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Electronic Supplementary Information

Apoferritin-CeO_2 Nanocomposite with Super ROS-Scavenging Activity

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Experimental Section

Apoferritin used in this work was prepared from ferritin by a reductive dissolution procedure.[1] Ferritin was obtained according to the methods described previously.[2, 3] The standard ferritin used in native PAGE assay was purchased from Sigma.

Preparation of nano-CeO_2: $(NH_4)_2Ce(NO_3)_6$ (2.74 g) and CH_3COONa (10 g) were dissolved in deionized water (70 ml) and then CH_3COOH (10 ml) was added to the solution. After stirring at room temperature for 1 h, the mixture was transferred to a Teflon-lined autoclave for hydrothermal treatment at 220 °C for 12 h. Finally, yellow precipitates were separated by centrifugation (6,000 g) for 10 min, washed with deionized water and ethanol several times, followed by drying at 60 °C in air overnight. The TEM image shows that the obtained particles are nanocubes with size at around 4.5 nm.

Preparation of AFt-CeO_2: Aqueous solution of apoferritin (5 ml, 18 μM) was mixed with nano-CeO_2 particles with a molar ratio of 1:5000 (protein/cerium). The pH value of the mixture was adjusted slowly to 2.0 with 1 M HCl, followed by continuously stirring at this pH for 30 min. Then, the pH value was slowly adjusted to 8.5 with 1 M NaOH. The resulting solution was stirred for 2 h at room temperature, subsequently centrifuged (12,500 g) at 4 °C for 20 min. After centrifugation, the supernatant was chromatographed with Sephadex G-25, and then concentrated. The cerium concentration of as-prepared AFt-CeO_2 was determined by ICP-OES (Perkin-Elmer Optima 5300DV), while the protein concentration was determined by Bradford method.

TEM characterization and PAGE analysis: For TEM observation, AFt-CeO_2 solution was dropped onto a carbon-coated copper grid and dried in air at room temperature, followed by negatively staining with phosphotungstic acid. TEM images were obtained using an FEI Tecnai G^2 Spirit microscope operating at an accelerating voltage of 120 kV. For HRTEM analysis, AFt-CeO_2 solution was dropped onto a copper grid covered with a thin carbon film

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and dried in air at room temperature. Lattice images and energy dispersive spectrum were recorded with an FEI Tecnai G^2 F30 S-TWIN microscope operating at an accelerating voltage of 300 kV. Native PAGE analysis of AFt-CeO_2, along with apoferritin and commercial ferritin was performed on a Bio-Rad electrophoresis system (Mini-PROTEAN 3 Cell) using 4% stacking gel and 6% resolving gel. After electrophoresis, the gel was stained overnight with 0.25% Coomassie Brilliant Blue R-250, followed by destaining with an aqueous solution containing 7% (v/v) acetic acid and 5% (v/v) methanol. After imaging, the blue protein bands on the gel were separately cut off, digested,[4] and analyzed with ICP-OES for cerium content.

EELS analysis: Samples were prepared following the same procedure as that of HRTEM. Spectra were acquired with a Gatan EELS spectrometer (776 Enfina) attached to a JEOL 2010F field emission gun (FEG) TEM operated at an accelerating voltage of 200 kV. The energy resolution at the zero-loss peak was 0.7 eV. The spectra were acquired with convergence and collection angles of 2 and 5 mrad, respectively. A 2 s acquisition time was used to limit the irradiation effect.

SOD mimetic activity assay: The superoxide anions ($O_2^{\cdot -}$) scavenging activity was assessed with a commercial SOD assay kit (Dojindo Laboratories, Japan). Briefly, a sample solution (20 μL) of SOD (Sigma), AFt-CeO_2, nano-CeO_2, apoferritin or "AFt + CeO_2" was mixed with WST-1 working solution (200 μL). With addition of xanthine oxidase solution (20 μL), the reaction was initiated and proceeded at 37 °C for 20 min. Subsequently, the absorbance of the final solution at 450 nm was determined with Infinite M200 microplate reader (Tecan). Deionized water (20 μL) was used as a vehicle control and the amount of scavenged $O_2^{\cdot -}$ was expressed as percentage of the total $O_2^{\cdot -}$ produced in the control. Each experiment was performed thrice. Results are given as mean ± s.d. (n = 3).

