Surface Chemistry

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Acquired Superoxide-Scavenging Ability of Ceria Nanoparticles**

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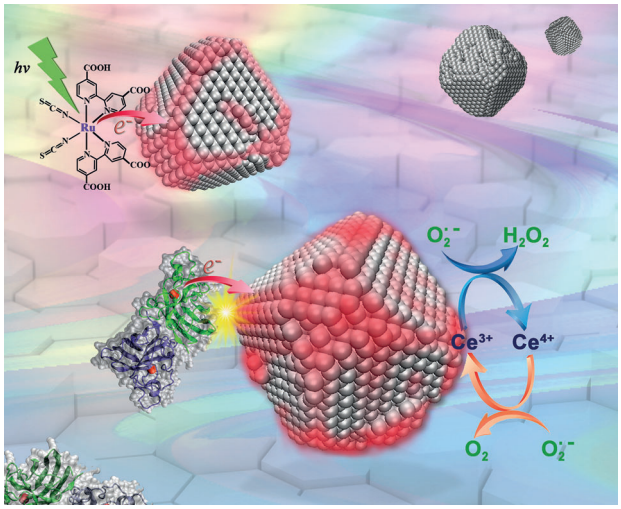
Abstract: *Ceria nanoparticles (nanoceria) are well known as a superoxide scavenger. However, inherent superoxide-scavenging ability has only been found in the nanoceria with sizes of less than 5 nm and with very limited shape diversity. Reported herein is a strategy to significantly improve the superoxide-scavenging activity of nanoceria sized at greater than 5 nm. The nanoceria with sizes of greater than 5 nm, with different shapes, and with a negligible Ce^{3+}/Ce^{4+} ratio can acquire remarkable superoxide-scavenging abilities through electron transfer. This method will make it possible to develop nanoceria-based superoxide-scavengers with long-acting activity and tailorable characteristics.*

Ceria nanoparticles are well known as high-performance catalysts because of the presence of the mixed valence states of cerium (Ce^{3+} and Ce^{4+}) and the presence of oxygen vacancies.[1] By coupling with the redox cycle between Ce^{4+} and Ce^{3+}, nanoceria scavenge superoxide radicals as a superoxide dismutase (SOD) mimetic.[2] Previous work has suggested that the SOD mimetic activity of nanoceria is determined by the fractions of Ce^{3+} on the particle surface.[1,2c] Nanoceria with sizes of greater than 5 nm have lower Ce^{3+}/Ce^{4+} ratios, and therefore exhibit negligible activity in comparison to smaller nanoceria.[2c,3] So far, inherent superoxide-scavenging ability has only been found in the nanoceria with sizes of less than 5 nm, and these bioactive nanoceria show very limited diversity with respect to shape.

The present study proposes a strategy to significantly improve the superoxide-scavenging activity of nanoceria sized at above 5 nm (Scheme 1). Our study began with



Scheme 1. Nanoceria acquire superoxide-scavenging ability after electron transfer.

a surprising observation: one of our nanoceria, having a size of (5.1 ± 0.4) nm, exhibited a negligible Ce^{3+}/Ce^{4+} ratio and low SOD mimetic activity (see Figures S1 and S2 in the Supporting Information), and exerted an antioxidant effect much stronger than that expected in human bronchial epithelial (16HBE) cells (details of the experiment are provided in the Supporting Information). After a 24 hour co-culture of cells with nanoceria at particle concentrations of 0.33–660 nM, dose-dependent decreases in the intracellular reactive oxygen species (ROS) and cell loss were observed (Figure 1 a,b). For each treatment, roughly 10% of the given nanoceria were taken up by cells (see Table S1) and resulted in increased SOD activity. To exclude possible cellular responses to nanoceria (e.g., enhanced SOD expression), untreated cells were first lysed and then incubated with 0.33–66 nM nanoceria for 1 hour. Dramatic increases in SOD activity were observed in the lysates after the incubation (Figure 1 c). However, if ATN-224 (a CuZn-SOD inhibitor) was added into the lysates before the 1 hour incubation with nanoceria, the increases in SOD activity were greatly diminished (see Figure S3).

