of the coverslips—Surfaces were characterized by contact angle measurements using an optical contact angle goniometer (KSV Instruments, Cam 200) and by XPS (Fisons 220i). XPS survey scans, as well as high-resolution N 1s and C 1s scans,

utilizing monochromatic Al K α excitation, were obtained according to our previously published procedures [2,14–17].

Isolation and culture of rat spinal cord—Spinal cords were isolated from euthanized adult rats (average age was 3–4 months) and the meninges were removed from the spinal cord. One single spinal cord from an adult rat weighs 1.10 g ($\pm$ 0.05). The harvested spinal cord was cut into small pieces and collected in cold Hibernate A [18] (www.BrainBits.com), GlutaMAX™, an antibiotic-antimycotic and B27 (Invitrogen). Next, the tissue was enzymatically digested for 30 minutes in papain (2mg/ml). The tissue was dissociated in 6 ml of fresh Hibernate A, GlutaMAX™, an antibiotic-antimycotic and B27. The 6 ml cell suspension was layered over a 4 ml step gradient (Optipep diluted 0.505: 0.495 (v/v) with Hibernate A/ GlutaMAX™ / antibiotic-antimycotic/ B27 and then made to 15%, 20%, 25% and 35% (v/v) in Hibernate A/ GlutaMAX™/ antibiotic-antimycotic/ B27) followed by centrifugation for 15 min, using 800g, at 4°C. The top 7 ml of the supernatant was aspirated. The next 2.75 ml from the major band and below was collected and diluted in 5ml Hibernate A/ GlutaMAX™/ antibiotic-antimycotic/ B27 and centrifuged at 600 g for 2 minutes. The pellet was resuspended in Hibernate A/ GlutaMAX™/ antibiotic-antimycotic/ B27, and after a second centrifugation, the pellet was resuspended in the culture medium. Approximately 12,000–13,000 live cells are harvested from one adult rat spinal cord. 1000 cells were plated on each coverslip (22 X 22 mm²) at 2 cells/mm². The culture medium was changed completely after the first 2 days in culture and thereafter half of the medium was changed every four days [2,19]. A molecular Probe's L-3224 Live/Dead Assay kit was used for the live-dead assays [16].

Immunocytochemistry for quantification of neuronal and glial cells in control and nanoparticle treated culture—Rabbit anti-neurofilament M polyclonal antibody, 150 kD, (Chemicon, AB1981, diluted 1:100) and mouse anti-GFAP monoclonal antibody (Chemicon MAB360, diluted 1:400), were used for staining the neuronal and glial cells. The method for immunostaining is described in detail elsewhere [2].

Electrophysiology of nanoparticle treated culture—Voltage clamp and current clamp experiments were performed with a Multiclamp 700A (Axon, Union City, CA) amplifier. Signals were filtered at 3 kHz and digitized at 20 kHz with an Axon Digidata 1322A interface. Data recording and analysis was performed with pClamp 8 (Axon) software. The detailed protocols are documented in our previous work [2,16].

Results and Discussion

A microemulsion process was used to synthesize the cerium oxide nanoparticles (Figure 1a) and they were characterized for morphology and surface chemistry by high-resolution transmission electron microscopy (HRTEM) and X-ray photoelectron spectroscopy (XPS). HRTEM indicated the formation of uniformly distributed, non-agglomerated nanoparticles of Cerium oxide in the range of 3–5nm as shown in the image in Figure 1b. Figure 1c shows a XPS spectrum that indicates a mixed valence state (Ce³⁺ and Ce⁴⁺) for the synthesized Cerium oxide nanoparticles. These results are similar to our previously published results [1,12,20].

The *in vitro* studies with the synthesized nanoparticles were carried out in a serum-free cell culture model of adult rat spinal cord (Figure 2) which has been shown to promote growth and long-term survival of dissociated adult rat spinal cord neurons [2]. This system consists of a patternable [15], non-biological, cell growth promoting organosilane substrate, N-1 [3-(trimethoxysilyl) propyl] diethylenetriamine (DETA) [16,17,21,22], coated on a glass surface combined with an empirically derived, novel serum-free medium and a reproducible cellular isolation and pre-plating methodology. The serum-free medium consisted of neurobasal A

supplemented with B27 [23], GlutaMAX™, acidic fibroblast growth factor, heparin sulphate, neurotrophin-3, neurotrophin-4, ciliary neurotrophic factor (CNTF), brain derived neurotrophic factor, glial derived neurotrophic factor, cardiotrophin-1, vitronectin and an antibiotic-antimycotic (Figure 2b). The quality of the surface modified coverslips used for cell culture was monitored using static contact angle measurements and XPS analysis as previously described [2,14,16,17]. Stable contact angles ($40.64^\circ \pm 2.9/\text{mean} \pm \text{SD}$) throughout the study indicated high reproducibility and quality of the DETA coatings and were similar to previously published results [2,14,16,17,24]. Based on the ratio of the N 1s (401 and 399 eV) and the Si 2p_{3/2} peaks, XPS measurements indicated that a monolayer of DETA (Figure 2c) was formed on the coverslips [2,14,16,17]. The cell isolation process from dissected adult rat spinal cord is briefly described in the methods.