Cell culture: Human hepatoma cell line HepG2 (supplied by ATCC) was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) newborn bovine serum (NBS) and 1% penicillin-streptomycin in a humidified incubator at 37 °C, 5% CO_2.

Quantification of intracellular AFt-CeO_2 and nano-CeO_2: HepG2 cells were initially grown to sub-confluence in cell culture dishes (3.5 cm diameter), and then co-incubated with 100 μM AFt-CeO_2 or nano-CeO_2 in 2 ml culture medium. After 12 h, the cells were extensively washed with PBS (pH 7.4) to remove all extracellular nanoparticles and digested with 1 ml nitric acid (16 M) for 24 h. Finally, the digested solution was diluted to 5 ml with deionized water, and the cerium concentration was determined with ICP-OES. Each experiment was performed thrice. Results are given as mean ± s.d. (n = 3).

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CLSM imaging: HepG2 cells were seeded in a Petri dish and grown to sub-confluence. AFt-CeO_2 or nano-CeO_2 was then added to the dish to a final cerium concentration of 100 μM. After 12 h incubation, the cells were washed three times with PBS (pH 7.4), then fixed in 3.7% paraformaldehyde for 30 min. Rhodamine-phalloidin (Invitrogen) was used to label microfilaments, while intracellular nanoparticles were observed by their reflected signal.[5] The corresponding images were taken by CLSM (SP5, Leica).

TEM observation: HepG2 cells were initially grown to sub-confluence, then co-incubated with 100 μM AFt-CeO_2 or nano-CeO_2 for 12 h. After incubation, the cells were rinsed with PBS (pH 7.4) and fixed using 2.5% glutaraldehyde at room temperature for 1 h. Subsequently, cells were post-fixed, treated with osmium, serially dehydrated with ethanol, and embedded in Epon. Finally, ultrathin sections were obtained with a Reichert Ultramicrotome (Leica) and mounted on 200-mesh copper grids. The samples were observed using a JEM-1400 (JEOL, Japan) electron microscope operating at a voltage of 80 kV.

Antioxidant experiment in vitro: HepG2 cells were seeded into six-well plates and grown to sub-confluence. Subsequently, the cells were incubated with nano-CeO_2 or AFt-CeO_2 for 12 h, at different cerium concentrations ranging from 0 to 200 μM. After incubation, the cells were rinsed three times with PBS (pH 7.4), then exposed to 600 μM H_2O_2 (Sigma) in culture medium for 12 h at 37 °C. Afterwards, the cells were rinsed again, followed by incubation with 20 μM DCFH-DA (Beyotime) at 37 °C for 30 min. At the end of incubation, the cells were washed thoroughly, and then harvested. The fluorescent intensity was analyzed with a CyAn flow cytometer (Dako), and data were acquired from 15,000 cells per sample. Cells without nano-CeO_2 or AFt-CeO_2 treatment before exposure to H_2O_2 were used as control (100%). The relative fluorescent intensity was expressed as a ratio of mean intensity of the experimental cells and the control cells. CLSM was also employed to study the intracellular antioxidant activity of nano-CeO_2 and AFt-CeO_2. HepG2 cells were treated following the same procedures as just mentioned above only except that the cerium concentration of nano-CeO_2 and AFt-CeO_2 were fixed at 25 μM. After incubation with DCFH-DA and subsequent washing, the cells were subjected to CLSM observation. The intracellular ROS level in each sample was reflected by the fluorescent intensity of the images. Each experiment was performed thrice. Results are given as mean ± s.d. (n = 3).