We narrowed our study to focus on the interactions between native CuZn-SOD and nanoceria. Electron-spin

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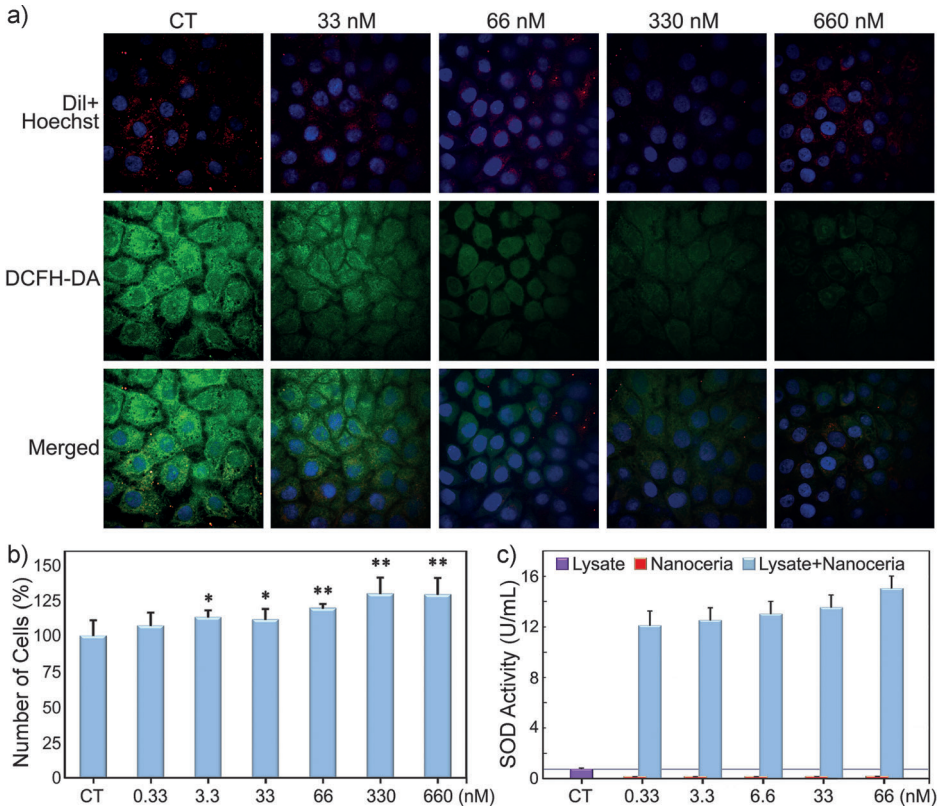

Figure 1. The antioxidant role of nanoceria in human bronchial epithelial (16HBE) cells. Cells were exposed to nanoceria at particle concentrations of 0.33-660 nM for 24 h. a) ROS measurement in cells using DCFH-DA staining. The cells were also stained with DiI (a lipophilic probe) and Hoechst 33258 (a nucleus probe). Each image covers a 100 μm by 100 μm area. b) Number of 16HBE cells after nanoceria exposure. The results are expressed as the mean ± S.D. ($n=6$; $^{*}P<0.05$, $^{**}P<0.01$ versus the control). Statistical analyses were done by one-way ANOVA and Tukey's test for post hoc analysis. c) SOD activity measurement in cell lysates after the lysates were incubated with nanoceria for 1 h. The results are expressed as the mean ± S.D. ($n=6$)