The outline of the cell isolation and cell plating is shown in Figure 2a and documented in detail in our previous work [2]. In each experiment, an equal volume of the cell suspension (1000 live cells at a density of 2 cells/mm²) was plated on each coverslip. Of the total number of coverslips plated with cells, half of the coverslips were used for control cultures and the other half received a single dose of 10nM nano-Ceria at the time of cell plating. At two different time intervals, day 15 and day 30, live-dead assays and neuron-glia immunostaining assays were conducted to quantify cell viability and the surviving cell types in both the control and nano-Ceria treated cultures. A student's T-test was used for statistical analysis. The results are expressed as mean $\pm$ SE, n = 6, where n stands for number of coverslips. The total number of coverslips used for these assays were drawn from six different experiments. Live-dead cell assays (Figure 3a) indicated a significantly higher cell survival at day 15 (617 ± 34 , n = 6) and at day 30 (472 ± 35 , n = 6) in nano-Ceria treated cultures as compared to the control cultures at day 15 (479 ± 37 , n = 6) and day 30 (328 ± 32 , n = 6). We also observed a significantly lower cell death at day 15 (59 ± 7 , n = 6) and day 30 (48 ± 7 , n = 6) in nano-Ceria treated cultures as compared to the control cultures on day 15 (110 ± 9 , n = 6) and day 30 (72 ± 8 , n = 6). Neurons and glial cells were identified by immunoreactivity for neurofilament 150 (neuronal marker) and glial fibrillary acidic protein (GFAP) (glial marker) antibodies respectively. The neuronal population was significantly higher in nano-Ceria treated cultures at day 15 (191 ± 40 , n = 6) and at day 30 (221 ± 12 , n = 6) compared to the control cultures on day 15 (71 ± 26 , n = 6) and day 30 (148 ± 9 , n = 6). There was no significant difference in glial cell population or populations of cells which stained for both neuron and glial markers in treated cultures compared to control cultures at either time interval (Figure 3b). Electrical activities of the nano-Ceria treated cultures were assessed using patch-clamp electrophysiology at day 30 in culture. The treated neurons expressed voltage dependent inward and outward currents (Figure 4a) and generated single action potentials (Figure 4b), similar to that observed for the controls and in other adult rat CNS cultures [2,25].

We propose that the presence of the mixed valence states of Ce³⁺ and Ce⁴⁺ on the surface of the nano-Ceria act as an anti-oxidant that allow the nanoparticles to scavenge free radicals from the culture system. Another complex set of surface chemical reactions [26] between the ions in the cell culture medium and the nano-Ceria then appear to be involved in reversing the oxidation state from Ce⁴⁺ to Ce³⁺. We believe that this is indicative of a cyclical regenerative, or auto-catalytic, reaction of the Ceria nanoparticles. The proposed mechanism is shown in Figure 5. To demonstrate the auto-catalytic property of the engineered nano-Ceria particles, we carried out a UV-visible spectroscopic study of a nano-Ceria sol treated with 10mM hydrogen peroxide (Figure 6). The UV-Visible spectrum of a sample of the nano-Ceria solution was used as a control (black trace in the graph). We added hydrogen peroxide to this solution and observed a shift in the spectrum to the right or to the lower energy portion of the spectrum (pink trace). In this reaction, hydrogen peroxide provides a source of hydroxyl radicals to mimic oxidative stress found *in vivo*. This shift is postulated to be due to a change in the oxidation state from Ce³⁺ to Ce⁴⁺ [27]. The nano-Ceria sample treated with hydrogen peroxide was then

kept in the dark for 30 days. UV-Visible spectra of these samples were then taken at day 15 and day 30 (blue and red traces for the day 15 and day 30 spectra, respectively). A gradual shift in the spectra to the left was seen over time. This gradual higher energy shift reflects the regeneration ($\text{Ce}^{4+} \rightarrow \text{Ce}^{3+}$) of the cerium oxide nanoparticles. When an additional hydrogen peroxide dose was administered to the solution on day 30, the UV-Visible spectrum again shifted to lower energy (green trace) which was followed by a gradual shift to the lower wavelength, as seen previously. The shift of the UV visible spectrum to a lower energy state on exposure to hydrogen peroxide with a recovery toward a higher energy state ($\text{Ce}^{3+} \rightarrow \text{Ce}^{4+} \rightarrow \text{Ce}^{3+}$). This indicates that nano-ceria exhibits a mechanism in which the engineered particle provides a new material for life science applications with unprecedented antioxidant activity and pseudo-infinite half-life. The auto-regenerative anti-oxidant property of these nanoparticles appears to be the key to its neuroprotective action.