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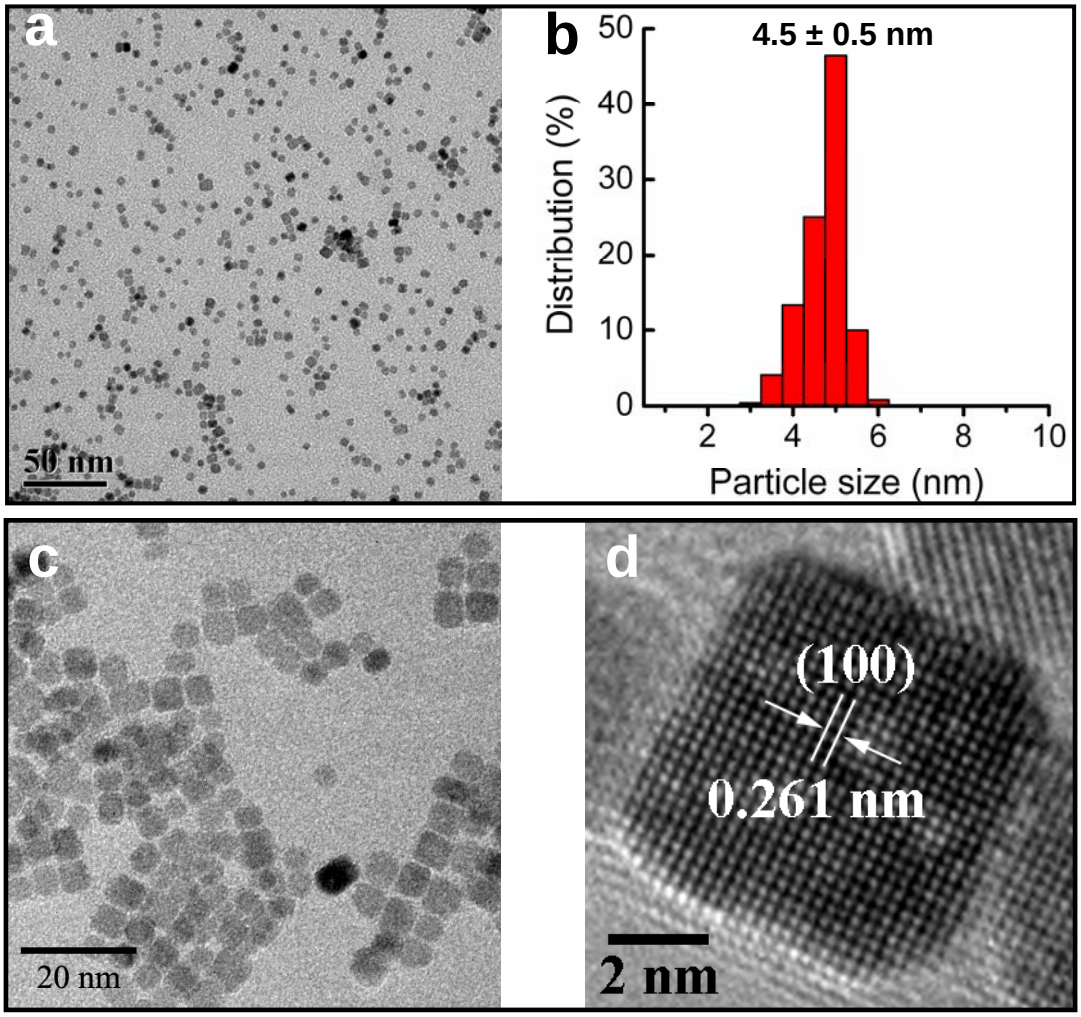

Fig. S1 TEM images of nano-CeO_2 particles (a, c) and the size distribution (b). (d) is the HRTEM image of a single nano-CeO_2 particle. It is clear from those pictures that the average size of the nano-CeO_2 particles is 4.5 nm, while the particles have a cubic structure with all the faces being (100) facet. It is reported that the (100) terminated ceria surface is more reactive and catalytically important than (111) and (110) surfaces.[6-9]

