

Unraveling Ros Conversion Through Enhanced Enzyme-Like Activity with Copper-Doped Cerium Oxide for Tumor Nanocatalytic Therapy

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Nanozyme catalytic therapy for cancer treatments has become one of the heated topics, and the therapeutic efficacy is highly correlated with their catalytic efficiency. In this work, three copper-doped CeO₂ supports with various structures as well as crystal facets are developed to realize dual enzyme-mimic catalytic activities, that is superoxide dismutase (SOD) to reduce superoxide radicals to H₂O₂ and peroxidase (POD) to transform H₂O₂ to •OH. The wire-shaped CeO₂/Cu-W has the richest surface oxygen vacancies, and a low level of oxygen vacancy (Vo) formation energy, which allows for the elimination of intracellular reactive oxygen species (ROS) and continuous transformation to •OH with cascade reaction. Moreover, the wire-shaped CeO₂/Cu-W displays the highest toxic •OH production capacity in an acidic intracellular environment, inducing breast cancer cell death and pro-apoptotic autophagy. Therefore, wire-shaped CeO₂/Cu nanoparticles as an artificial enzyme system can have great potential in the intervention of intracellular ROS in cancer cells, achieving efficacious nanocatalytic therapy.

attention due to their advantages such as improved stability, higher cost-effectiveness, and less strict storage conditions in contrast with natural enzymes.^[3,4] Particularly, incorporating their distinctive properties and catalytic abilities, nanozymes have great potential in various applications, ranging from diagnosis in vitro to substitutive use in certain biosystems.^[5,6] Nanozymes have been explored for cancer treatment by modulating intracellular reactive oxygen species (ROS).^[7]

ROS is essential for living organisms due to its important function in regulating various physiological functions. The intrinsic biochemical properties of ROS can demonstrate mechanisms for the physiological activities of living organisms, which are worth being studied in depth.^[8,9] In cancer cells, intracellular electron transport chains and

NADPH oxidases result in continuous generation of O₂^{•−}.^[10] O₂^{•−} can be steadily converted into H₂O₂ catalyzed by superoxide dismutase (SOD) and be further transformed to hydroxyl radicals through the peroxidase-like (POD) reaction.^[11] To improve the catalytic ability of nanozyme, Ir-N₅ SA/Cer was prepared by introducing axial N coordination in SA enzymes. It has been reported that the Ir-N₅ SA can activate natural oxidase, peroxidase,

1. Introduction

Despite the development of multiple anti-cancer strategies, chemotherapy is still an indispensable therapeutic method for different malignant tumors.^[1,2] Nanozyme catalytic therapy is an emerging chemotherapeutic method. Nanozymes, nanomaterials with enzyme-mimicking properties, have drawn increasing

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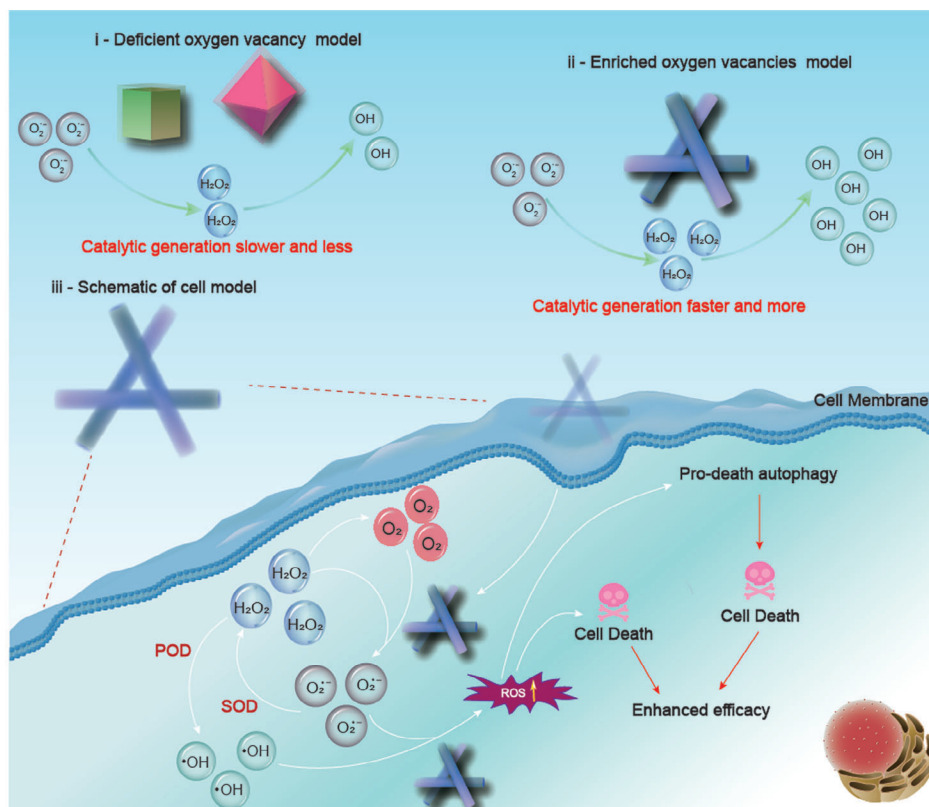