resonance (ESR) spectroscopy revealed an enhanced SOD activity immediately after 33 nM of nanoceria was mixed with 20 U mL^{-1} of CuZn-SOD in PBS (0.01M, pH 7.4; Figure 2a). Preincubation of nanoceria with native SOD resulted in an even higher level of SOD activity in the mixture (Figure 2a). Similar results were found by using a ferricytochrome C reduction assay:[4] preincubation of 0.033 nM nanoceria with 1 U mL^{-1} of CuZn-SOD (about 7 nM) in PBS improved the SOD activity of a mixture by 6- to 12-fold compared to native SOD alone (Figure 2b,c). We also found that the mixing and preincubation of nanoceria with inactivated CuZn-SOD in a PBS solution could not promote the SOD activity of the mixture. This observation led us to speculate that the SOD mimetic activity of nanoceria was activated by the redox-active SOD, or that nanoceria could be acting as a coenzyme to promote the activity of CuZn-SOD.

To reveal the respective roles of nanoceria and CuZn-SOD in superoxide scavenging, fresh CuZn-SOD was pre-incubated with nanoceria for 15 minutes, and then inactivated by rapid heating. Immediately after the heating, the residual SOD activity of the mixture was greater than 5.0 U mL^{-1} (Figure 2c). This result implies that the SOD mimetic activity of nanoceria, rather than the activity of the native SOD enzyme itself, is being enhanced during the 15 minute preincubation. This result also suggests that the activity of the mixture would gradually decrease to a negligible level after the inactivation of CuZn-SOD.

We also determined the dynamic changes in the SOD mimetic activity of the CuZn-SOD/nanoceria mixture over a 24 hour period. Those results showed a rapid increase in the superoxide-scavenging activity immediately after the mixing of CuZn-SOD and nanoceria, and, 3 hours later, a slow decline results from the gradual inactivation of CuZn-SOD in PBS at 37 °C (Figure 3a; see Figure S4). However, if fresh CuZn-SOD was added periodically, the nanoceria could repeatedly acquire superoxide-scavenging abilities (Figure 3b). Therefore, we speculate that the nanoceria could become a long-acting superoxide scavenger in the cytosol, where active CuZn-SOD enzyme is routinely present.

By what mechanism did nanoceria acquire this ability to scavenge superoxides? Previous work has suggested that the Ce^{3+}/Ce^{4+} ratio determines the SOD mimetic activity of nanoceria;[2c,5] therefore we speculate that the redox-active CuZn-SOD could induce the reduction of Ce^{4+} to Ce^{3+}. CuZn-SOD can accelerate the spontaneous reaction of dismutation of superoxide by a cyclic oxidation/reduction of the Cu ion.[6] During the interactions between CuZn-SOD and nanoceria, there might be a certain probability of electron transfer from the enzyme to nanoceria coupled with the reduction of Cu^{II}. Nanoceria can act as an electron sponge,[7] thus storing electrons and thereby regenerating the reactive sites necessary for superoxide scavenging.

To validate our hypothesis, we tested whether the nanoceria could acquire superoxide-scavenging ability after the electron transfer from other electron donors. One of the electron donors we tested was $[Ru(dcbpy)_2(NCS)_2]$ (abbreviated as RuN_3), a well-known sensitizing dye which exhibits ultrafast electron-donating capacity energized by visible light. Our results showed that the mimetic SOD activity of the mixture of 0.033 nM nanoceria and 33 nM RuN_3 would rise to 15 U mL^{-1} under visible-light irradiation (Figure 4). After turning off the light, we found the mixture exhibited a time-dependent decrease in the SOD activity. These results confirm that nanoceria acquire their superoxide-scavenging ability after interfacial electron transfer.

