Published in final edited form as:

Angew Chem Int Ed Engl. 2009 ; 48(13): 2308–2312. doi:10.1002/anie.200805279.

Oxidase Activity of Polymer-Coated Cerium Oxide Nanoparticles**

Mr. Atul Asati, Dr. Santimukul Santra, Mr. Charalambos Kaittanis, Dr. Sudip Nath, and Prof. J Manuel Perez

Nanoscience Technology Center, Department of Chemistry, and Burnett College of Biomedical Sciences, University of Central Florida, 12424 Research Parkway, Suite 400, Orlando, FL 32826 (USA), Fax: (+1) 407-882-2843, <http://www.nanoscience.ucf.edu/faculty/perez.php>

J Manuel Perez: jmperez@mail.ucf.edu

Keywords

Polymer coated nanoceria; ELISA; Oxidant; pH-dependent; Oxidase

Unique catalytic activities have been reported in nanoscale materials in recent years.[1] These size-dependent properties, which often times are absent in the bulk materials, are the basis for designing novel catalysts with multiple applications in energy storage, chemical synthesis and biomedical applications.[2,3] Cerium oxide has been extensively used in catalytic converters for automobile exhaust systems, as an ultraviolet absorber and as an electrolyte for fuel cells.[4–7] Most recently, it has been found that nanosized cerium oxide (nanoceria) possesses antioxidant activity at physiological pH and their potential use in biomedical applications such as protection against radiation damage, oxidative stress and inflammation has been reported.[8–12] The ability of these nanoparticles to act as antioxidant resides on their ability to reversibly switch from Ce^{+3} to Ce^{+4} . [11] Furthermore, the synthesis of a biocompatible dextran-coated nanoceria (DNC) preparation and enhanced stability in aqueous solution has been recently reported.[12]

Herein, we report that nanoceria has an intrinsic oxidase activity at acidic pH as it can quickly oxidize a series of organic substrates without any oxidizing agent e. g. hydrogen peroxide. The observed activity is not only pH-dependent, but also dependent on the size of the cerium oxide nanoparticles, as well as the thickness of the polymer coating. Based on these findings, we have designed an immunoassay in which a folate-conjugated cerium oxide nanoparticle provides dual functionality of binding to folate expressing cancer cells and detection via catalytic oxidation of sensitive colorimetric substrates/dyes. The unique pH-dependent oxidase activity of cerium oxide nanoparticles in aqueous media makes them a powerful tool for wide range of potential applications in biotechnology and environmental chemistry.

For our first set of experiments, we investigated if a DNC preparation[12] could facilitate the oxidation of a series of organic dyes at low pH. In these experiments, we selected 3,3', 5,5'-tetramethyl benzidine (TMB) and 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (AzBTS), which upon oxidation develop either a blue (TMB) or green (AzBTS) color in aqueous solution.[13,14] These dyes are typically used as horseradish peroxidase (HRP) substrates in various bioassays and most recently they have been used to demonstrate the

**The authors acknowledge funding by the National Institutes of Health (CA101781) and an UCF-NSTC Start Up Fund all to JMP.

Correspondence to: J Manuel Perez, jmperez@mail.ucf.edu.

peroxidase activity of iron oxide nanoparticles.[15] However, in these peroxidase-catalyzed reactions, hydrogen peroxide (H_2O_2) is required as the electron acceptor or oxidizing agent. In contrast, we have found that DNC catalyzes the fast oxidation (within minutes) of both TMB and AzBTS in the absence of hydrogen peroxide, as judged by the appearance of the characteristic color upon addition of the dyes to citrate-buffered solutions (pH 4.0) of the nanoparticles and by the corresponding UV-visible spectrum (Figure 1a and Supporting Information 1). Meanwhile, at pH 7.0, no significant oxidation of TMB or AzBTS was observed, even in the presence of hydrogen peroxide or upon overnight incubation, as judged by the absence of color development upon addition of nanoceria at this pH (Supporting Information 2). Furthermore, pH-dependent studies of the DNC-catalyzed oxidation of TMB show that as the pH of the buffered solution increases from pH 4.0 to 7.0, the ability of DNC to oxidize the dye decreases (Figure 1b). These results suggest that DNC behaves as an oxidizing agent in a pH-dependent manner, being most optimal at acidic pH.

To further verify the ability of nanoceria to behave as an oxidation nanocatalyst, we chose dopamine (DOPA), a catecholamine difficult to oxidize at low pH.[16] Results showed that DNC facilitated the oxidation of DOPA in citrate within minutes, producing the characteristic orange color corresponding to aminochrome, one of the major oxidation products of DOPA (Figure 1a). The formation of aminochrome by DNC was confirmed by UV-vis studies which show the appearance of the characteristic band at 475 nm (Supporting Information 3). However, in the absence of DNC, no apparent oxidation of DOPA occurs at pH 4.0, even after days of incubation. This contrast to DOPA solutions in water or citrate buffer pH 7.0, where DOPA slowly auto-oxidizes, developing the characteristic reddish-brown color after overnight incubation. Taken together, our results demonstrate that DNC is able to catalyze the oxidation of various organic molecules at acidic pH.