Figure 1. The mechanism for CeO₂/Cu to mediate the relay transformation of O₂^{•−} → H₂O₂ → OH to induce cancer cell death.

and catalase, as well as catalyzing NAHD to H₂O₂.^[12] However, it is challenging to promote the production of •OH by intracellular SOD and POD, thus, constant production of •OH inside cancer cells has been attempted by various approaches to enhance the cytotoxicity of ROS for anti-tumor treatment.^[13] Nanozymes could be developed to continuously convert endogenous ROS (O₂^{•−} and H₂O₂) into •OH inside cancer cells.

CeO₂ possesses sufficient surface oxygen vacancies, which are critical for converting O₂^{•−} hydrogenation and H₂O₂ resolving.^[14] CeO₂ with morphology of different exposed crystal facets has been found to be essential in a series of reactions. Herein, we have fabricated CeO₂ of wires (CeO₂/Cu-W), cubes (CeO₂/Cu-C), and octahedrons (CeO₂/Cu-O) as the support for the Cu metal. The CeO₂/Cu-W exhibits a high SOD and POD activity in contrast with CeO₂/Cu-C and CeO₂/Cu-O and facilitates continuous catalysis of O₂^{•−} → H₂O₂ → •OH in cancer cells via the relay reaction supported by oxygen vacancies, eventually achieving constant production of •OH, triggering the apoptosis of cancer cells (Figure 1).

2. Results and Discussion

To verify the crystal characteristics of CeO₂ and CeO₂/Cu, powder X-ray diffraction (XRD) experiments were conducted. Figure S1 (Supporting Information) displays the XRD patterns of CeO₂ and CeO₂/Cu with different morphologies. Scanning electron microscopy (SEM) images show that CeO₂ with different shapes are uniformly arranged on the substrate with a smooth sur-

face (Figure S2, Supporting Information), indicating the well-prepared CeO₂ works as a template for the following reaction. Transmission electron microscopy (TEM) images in Figure S3 (Supporting Information) exhibit the same smooth surface as SEM images. Mapping analysis by Energy-dispersive X-ray spectroscopy verifies uniform distribution of Cu, Ce, and O in CeO₂/Cu, indicating that Cu components are doped into nanostructure rather than coating, exhibited in Figure S4 (Supporting Information). Figure 2 shows TEM and SEM images, which demonstrate the exposed lattice plane of CeO₂/Cu samples with different morphologies. For wire-shaped CeO₂/Cu-W (Figure 2a–c), which have a length of 100–300 nm and a width of ≈10 nm, and the interplanar spacing in CeO₂/Cu-W is 0.189 and 0.309 nm, corresponding to (110) and (111) crystal plane. CeO₂/Cu-C has a shape of cubes at a bulk with 200 nm and the (100) crystal facet exhibits a distinct interplanar spacing of 0.272 nm, revealed by Figure 2d,f. Based on Figure 2g, CeO₂/Cu-O particles exhibit a morphology of octahedrons with a length of 200–300 nm. The (111) facet with an interplanar spacing of 0.311 nm is selectively exposed due to its thermodynamical stability (Figure 2i).

The selectivity by crystal facets was confirmed by the electro-sorption of hydroxide (OH_{ads}) studies.^[15] Cyclic voltammetry results, as shown in Figure 3a, indicated that different facets exhibit different OH[−] adsorption/desorption peaks in the C–V curves caused by the exposed crystal facets of CeO₂/Cu catalysts with different morphologies.^[16] Peaks at −0.64, −0.59, and −0.54 V_{Ag/AgCl} in the C–V curves represent (100), (110), and (111)