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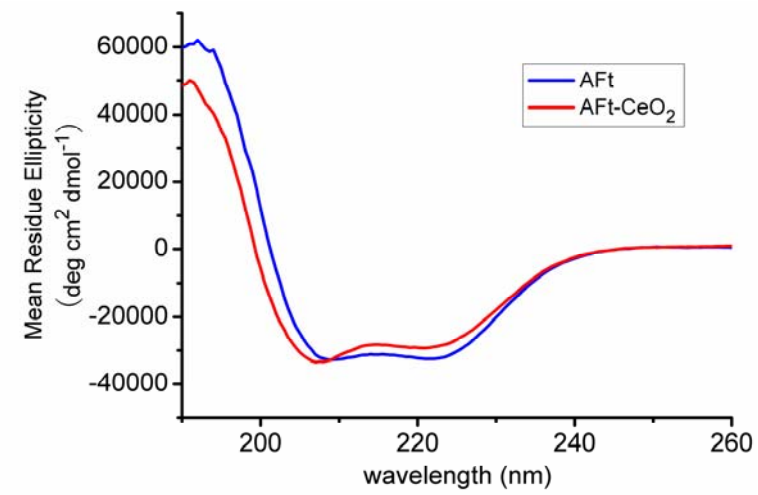

Fig. S2 CD spectra of AFt and AFt-CeO_2. CD spectra were recorded with a MOS-450 AF/CD multi-purpose spectrophotometer (Bio-Logic, France) in a quartz cuvette (1 mm) at room temperature.

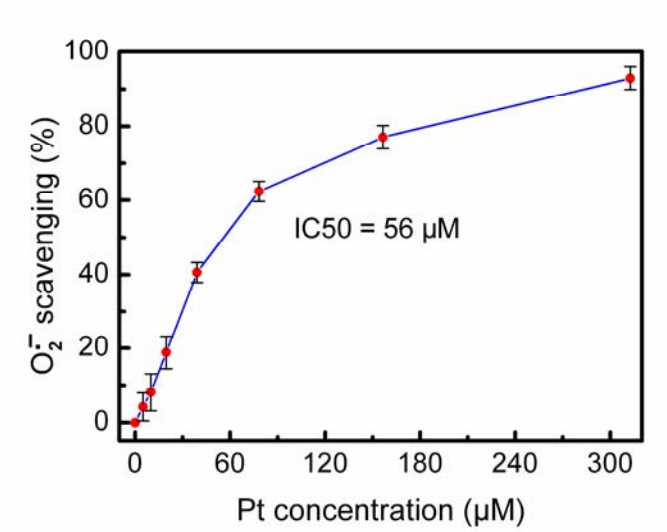

Fig. S3 Dose-dependent $O_2^{\cdot-}$ scavenging by AFt-Pt analyzed with the same SOD assay kit as used in the analysis of $O_2^{\cdot-}$ scavenging by AFt-CeO_2. Results are given as mean ± s.d. (n = 3).

AFt-Pt in the manuscript was used only as a control to illustrate that apoferritin-encapsulated non-noble metal oxide (i.e. AFt-CeO_2) possessed a much better ROS-scavenging activity than apoferritin-encapsulated noble metal nanoparticle (AFt-Pt), although it is generally considered that the redox activity of noble metal Pt nanoparticles is very high. This control not only demonstrated the superiority of AFt-CeO_2 in ROS-scavenging but also showed the better feasibility of AFt-CeO_2 as CeO_2 was much cheaper than platinum.