The auto-catalytic properties of the ceria oxide particles were further demonstrated in a hydrogen peroxide-induced oxidative injury model utilizing the adult spinal cord model system. A 100 mM hydrogen peroxide solution was added for 1h to both a control culture and a nano-Ceria treated culture at day 30. After 1h of hydrogen peroxide treatment, the cell viability was assayed using a live-dead assay kit. The nano-Ceria treated cultures had a significantly higher number of live cells (82 ± 18 , $n = 6$) as compared to the control (29 ± 6 , $n = 6$). We did not observe any significant difference in the number of dead cells between nano-Ceria treated (362 ± 73 , $n = 6$) and control (309 ± 44 , $n = 6$) cultures after hydrogen peroxide treatment. This result indicates that the nano-Ceria treated cultures had a significantly higher peroxide detoxification ability (Figure 7) and this may also be a significant indicator of its potential protection abilities after ischemic insult.

Spinal cord neurons and other CNS neurons are prone to damage due to oxidative stress [28, 29] both *in vitro* [30] and *in vivo* [31–33]. To maintain healthy *in vitro* cultures of spinal neurons and other CNS neurons, several antioxidants are generally used in culture medium. The major source of anti-oxidant molecules in serum-free neuron culture medium is the B27 supplement [23,34]. B27 contains five antioxidants; vitamin E, vitamin E acetate, superoxide dismutase, catalase, and glutathione [23]. However, the half-life of these antioxidants is limited and they have to be replenished each time the medium is changed to maintain a healthy culture [34]. In nano-Ceria treated cultures, we observed a significant rise in neuron survival as compared to the control culture, which were supplemented only with B27. The auto-regenerative antioxidant properties of a single dose of the autocatalytic nano-Ceria in this *in vitro* model is the most probable explanation of the significant neuroprotective effect observed in the treated culture.

Conclusions

Based on our cell culture assays, UV-visible spectroscopic studies, hydrogen peroxide-induced oxidative injury assay and our proposed working hypothesis, we conclude that the supplementation of neuronal cultures with a **single dose** of the Ceria nanoparticles has a significant synergistic effect in a realistic model system of spinal cord injury. Future studies will focus on elucidating the biological mechanism of action of the nano-Ceria. The use of nano-Ceria with other antioxidants may, in the future, prove beneficial for the *in vivo* mitigation of ischemic events after spinal cord injury as well as possibly being a new therapeutic agent for oxidation injury in the other neurodegenerative diseases or injury.

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Figure 1. Synthesis and Characterization of Cerium oxide Nanoparticles. (a) Outline of the microemulsion technique to synthesize the nano-Ceria (b) XPS analysis of synthesized Cerium oxide nanoparticles showing the presence of both Ce^{3+} and Ce^{4+} valence states (c) HRTEM image of the synthesized nanoparticles showing uniform particle size in the range of 3–5 nm.

Figure 2. Adult Rat Spinal Cord Culture (a) Isolation of adult rat spinal cord cells from the whole cord (b) Development of serum-free culture medium using various growth factors (c) Surface modification of the glass cover slips for cell culture.

Figure 3.

Live-Dead Assay and Neuron-Glial Cell Assay Studies of Control and nano-Ceria Treated Cultures of Adult Rat Spinal Cord (a) Live-dead cell assays indicated that nano-Ceria treated cultures had significantly higher cell survival and significantly less cell death at day 15 and day 30 in culture as compared to the control cultures (b) Neuron-glial cell assays indicated that a significantly high neuronal survival in treated cultures at day 15 and day 30 as compared to the control cultures. (Data are presented as Mean $\pm$ SE, n = 6; * p < 0.05; ** p < 0.01).

Figure 4. Voltage-clamp recording from a treated culture at day 30 (left) Current clamp recording indicating a single action potential in a nano-Ceria treated culture at day 30 (right).

Figure 5. Schematic Detailing the Proposed Regenerative Properties of nano-Ceria and probable mechanism of Cerium oxide nanoparticles' free radical scavenging property and auto-catalytic behavior.

Figure 6. UV-visible study of Cerium Oxide nanoparticles treated with hydrogen peroxide at different time intervals.

Figure 7. Results After Hydrogen Peroxide-Induced Oxidative Injury in Control and Treated Cultures of Adult Rat Spinal Cord at day 30. Live-dead cell assay after hydrogen peroxide treatment indicates that nano-Ceria treated cultures had a significantly higher number of surviving cells as compared to the control (Data are presented as Mean $\pm$ SE, n = 6; * p < 0.05; ** p < 0.01).