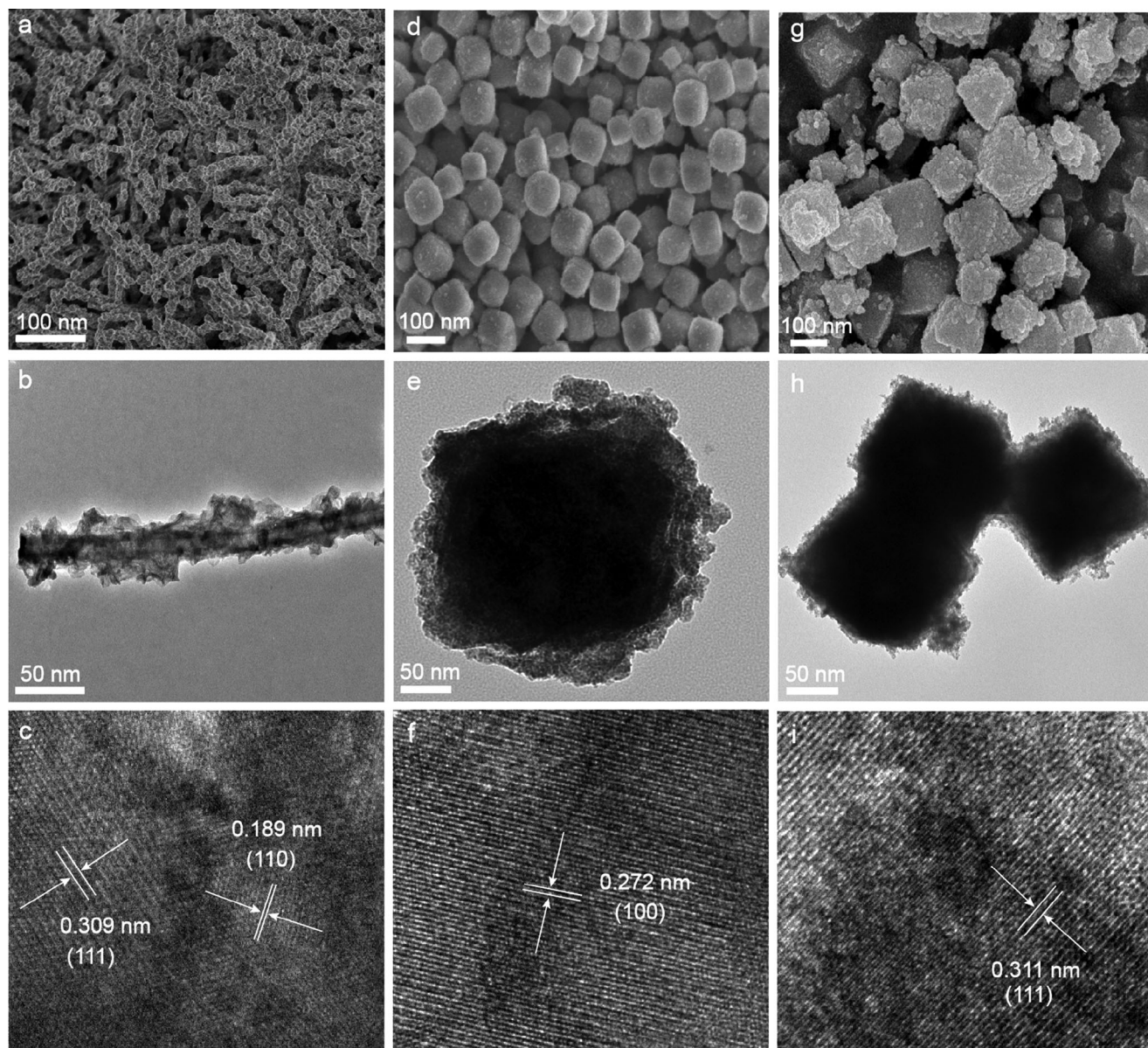


Figure 2. SEM and TEM images of CeO₂/Cu-W a–c), CeO₂/Cu-C d–f), and CeO₂-C g–i).

planes, respectively. In addition, three distinguished adsorption peaks can be found, which indicates a similar distribution trend on the surface of catalysts. The relative ratio of the crystal planes could be obtained by the integration of three adsorption peaks (Figure 3b–d). To further illustrate the existence of oxygen vacancies, electron paramagnetic resonance spectra were obtained (Figure 3e). As a result, CeO₂/Cu-C and CeO₂/Cu-O present weakly unpaired electrons, while CeO₂/Cu-W exhibits a stronger opposite peak at $g = 1.998$, and the increased intensity of the opposite peak is ascribed to the increase in the oxygen vacancy density, suggesting that CeO₂/Cu-W has the richest surface oxygen vacancies.^[17] The actual Cu/Ce ratio in different samples can be confirmed using an inductively coupled plasma mass spectrometry (ICP-MS) (Table S1, Supporting Information).

The valence states of Ce and Cu was investigated using an X-ray photoelectron spectroscopy (XPS). Figure 3f indicates that Ce 3d can be fitted into 10 peaks, which corresponds to the Ce 3d_{5/2} and Ce 3d_{3/2} states.^[18] The characteristic peaks of ν_o , ν' , μ_o , and μ'' are generated due to the existence of Ce³⁺ species while other six peaks can be ascribed to Ce⁴⁺ species.^[19] The surface density of Ce³⁺ can be determined according to Ce⁴⁺ and Ce³⁺ peaks areas. The ratios of Ce³⁺ and Ce⁴⁺ species regarding all obtained samples are showed in Table S2 (Supporting Information). The Ce³⁺ concentration is related to the surface oxygen vacancies, and their formation is associated with the existence of Ce³⁺ species.^[20] Our calculations suggest that the correlation could be challenged by the Cu²⁺ substitution. In addition, the substitution of one Ce³⁺ next to a V_O by one Cu²⁺ can be due to the conversion of Ce³⁺ to Ce⁴⁺. Consequently, an increase in