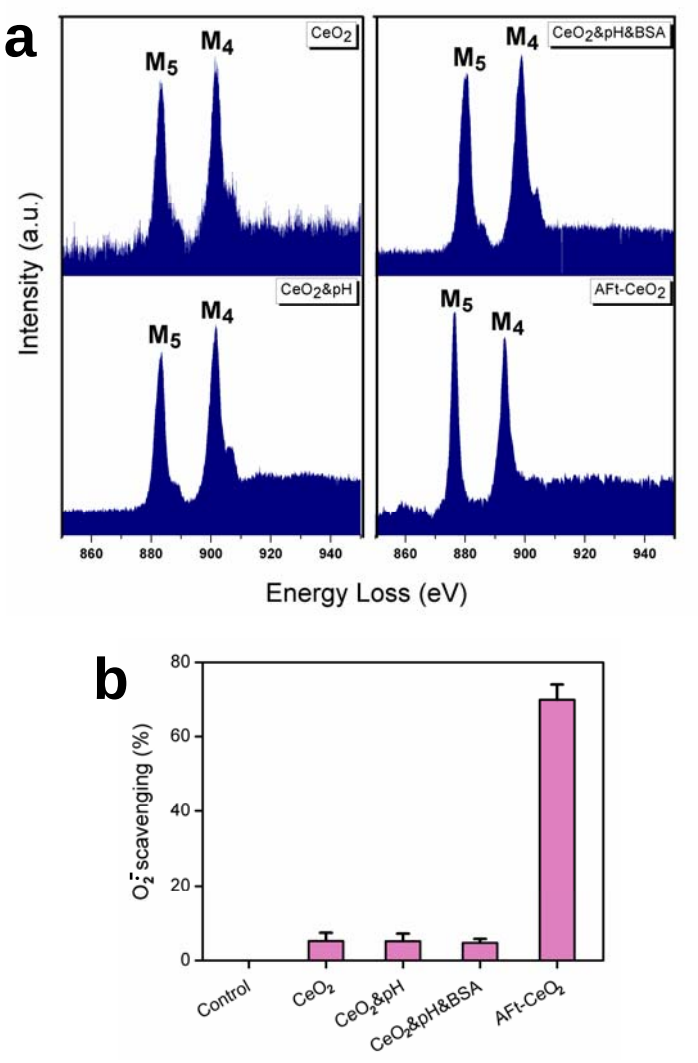

Fig. S4 (a) EELS profiles of pristine CeO_2, CeO_2&pH, CeO_2&pH&BSA, and AFt-CeO_2. (b) Comparison of $O_2^{\cdot-}$ scavenging activity of pristine CeO_2, CeO_2&pH, CeO_2&pH&BSA, and AFt-CeO_2.

To check whether the pH value adjustment will change the valence state and ROS-scaveging activity of nano-CeO_2, we carried out two control experiments. One is that pristine nano-CeO_2 particles were treated under the same conditions as in the preparation of AFt-CeO_2 but without using apoferritin (termed as "CeO_2&pH"). The other is that nano-CeO_2 particles were treated under the same conditions used in the preparation of AFt-CeO_2 except that apoferritin was replaced by BSA (termed as "CeO_2&pH&BSA"). Subsequently, the valence states of the cerium in "CeO_2&pH" and "CeO_2&pH&BSA" were analyzed with EELS. EELS results showed that the Ce valance states in the two control samples were both + 4, which demonstrated that the valance state change of AFt-CeO_2 was not due to the pH value adjustment but the interaction (charge-transfer effect) between nano-CeO_2 and outside apoferritin. Moreover, as shown in Fig. S4 b, "CeO_2", "CeO_2&pH" and "CeO_2&pH&BSA" all showed very little ROS scavenging activity, nothing comparable to AFt-CeO_2.

