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Highly sensitive and robust peroxidase-like activity of porous nanorods of ceria and their application for breast cancer detection

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ABSTRACT

Porous nanorods of ceria (*PN*-Ceria), a novel ceria nanostructure with a large surface area and a high surface Ce^{3+} fraction, exhibited strong intrinsic peroxidase activity toward a classical peroxidase substrate in the presence of H_2O_2. Peroxidase-like activity of ceria originated from surface Ce^{3+} species as the catalytic center, thereby explaining the high performance of *PN*-Ceria as an artificial enzyme mimicking peroxidase. Compared with the natural enzyme horseradish peroxidase (HRP), *PN*-Ceria showed several advantages such as low cost, easy storage, high sensitivity, and, prominently, chemical and catalytic stability under harsh conditions. Importantly, the enzymatic activity of *PN*-Ceria remained nearly constant and stable over a wide range of temperature and pH values, ensuring the accuracy and reliability of measurements of its peroxidase-like activity. A *PN*-Ceria based novel diagnostic system was developed for breast cancer detection with a higher sensitivity than the standard HRP detection system. Our work has laid a solid foundation for the development of *PN*-Ceria as a novel diagnostic tool for clinical use.

1. Introduction

The construction of efficient artificial enzymes, as a robust and cost-effective alternative to natural enzymes, has been an increasingly important focus in the field of biomimetic chemistry. Artificial enzymes are superior to natural enzymes in that the latter is plagued by intrinsic drawbacks such as unstable catalytic activity, low physical/chemical stability after exposure to extremes of pH and temperature, susceptibility to protein denaturation, and high cost associated with production. To date, many artificial enzymes, including metal complexes, bio-molecules, polymers, supramolecules, and inorganic nanostructures, have been developed as promising alternatives for bio-sensing [1–3], immunoassays [4–6], clinic diagnostics [6–9], neuroprotection [10–14], and disease therapy [15–18].

Among various artificial enzymes, nanomaterial-based products (nanozymes) have received considerable attention over the past few years due to their unique size, shape, and composition as well as structure-dependent properties [15–18]. Since the discovery of Fe_3O_4 nanoparticles with peroxidase-like activity [6], there has been an explosive interest in using various nanomaterials as nanozymes to mimic peroxidase activity, including carbon dots, carbon nanotubes, graphene oxides, TiO_2, Co_3O_4, MnO_2, V_2O_5, CuO, Au and Au/Pt [6,8,19–29]. These studies show that nanomaterial-based peroxidase mimetics are of low cost, simple storage, and good tunability in catalytic activity. Despite the advantages of nanozymes over natural enzymes, the robustness of artificial enzymes in a wide range of temperature and pH media is still unsatisfactory for practical applications [7,28,29]. For example, previous studies have demonstrated the loss or significant reduction in the catalytic activity of the majority of nanozymes at extremes of temperature and pH, which may be required for the survival of targets or measurements [1,7]. Metal oxides (*e. g.* CuO, Co_3O_4) can be chemically etched in low pH reaction media. Surface properties of nanozymes can be changed during their storage under ambient conditions (influenced by humidity, oxygen, *e.g.*). It is still a challenge to develop chemically and catalytically stable nanozymes with highly robust peroxidase-like activity for practical applications.

Ceria, the oxide of the second element in lanthanide group, has the potential to be developed as nanozymes for biomedical applications due to its low toxicity, biocompatibility, and ability to scavenge radicals for the protection against cellular damage [30–35]. The ability to switch reversibly between the redox pair

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Ce^{3+}/Ce^{4+} endows nanoceria with the property of radical scavenging, a function reminiscent of biological antioxidants such as catalase and superoxide dismutase (SOD) [33,36–39]. Although nanoceria has been studied extensively as SOD and catalase mimetics, research on its peroxidase-like activity remains scant.

Herein, we demonstrated a highly active and robust peroxidase mimetic made of porous nanorods of ceria (*PN*-Ceria) and its application in efficient detection of breast cancer cells through a nanostructure based enzyme-linked immunosorbent assay (ELISA). Our studies revealed a relationship between surface properties of nanoceria and its peroxidase-like activity, in which a higher surface Ce^{3+} fraction and larger surface area lead to an improved catalytic activity of ceria. Then, the highest peroxidase-like activity of *PN*-Ceria with the largest surface area and the highest surface Ce^{3+} fraction was observed, compared with other nanoceria. Specifically, the almost constant activity of *PN*-Ceria measured under a wide range of measuring reaction temperature (4–60 °C) or treated at various temperatures (4–200 °C) or in aqueous media with pH value (0–14) ensures the accuracy and reliability (physical/chemical stability) of the measurements. The high chemical and catalytic stability indicate its promise for future practical applications under various conditions.

2. Materials and methods

2.1. Materials

Sodium hydroxide and hydrogen peroxide were obtained from Alfa Aesar. Cerium nitrate, 3,3′,5,5′-Tetramethylbenzidine (TMB), horseradish peroxidase (HRP), acetic acid and sodium acetate were purchased from Sigma–Aldrich. The primary anti-body of goat anti-rabbit CA15-3, the secondary anti-body of rabbit anti-CA15-3 and the antigen of CA15-3 were from Shanghai Anyan Bioreagent Inc (China).