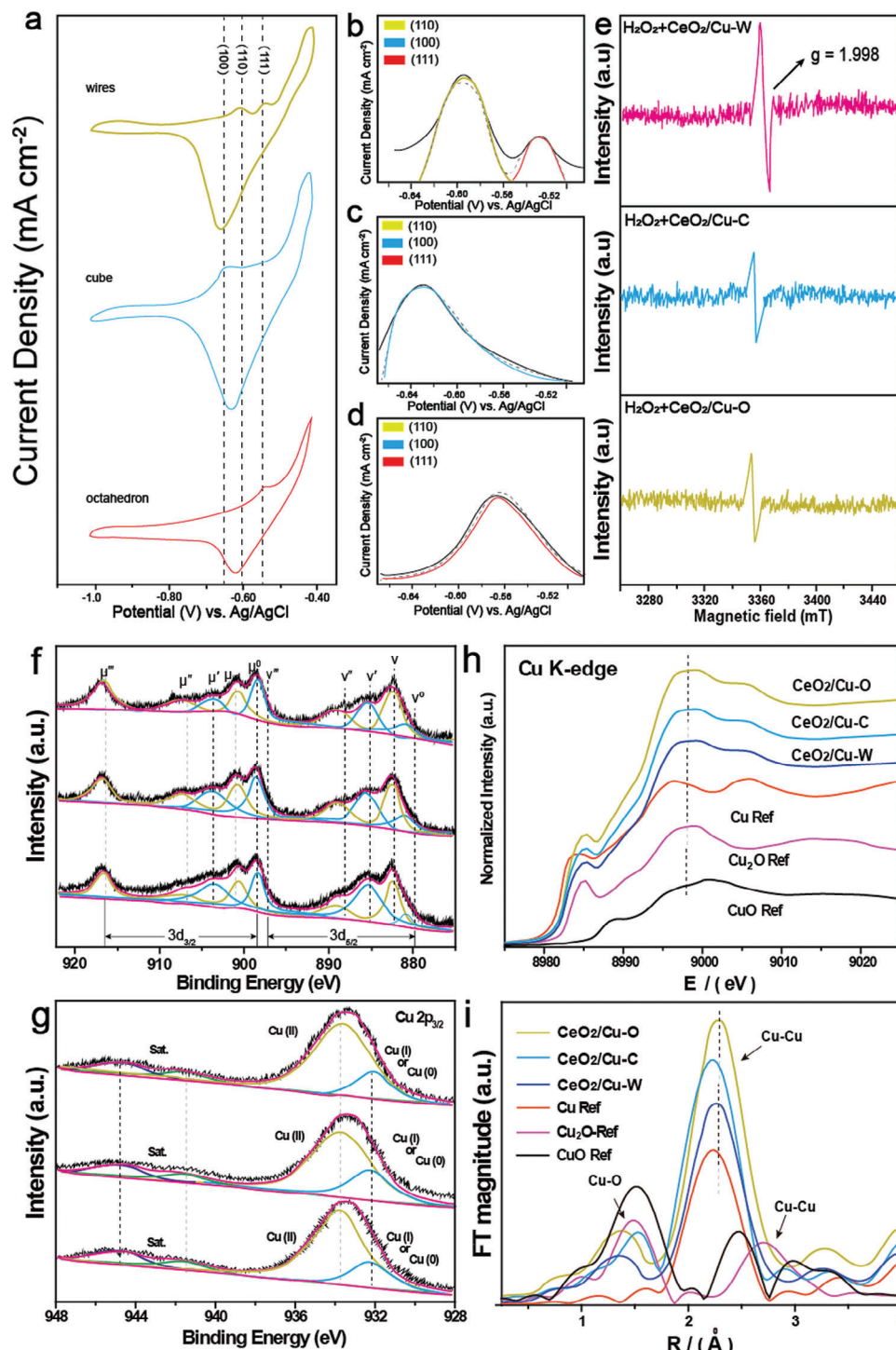


Figure 3. a) C–V curves of NWs, NPs, and NSs in Ar-saturated KOH (1.0M) at a scanning rate of 50 mV s^{-1} . b–d) The OH^- adsorption peaks of the $\text{CeO}_2/\text{Cu-W}$ (b), $\text{CeO}_2/\text{Cu-C}$ (c), and $\text{CeO}_2/\text{Cu-O}$ (d) were fitted to the corresponding crystal plane proportions in the inset. e) EPR spectra of $\text{CeO}_2/\text{Cu-W}$, $\text{CeO}_2/\text{Cu-C}$ and $\text{CeO}_2/\text{Cu-O}$. f,g) XPS characterization of Ce 3d f) and Cu $2p_{3/2}$ g) of CeO_2/Cu . h) XANES spectra at the Cu K-edge. i) The corresponding K^3 -weighted Fourier-Transform (FT) spectra in R-space.