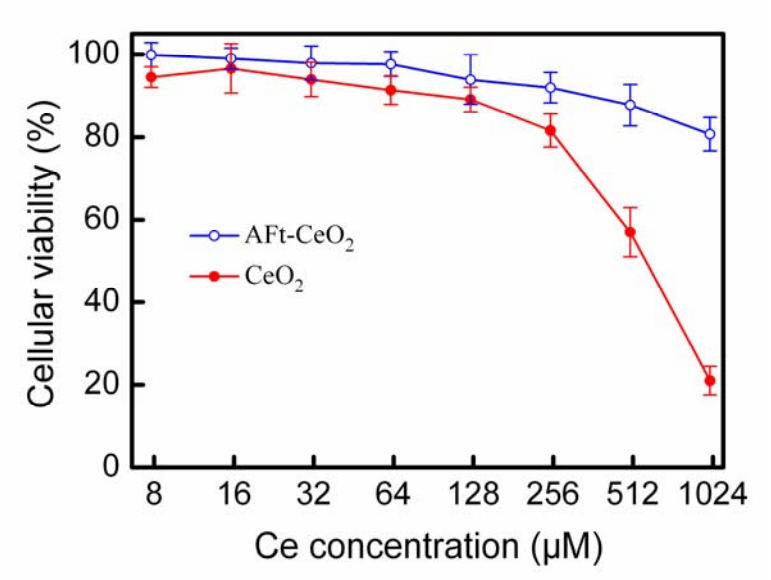

Fig. S5 Cytotoxicity analysis of AFt-CeO_2 and nano-CeO_2 to HepG2 cells. Results are given as mean ± s.d. (n = 3). A Neutral Red Cell Proliferation and Cytotoxicity Assay Kit (Beyotime, China) was employed to evaluate the toxicity of AFt-CeO_2 and nano-CeO_2 according to the manufacturer's instructions. Briefly, HepG2 cells were seeded into 96-well plate and grown to sub-confluence. Then serial dilutions of AFt-CeO_2 or nano-CeO_2 were added into the wells. The final concentration of cerium in each well ranged from 0 to 1000 μM. After 24 h incubation, the cells were stained with neutral red solution for 2 h, followed by rinsing twice with PBS, and then lysed for 10 min at the room temperature. The absorbance of the final cell lysates at 540 nm was recorded by microplate reader (Infinite M200, Tecan). The cellular viability in each well was expressed as percentage of control cells.

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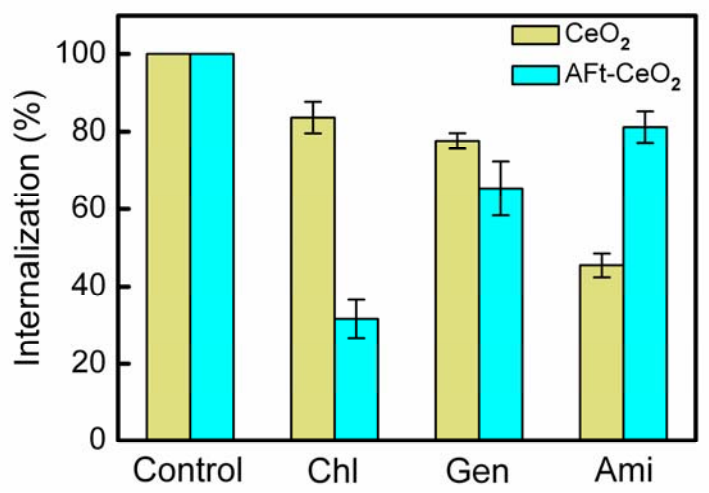

Fig. S6 Evaluation of internalization pathways of AFt-CeO_2 and nano-CeO_2 in HepG2 cells. To study the uptake pathways of AFt-CeO_2 and nano-CeO_2 in HepG2 cells, the cells were initially grown to sub-confluence and then preincubated with Chl (30 μM, Merck), Gen (200 μM, Merck) and Ami (330 μM, Merck) for 30 min separately, prior to subsequent co-incubation with 100 μM AFt-CeO_2 or nano-CeO_2 for 12 h. Chl, Gen and Ami were known as the specific inhibitors of clathrin-mediated endocytosis, caveolae and macropinocytosis, respectively.[10] After co-incubation, the cells were extensively washed with PBS (pH7.4) to remove all extracellular nanoparticles and digested with 1 ml nitric acid (16 M) for 24 h. Finally, the digested solution was diluted to 5 ml with deionized water, and the cerium concentration was determined with ICP-OES (Perkin-Elmer Optima 5300DV). The data were expressed as percent internalization relative to the controls which were in the absence of inhibitors (Chl, Gen, Ami). A marked decrease (about 70% compared to the control) of the intracellular AFt-CeO_2 was observed after preincubation of the cells with Chl. Preincubation with Gen or Ami also resulted in slight decreases by 35% and 19%, respectively. As for nano-CeO_2, the internalized amount was greatly reduced to 45% by preincubating the cells with Ami, whereas preincubating with Gen and Chl only reduced the internalized amount to 78% and 84%, respectively. Results are given as mean ± s.d. (n = 3).

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