2.2. Synthesis of PN-Ceria

PN-Ceria was prepared through a two-step hydrothermal process [40]. Briefly, 5 mL of 0.8 M cerium nitrate were added to 75 mL of sodium hydroxide aqueous solution (containing 19.2 g sodium hydroxide) under vigorous stirring. After aging for 30 min at room temperature, the mixture was hydrothermally treated in a Pyrex bottle at 100 °C for 24 h. The products were centrifuged off, washed with ethanol and water alternatively for three times and dried at 60 °C overnight. Then, the obtained nanostructures were re-dispersed in water with a concentration of 1.0 mg/mL and further hydrothermally treated in a Teflon-lined autoclave at 160 °C for 12 h. The light yellow precipitates were centrifuged off and dried at 60 °C to obtain *PN*-Ceria.

2.3. Synthesis of other ceria nanostructures

Ceria nanoparticles were obtained by calcining cerium nitrate at 500 °C for 2 h. Ceria cubes were synthesized *via* a hydrothermal method. Briefly, 0.868 g of cerium nitrate and 9.8 g of sodium hydroxide were dissolved in 5 and 35 mL of deionized water, respectively. After aging of the mixture solutions of cerium nitrate and sodium hydroxide for 30 min, the reaction was subjected to hydrothermal treatment at 180 °C for 24 h. The cubes were collected by centrifugation after thoroughly washing with copious amount of water. The synthesis of non-porous ceria nanorods was similar to that of ceria cubes, but at a low temperature of 100 °C. Ceria octahedrons were prepared by hydrothermal process, in which the mixture of cerium nitrate (4.3 mg) and sodium phosphate (1.6 mg) in 40 mL of deionized water was transferred into the Teflon-lined autoclave and maintained at 170 °C for 12 h. The precipitates were centrifuged off, washed with ethanol for twice, dried at 60 °C and then calcinated at 400 °C for 4 h [35,41–43].

2.4. Characterization

The transmission electron microscopy (TEM) images were taken by a Hitachi HT-7700 field-emission transmission electron microscope with an accelerating voltage of 120 kV. X-ray diffraction (XRD) measurements were performed on a Shimadzu X-ray diffractometer (Model 6000) at a scanning rate of 10°/min in the 2θ range from 10 to 90°, with CuKα radiation. The oxidation states of nanoceria were analyzed by X-ray photoelectron spectroscopy (XPS), a Thermo Electron Model K-Alpha with AlKα as the excitation source. Nitrogen adsorption and desorption measurements were performed on ASAP 2020 HD88 (Micromeritics). The samples were degassed at 200 °C under vacuum to ensure a clean dry surface. The surface areas were calculated by the Brunauer–Emmett–Teller (BET) method.

2.5. Peroxidase mimetics activity of nanoceria

The reaction kinetics measurements were carried out in time course mode by monitoring the absorbance change at 652 nm with a 10-min interval using a Perkin Elmer UV spectrophotometer. The experiments were carried out at various conditions in 1.0 mL reaction buffer (0.2 mol L^{-1} CH_3COONa, pH = 4.0) with TMB as substrate and H_2O_2 (100 mM) as the oxidant. The various forms of nanoceria (200 ng) were used as catalysts with reference to the use of 1.0 ng HRP in otherwise identical reactions.

The Michaelis–Menten constant was calculated according to the Michaelis–Menten eqn: $V = (V_{max} \times [S]/K_m + [S])$where V is the initial rate, V_{max} is the maximal reaction rate, [S] is the concentration of substrate and K_m is the Michaelis constant. The details of calculation can be found in Supporting Information.

2.6. Immunoassay protocol and detection

The second antibodies (rabbit anti-CA15-3) were immobilized directly on the *PN*-Ceria surface to obtain surface modified *PN*-Ceria. Typically, 10 mg of *PN*-Ceria were dispersed in 10.0 mL of 100 ng/mL second anti-body (Tris–HCl buffer, 10 mM, pH 9.0) and sonicated for 10 min at room temperature. After incubation at 37°C for 2 h, 100 μL of 5% BSA (diluted in PBS, pH 7.0) was added for another 30 min to prevent non-specific interaction. Finally, surface modified *PN*-Ceria was centrifugated off, re-dispersed into Tris–HCl buffer (10 mM, pH 7.4, 150 mM NaCl) with a concentration of 100 ng/mL and was stored at 4 °C for future use.

ELISA detection of breast cancer was performed in 96-well polystyrene plates. Each well of the 96-well plates was coated with 100 μl of primary anti-body (goat anti-rabbit CA15-3) at a concentration of 1.0 μg/mL and incubated at 4 °C for 6 h. The unbound anti-body was removed from the plate by thoroughly washing with PBST buffer (pH 7.4). After that, each well was blocked with 5% BSA (diluted in PBS, pH 7.0) for 1 h at 37 °C to avoid the non-specific interaction with plate surface. The CA15-3 antigen (a well-known breast cancer biomarker) was used as model antigen. The aqueous solution of CA15-3 antigen in PBST buffer (pH 7.4) with the concentrations from 10^5 to 10^{-3} ng/mL was added to plate and incubated at 37 °C for 1 h. After removal of unbound antigen, 100 ng/mL surface modified *PN*-Ceria was added in the well and incubated for 1 h at 37°C. The plates were washed three times with PBST buffer (pH 7.4) to remove the unbound anti-body modified *PN*-Ceria. The color development was initiated by adding the TMB/H_2O_2 mixture solution (NaAc buffer, pH 4.0). After 10 min