the Cu^{2+} doping content leads to a decline in the $\text{Ce}^{3+}/\text{Ce}^{4+}$ ratio of the initial CeO_2 , which is in accordance with lower copper proportion from XPS data. $\text{CeO}_2/\text{Cu-W}$ exhibits the highest concentration of Ce^{3+} (0.49) among all samples while the $\text{CeO}_2/\text{Cu-C}$ and $\text{CeO}_2/\text{Cu-O}$ are 0.38 and 0.36, respectively. Therefore, the assumption that surface oxygen vacancies of $\text{CeO}_2/\text{Cu-W}$ possess the highest density is made. The peaks of 933.6 and 932.5 eV in spectra of Cu $2p_{3/2}$ were assigned to Cu^{2+} and $[\text{Cu}^0+\text{Cu}^{1+}]$ (Figure 3g).^[21] The low oxidation potential of $[\text{Cu}^0+\text{Cu}^{1+}]$ suggest that oxygen vacancies are generated around the doping sites of Cu atoms.^[22] The concentration of the oxygen vacancy can be determined by O 1s spectra. As depicted in Figure S5 (Supporting Information), deconvoluted result showed three peaks at 529.3, 531.4, and 534.0 eV, of which 529.3 and 531.4 eV demonstrated the existence of lattice oxygen (O_a : O^-) and surface oxygen (O_s : O^- , O_2^{2-} or O^{2-}), respectively. Meanwhile, the peak at 534.0 eV verifies other oxygen species (O_γ) with weak bound including CO_3^{2-} , adsorbed H_2O , and OH^- .^[23] The abundance of oxygen vacancies (V_O) can be calculated based on the ratio of O_β and ($\text{O}_a+\text{O}_\beta+\text{O}_\gamma$), in line with Ce 3d spectra. The above results indicates that the amount of surface oxygen vacancies is proportional to the number of Ce^{3+} . It could be suggested that CeO_2 with different morphologies exposes different crystal facets that possess distinct V_O formation energy, thereby exhibiting various densities of V_O .

The atomic structure of obtained samples was confirmed by X-ray absorption near-edge structure (XANES) and extended X-ray absorption fine structure (EXAFS) (Figure 3h,i). In contrast with standard CuO sample (CuO-ref, black curve), the peaks of all Cu-doped CeO_2 samples display a distinct shift toward the lower energy side, which results from the low oxidative potential of $[\text{Cu}^0+\text{Cu}^{1+}]$. The characteristic peak shapes of the three samples are similar to those of standard Cu_2O (Cu₂O-ref, purple curve) at ≈ 899.6 eV. The XANES spectrum of CeO_2/Cu is between two standard spectra, indicating the coexistence of +1 and +2 valence states of Cu atoms. The conversion of EXAFS at the Cu K-edge to R-space (Figure 3i) gives more structural information of the three samples. The three peaks in dotted lines represent the Cu–O and Cu–Cu bonds. Compared to standard Cu and Cu_2O samples, the three samples exhibit a main peak at 2.14 Å (Cu–Cu bond) and a minor one at 1.55 Å, indicating a high percentage of Cu–Cu bonds and a low percentage of Cu–O bonds. In Figure S6 (Supporting Information), the coordination peak of the Cu–Cu bond of $\text{CeO}_2/\text{Cu-C}$ and $\text{CeO}_2/\text{Cu-O}$ centered at 2.12 and 2.15 Å. However, $\text{CeO}_2/\text{Cu-W}$ exhibited a shift for that of the Cu–Cu bond at 2.14 Å, which could be attributed to the generation of oxygen vacancies. The enhanced oxygen vacancies of the three samples indicate that it can significantly enhance the chemocatalysis capability of $\text{O}_2^{\cdot-}$.^[24] Furthermore, the Cu LMM Auger spectrum was utilized to determine the oxidation state of copper for the $\text{CeO}_2/\text{Cu-W}$ sample. Two final-state terms split from the L–S coupling ^1G and ^3F have been previously reported. The ^1G peaks are detected for different valence states of Cu: 917.1 (Cu^{2+}), 915.8 (Cu^{1+}), and 918.0 eV (Cu^0), while the ^3F peak represents the existence of Cu^0 .^[25] Displayed in Figure S7 (Supporting Information), the ^1G peak at 916.5 eV of $\text{CeO}_2/\text{Cu-W}$ suggests that Cu is in a valence state of between +1 and +2. On the other hand, the distinct ^3F peak confirms the presence of Cu^0 , which corresponds to the XAS results.