It has been well established that the catalytic properties of nanomaterials often depend upon the size of the nanocrystal.[17] However, studies on the effect of polymer coating thickness surrounding the nanoparticles are less common. This motivated us to study if the nanoceria-catalyzed oxidation of these dyes is also size- and polymer-coating-thickness-dependent. Our previously reported dextran-coated nanoceria (DNC) preparation was synthesized via an in-situ procedure[12], in which the dextran (10 kDa) is present in solution at the time of the initial formation of the cerium oxide nanocrystals. Under these conditions, the polymer influences both the nucleation and growth of the initial nanocrystal, resulting in nanoparticles with a small nanocrystal core surrounded by a thin polymeric coating. In the case of DNC, we have obtained nanoparticles with a cerium oxide core of 4 nm surrounded by a thin coating of dextran for a total nanoparticle size (hydrodynamic diameter) of 14 nm. Meanwhile, a step-wise procedure in which the polymer is added at a specific time after initial formation of the nanocrystals can be adopted for the synthesis of nanoceria. This method has been previously reported for the synthesis of polymer-coated iron oxide nanoparticles, yielding nanoparticles with a thicker polymer coating as compared to the in-situ process.[18] In addition, slightly larger nanocrystals cores are also obtained using this method. Therefore, to study the effect of the polymeric coating thickness on the catalytic activity of nanoceria, we synthesized the DNC nanoparticles using a step-wise method. In this method the dextran polymer was added 60 seconds after initial formation of the nanocrystals, to yield a stepwise DNC (*sw*DNC) nanoparticle preparation with average hydrodynamic diameter of 100 nm, ~10 times bigger than the DNC nanoparticles obtained with the in-situ method (*is*DNC). In addition, another set of polymer-coated nanoceria was synthesized using polyacrylic acid (1.8 kDa). The use of a smaller molecular weight polymer in the synthesis of polymer-coated nanoceria is advantageous because it would allow the formation of nanoparticles with an even thinner coating than those obtained with dextran (10 kDa) using either the in-situ or step-wise method. Dynamic light scattering experiments show that for the in situ polyacrylic-acid-coated nanoceria preparations

(*is*PNC), the average hydrodynamic diameter of the nanoparticles was 5 nm. Meanwhile, for the stepwise preparation (*sw*PNC), a value of 12 nm was obtained (Supporting Information 4). As hypothesized, smaller nanoparticles with a thinner polymer coating were obtained using the 1.8kDa polyacrylic acid polymer. The average zeta potential value (ξ) for the DNC preparation was -0.78 ± 0.4 m, while for PNC was -27.8 ± 2.4 mV.

Next, we used these preparations of nanoceria to perform various kinetic studies and assess the effect of the coating thickness and nanoparticle size on the catalytic activity of nanoceria. Results show that nanoceria's ability to oxidize TMB varies with nanoparticle size in the order *is*PNC (5 nm) > *sw*PNC (12 nm) > *is*DNC (14 nm) > *sw*DNC (100nm). Interestingly, the nanoparticles composed of a thin polyacrylic acid coating (*is*PNC) have a higher catalytic activity than those composed of a thicker dextran coating (*sw*DNC) (Figure 2). This might be attributed to the fact that nanoceria with a thin and permeable polyacrylic acid-coating can facilitate the transfer of molecules in and out of the nanoceria core surface faster than a thicker coating. Similar experiments were performed with AzBTS which show similar behavior to TMB (Supporting Information 5).

Next, we determined the steady state kinetic parameters for the nanoceria-catalyzed oxidation of TMB. Typical Michaelis-Menten curves were obtained for both PNC and DNC (Supporting Information 6 and 7). Results show that as the hydrodynamic diameter of the nanoparticles increases, lower K_m and V_{max} values are obtained (Table 1). Similar results were observed with AzBTS. The fact that the nanoceria preparation with the smaller hydrodynamic diameter and thinner coating (*is*PNC) displays the fastest kinetics (contrary to *sw*DNC) suggests that the thickness of the polymer coating plays a key role in the rate of oxidation of the substrate. Furthermore, kinetic studies of nanoceria (*is*PNC) at various pH indicate faster kinetics at acidic pH (K_m 3.8, V_{max} 0.7) and much slower kinetics at neutral pH (K_m 1.3, V_{max} 0.1) (Table 2) as expected. These results contrast to those obtained using the enzyme HRP or iron oxide nanoparticles where slower kinetics are reported even in the presence of hydrogen peroxide.[15]

The oxidase activity of nanoceria in slightly acidic aqueous solution makes them potentially useful as aqueous redox catalyst and as aqueous oxidants of water pollutants. [19] An immediate and equally important application of this technology is in the design of more robust and reliable TMB-based immunoassays using surface-modified cerium oxide nanoparticles. In traditional ELISA, a horseradish peroxidase (HRP) labeled secondary antibody is utilized to assess the binding of a specific primary antibody to a particular target or surface receptor (Figure 3a). This binding event is assessed by the ability of HRP to oxidize a chromogenic substrate such as TMB in the presence of hydrogen peroxide. In traditional ELISA, high rate of negative results is mainly attributed to (1) the instability of the target, (2) the instability of HRP that when denatured loses its' peroxidase activity or (3) the instability of hydrogen peroxide which upon prolonged storage decomposes and losses its ability to oxidize the substrate TMB in the presence of HRP. We hypothesized that a nanoceria-based detection approach shall be more robust than current HRP-based assays, as no enzyme or hydrogen peroxide would be needed for detection (Figure 3b). The oxidase activity of the nanoceria, by itself, shall facilitate the oxidation and corresponding color development. Using this assay, one can perform an immunoassay and identify the presence and concentration of a target faster and cheaper than using traditional ELISA.