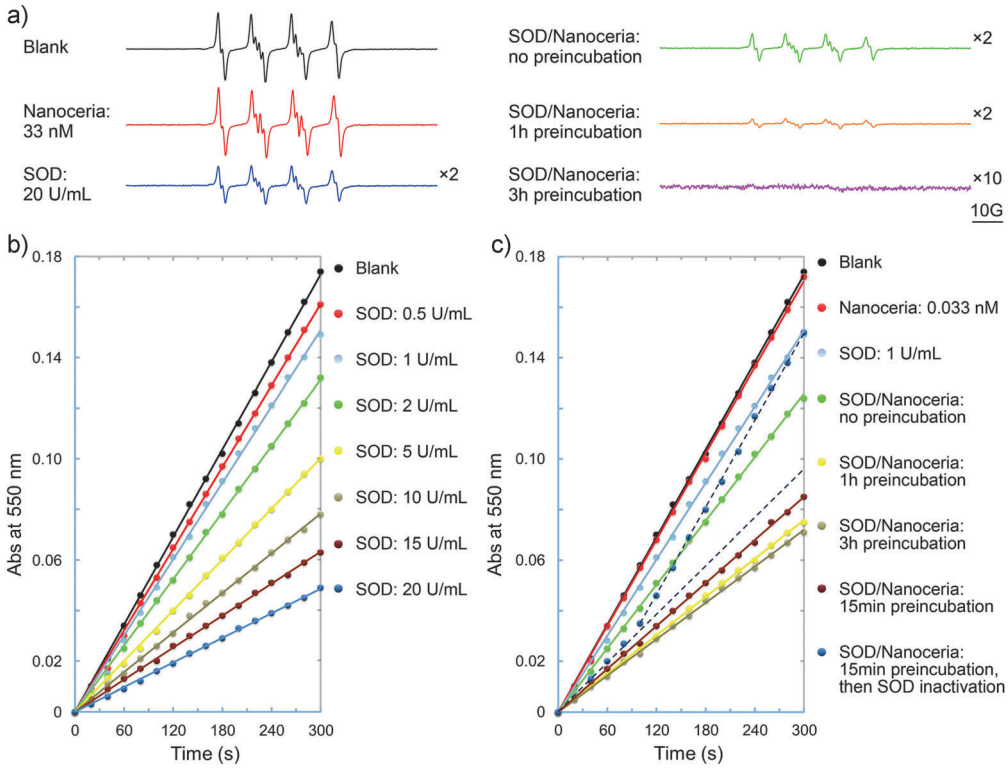

Figure 2. The measurement of SOD mimetic activity. a) Effect of SOD/nanoceria on superoxide anions from KO_2 determined by ESR measurement. The concentrations of CuZn-SOD and nanoceria used were 20 U mL^{-1} and 33 nM, respectively. All ESR measurements were carried out using the following settings: 10 mW microwave power; 100 G scan range and 1 G field modulation; and 40 s scan time. The ESR spectra were recorded 1.5 min after addition of KO_2. Some of the peak signals are shown in a magnified scale at ×2 and ×10. b) Effect of native CuZn-SOD on superoxide anions from hypoxanthine/xanthine oxidase system as determined by a ferricytochrome C assay. c) Effect of CuZn-SOD/nanoceria on superoxide anions from hypoxanthine/xanthine oxidase system as determined by ferricytochrome C assay. The concentrations of CuZn-SOD and nanoceria used were 1 U mL^{-1} and 0.033 nM, respectively.

In a subsequent study, using other shapes of nanoceria and with sizes greater than 5 nm, we proved that even the octahedral nanoceria sized at 25 nm and the rod-shaped nanoceria [width: (8.5 ± 1.3) nm, length: (114.8 ± 39.4) nm] can efficiently remove superoxide radicals after interfacial electron transfer (see Figure S5). We succeeded in improving the superoxide-scavenging activity of the nanoceria, and obtained octahedral and rod-shaped bioactive nanoceria. Our method will make it easier to tailor the properties of bioactive nanoceria for the purpose of regulating their transport and metabolism in organisms.