The catalytic capability of SOD and POD is essential in catalyzing the endogenous peroxides and superoxide into hydroxyl radicals. The SOD and the POD units processed by CeO_2/Cu work in concert to achieve the cascade relay catalysis that promotes the serial transformation of $\text{O}_2^{\cdot-} \rightarrow \text{H}_2\text{O}_2 \rightarrow \bullet\text{OH}$.^[26] The POD-like enzymatic capability of CeO_2/Cu at pH 4.4, 5.0, and 6.0 were measured via the classic $\text{H}_2\text{O}_2/\text{TMB}$ system. $\text{CeO}_2/\text{Cu-W}$ possesses a higher and more sustainable POD-like activity at different pH levels than $\text{CeO}_2/\text{Cu-C}$ and $\text{CeO}_2/\text{Cu-O}$ according to the time course curves, also evidenced by a higher concentration of $\bullet\text{OH}$ based on the absorbance value at 650 nm (Figure 4a–c; Figure S8, Supporting Information).^[27] To examine the SOD-like activity of CeO_2/Cu samples, the experiment of $\text{O}_2^{\cdot-}/\text{NBT}$ (Nitrobluetetrazolium) inhibition assay was conducted.^[28] The generation of $\text{O}_2^{\cdot-}$ is triggered by riboflavin illumination, which can be detected by the NBT, and the formation of NBT-monoformazan can be revealed by the absorption peak at 540 nm. Due to the SOD-like activity of $\text{CeO}_2/\text{Cu-W}$, the reduced level of $\text{O}_2^{\cdot-}$ results in a reduction in the absorption value at 540 nm. However, the $\text{CeO}_2/\text{Cu-C}$ and $\text{CeO}_2/\text{Cu-O}$ samples have a negligible effect on the absorption value. At pH 6.0 and 7.4, riboflavin illumination results in a continuous increase in the absorbance with the addition of the illumination time (Figure S9, Supporting Information). Nevertheless, $\text{CeO}_2/\text{Cu-W}$ curbs the increase of the absorbance to a great extent compared to $\text{CeO}_2/\text{Cu-C}$ and $\text{CeO}_2/\text{Cu-O}$, suggesting its superior SOD-like activity (Figure 4d–f). The above experimental results confirm the superior POD- and SOD-like activities of $\text{CeO}_2/\text{Cu-W}$ that can improve the catalytic efficiency of the reduction process. The xanthine/xanthine oxidase system (Xan+XOD) was also employed to verify the catalytic activity of $\text{CeO}_2/\text{Cu-W}$.^[29] Similar to the results of inhibition assay of $\text{O}_2^{\cdot-}/\text{NBT}$, the $\text{CeO}_2/\text{Cu-W}/(\text{Xan}+\text{XOD})/\text{DMPO}$ system exhibits a weaker ESR signal of $\text{DMPO}/\text{O}_2^{\cdot-}$, indicating that $\text{CeO}_2/\text{Cu-W}$ can catalyze $\text{O}_2^{\cdot-}$ to H_2O_2 more efficiently (Figure 4g). The stronger ESR signal of $\text{DMPO}/\bullet\text{OH}$ using the $\text{CeO}_2/\text{Cu-W}/\text{H}_2\text{O}_2/\text{DMPO}$ system suggests that $\text{CeO}_2/\text{Cu-W}$ promotes the catalytic process of $\text{H}_2\text{O}_2 \rightarrow \bullet\text{OH}$ at pH = 6.0 (Figure 4h). Therefore, it can be concluded that $\text{CeO}_2/\text{Cu-W}$ can facilitate the overall reaction of $\text{O}_2^{\cdot-} \rightarrow \text{H}_2\text{O}_2 \rightarrow \bullet\text{OH}$ to achieve a better tumor inhibition efficacy.

We further reveal the mechanism of oxygen vacancy formation through DFT calculations and elucidate that Cu doping can effectively promote the oxygen supply capacity of CeO_2 . Previous theoretical and experimental results suggested that CeO_2 (111) has a lower surface energy and is more likely to form stable structures, which is consistent with our experimental results.^[30] The effect of the Cu-doped $\text{CeO}_2(111)$ surface on the formation of oxygen vacancies was then examined. Figure 5a plots the optimized CeO_2 bulk at a relaxed Ce–O bond length of 2.366 Å. The O vacancy formation energy in the stable CeO_2 bulk is 3.87 eV. Although the coordination number of the O atom on the surface of $\text{CeO}_2(111)$ is decreased to three, the bond length of the Ce–O remains unchanged (Figure 5b). It can be attributed to the dangling bonds on the $\text{CeO}_2(111)$. However, the forming energy of oxygen vacancy on the surface of $\text{CeO}_2(111)$ is only 2.45 eV.

It is worth noting that the doped Cu atom can effectively lower the oxygen vacancy formation energy. Figure 5c shows the doped Cu atom is unsettled, leading to the breakage of the Cu–O bond. The passive oxygen atom on the surface of $\text{CeO}_2(111)$ has