For this purpose, polyacrylic acid coated nanoceria (*is*PNC) was conjugated to folic acid using click chemistry[20–22](Supplementary Scheme 1 and Supporting Information 8). Folic acid is the ligand for the folate receptor, which is overexpressed in many tumors and cancer cell lines.[23,24]We hypothesized that a nanoceria conjugate with folic acid instead of an anti-folate receptor antibody will make a more robust nanoprobe for our immunoassay.

Experiments were performed using the lung cancer cell line (A-549) which over express the folate receptor.[23,24] In control experiments, cardiac myocytes (H9c2) that do not over express the folate receptor were used.[25] In our first set of experiments either A-549 or H9c2 cells (6000 cells) were incubated with increasing amount of folate-cerium oxide nanoparticles in 96-well plate for three hours, followed by incubation with TMB (0.04 mM) for 30 minutes and monitoring of product formation at 652 nm using a microtiter plate reader. As expected, a folate-nanoceria dependent binding was observed for the lung carcinoma cell line (A-549) compared to cardiac myocytes (H9c2) judged by an increase in absorbance at 652 nm with increasing amount of folate-nanoceria (Figure 4). In another set of experiments, increasing number of folate-positive lung carcinoma cells (1500 to 6000 cells) were treated with a constant amount of folate-ceria (5.0 μ M). Results show an increase in TMB oxidation product formation (652 nm absorbance) with increasing number of A549 cells (Figure 5). This was expected as an increasing number of A549 translates into an increasing number of surface folate-receptors available for binding to the folate-nanoceria. These results demonstrate the utility of cerium oxide nanoparticles as a detection tool due to their dual functionality. Nanoceria-based assays outperform the traditional sandwich ELISA, which requires hydrogen peroxide and an additional step to introduce an antibody carrying horseradish peroxidase (HRP-antibody) to allow detection..

In conclusion, we report that nanoceria possess unique oxidase activity as it can facilitate the fast oxidation of organic dyes and small molecules in slightly acidic conditions without the need of hydrogen peroxide. When compared to other systems that require peroxides or proteins (such as oxidases and peroxidases), our polymer coated nanoceria is a more robust and water-soluble redox nanocatalyst, as it is not susceptible to denaturation or decomposition. Furthermore, conjugation with targeting ligands makes nanoceria an effective nanocatalyst and detection tool in immunoassays. Taken together, these results demonstrate that this unique aqueous oxidase activity of nanoceria can be used in a wide range of new potential applications in biotechnology, environmental chemistry and medicine.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Formation of color product due to substrate oxidation is pH dependent. a) Photographs show production of color product upon addition of nanoceria to TMB, AzBTS and DOPA at pH 4.0. b) Oxidation of TMB is pH dependent with the optimum activity at pH 4.0. buffer pH 4.0

Figure 2. Nanoceria-promoted oxidation of TMB is size-dependent. At pH 4.0, smaller size ceria nanoparticles show higher oxidase activity than larger size nanoparticles.

Figure 3. Comparison between traditional ELISA (a) and nanoceria-based ELISA (b). In traditional ELISA, a HRP-antibody is utilized as secondary antibody that upon hydrogen peroxide treatment facilitates the oxidation of TMB, resulting in color development. Meanwhile, in a nanoceria- based ELISA, the oxidase activity of nanoceria facilitates the direct oxidation of TMB without the need of HRP or hydrogen peroxide.

Figure 4. Nanoceria-mediated ELISA detection of folate receptor expressing cells. Folate-nanoceria associating with A-549 cells which overexpress the folate receptor effectively oxidizes TMB.

Figure 5. Nanoceria based immunoassay is sensitive to the number of folate positive cells (folate receptor expressing cells A-549).

Table 1Comparison of nanoceria's size-dependent kinetic parameters^[a]

Nanoceria	Size (nm)	K _m (mM)	V _{max} (μM/s)
<i>is</i> PNC	5	3.8	0.7
<i>sw</i> PNC	12	1.9	0.6
<i>is</i> DNC	14	1.8	0.5
<i>sw</i> DNC	100	0.8	0.3

^[a]Data obtained at pH 4.0

Table 2Nanoceria's oxidase kinetics are pH-dependent^[a]

pH	K _m (mM)	V _{max} (μM/s)
4.0	3.8	0.70
5.0	6.9	0.36
6.0	2.4	0.10
7.0	1.3	0.10

^[a]Data obtained with *is*PNC.