Our improved understanding of nanoceria may provide a way to avoid the inactivation of nanoceria under physiological conditions. We acknowledge that the stability of Ce^{3+} at room temperature and

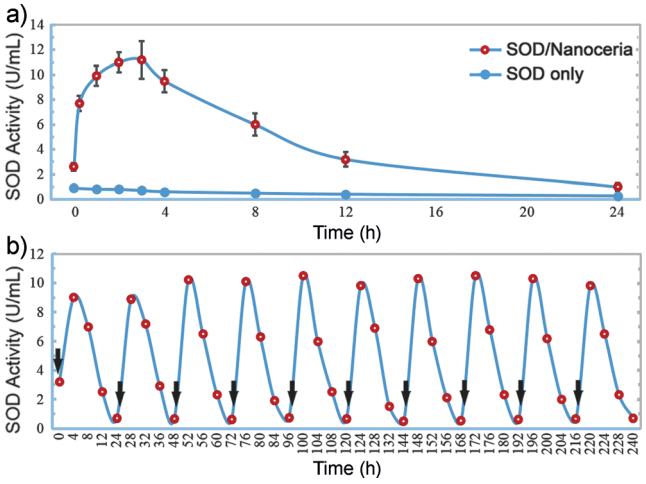

Figure 3. The SOD mimetic activity of the CuZn-SOD/nanoceria mixture. a) The SOD mimetic activity after the mixing of CuZn-SOD (final concentration of 1 U mL^{-1}) and nanoceria (final concentration of 0.033 nM) in a 24 h period. b) The periodic changes in the SOD mimetic activity of the CuZn-SOD/nanoceria mixture. The initial concentration of nanoceria was 0.033 nM and the black arrow represents the time at which fresh CuZn-SOD was added to reach a final enzymatic concentration of 1 U mL^{-1}.

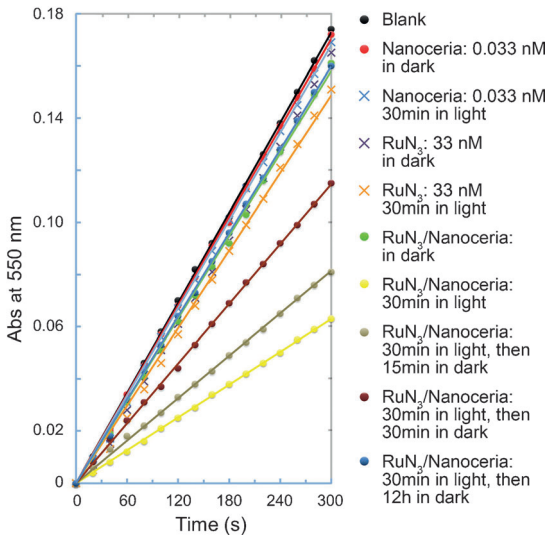

Figure 4. Effect of RuN_3/nanoceria on superoxide anions from hypoxanthine/xanthine oxidase system as determined by a ferricytochrome C assay. The concentrations of RuN_3 and nanoceria used were 33 nM and 0.033 nM, respectively.

aerobic conditions remains controversial.[8] Without the coating of surface ligands to stabilize the oxygen vacancies, nanoceria sized at greater than 3 nm could not maintain a substantially higher Ce^{3+}/Ce^{4+} ratio under ambient conditions when compared to their bulk counterpart.[8a] Therefore, even nanoceria sized at less than 5 nm would lose their inherent SOD mimetic activity because of Ce^{3+} oxidation, and the time required to regenerate that activity usually takes days and weeks.[4,9] In addition, the abundance of phosphate in biological systems may greatly decrease the activity of nanoceria because of the binding of phosphate anions to cerium.[10] But in the present work, the SOD mimetic activity of nanoceria was activated within minutes by incubation with CuZn-SOD or RuN_3 in PBS. The activity of nanoceria could be regenerated again and again, or maintained for a long time when there was continuous electron transfer. By establishing a strategy by which nanoceria of diverse sizes, shapes, and surface chemistries can acquire remarkable superoxide-scavenging abilities, we open the door to more practical and attractive biomedical applications for these nanoparticles.

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