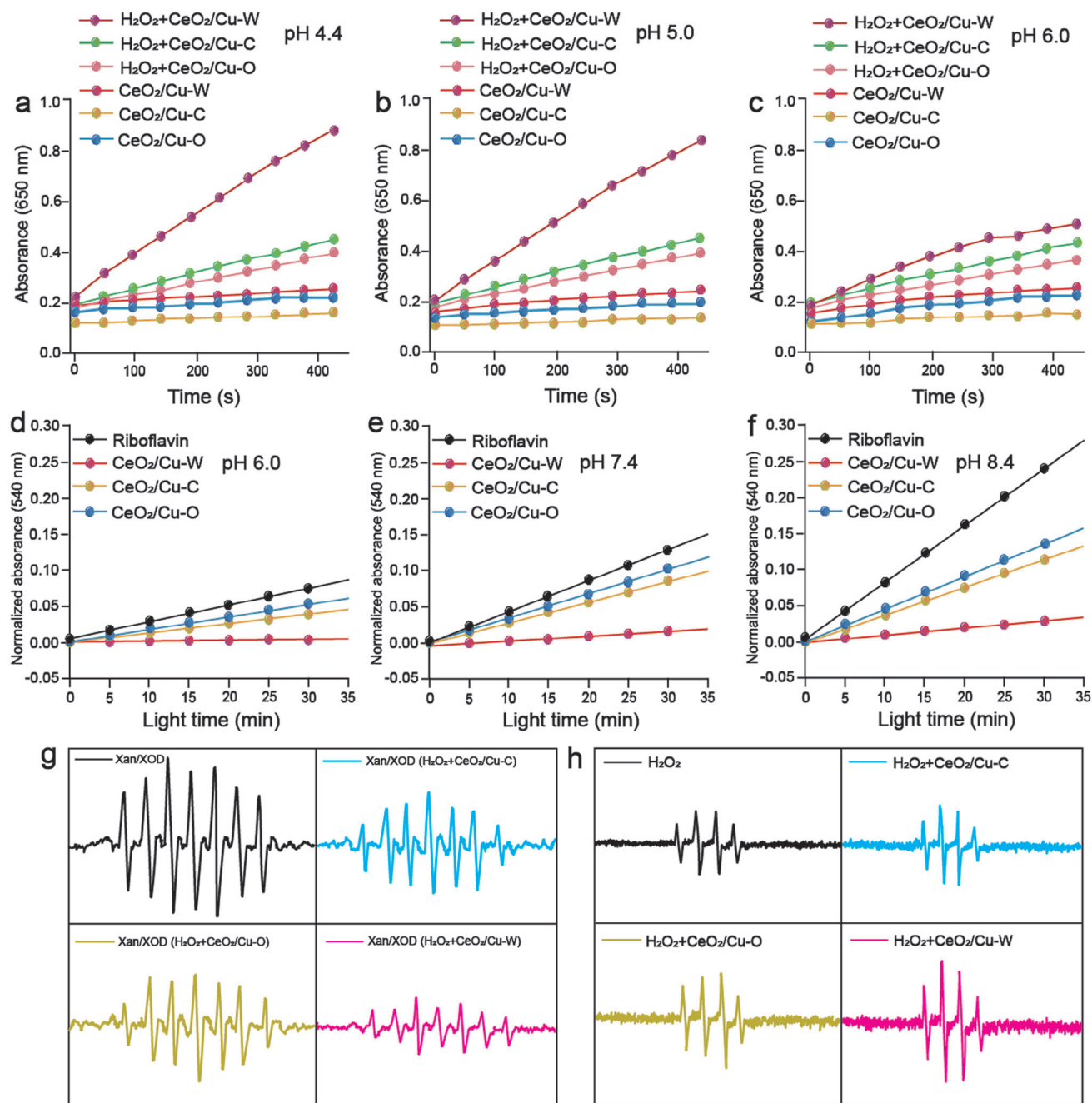


Figure 4. a–c) Temporal absorbance variations at a wavelength of 650 nm in different shaped CeO₂/Cu/TMB/H₂O₂ systems at pH of 4.4, 5.0, and 6.0. d–f) Normalized temporal absorbance variations at 540 nm wavelength for different samples. g, h) ESR spectra in two different solution systems.

to shorten the residual Ce–O bond length (2.179, 2.196 Å) to maintain structural stability. The oxygen atoms on the surface have a lower coordination number (2), leading to an ultra-low oxygen vacancy formation energy of 0.01 eV. Thus, the unknotted O atoms are more likely to migrate, providing rapid supply of oxygen to the surface of CeO₂(111). Moreover, the defect structure caused by Cu doping enables the oxygen atoms in the subsurface layer to have a low oxygen defect formation energy. From Figure 5d, the oxygen vacancy formation energy is 2.34 eV,

which is 0.11 eV smaller than that of the O atom on the surface of Ce (111). The charge density difference of CeO₂(111)/Cu (Figure 5e) exhibits that the doped Cu atom transfers electrons to the oxygen atom, resulting in the reduction of the oxygen atoms. In addition, it can be seen from the density of states (DOSs) (Figure 5f–i) that the doping of the Cu atom leads to the gain of electrons by the antibonding orbitals of oxygen atoms to reach the Fermi energy level, which finally causes the activation of oxygen atoms.

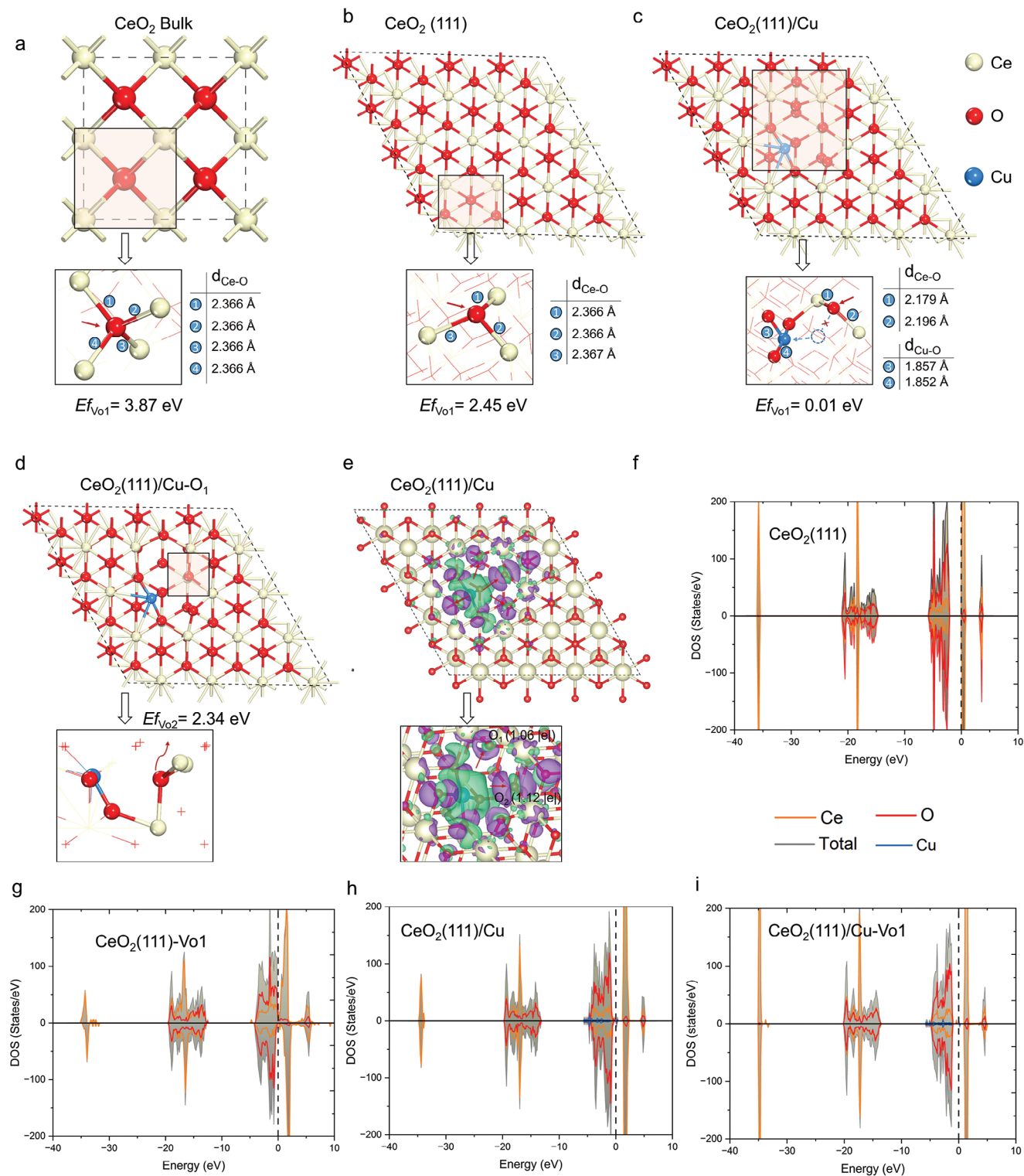


Figure 5. DFT calculations for analyzing the mechanism of O vacancies formation in the CeO_2 . a) Optimized geometry of the CeO_2 bulk. Key Ce—O bonds and O vacancy formation energies are displayed. b) The geometry of CeO_2 (111) can be optimized by using the Ce—O bond length and the oxygen vacancy formation of surface oxygen atoms. c) Optimized geometry of $\text{CeO}_2(111)/\text{Cu}$ with Ce—O, Cu—O bond lengths, and oxygen vacancy formation energies of the oxygen atom in the surface layer. d) Optimized geometry of $\text{CeO}_2(111)/\text{Cu-Vo1}$ with the oxygen vacancy formation energy of the oxygen atom in the subsurface layer. e) Charge density differences of $\text{CeO}_2(111)/\text{Cu}$. The Bader charge of the O atoms in the surface and subsurface of $\text{CeO}_2(111)$ are shown. f–i). Density of states (DOS) of $\text{CeO}_2(111)$, $\text{CeO}_2(111)\text{-Vo1}$, $\text{CeO}_2(111)/\text{Cu}$, and $\text{CeO}_2(111)/\text{Cu-Vo1}$, respectively.

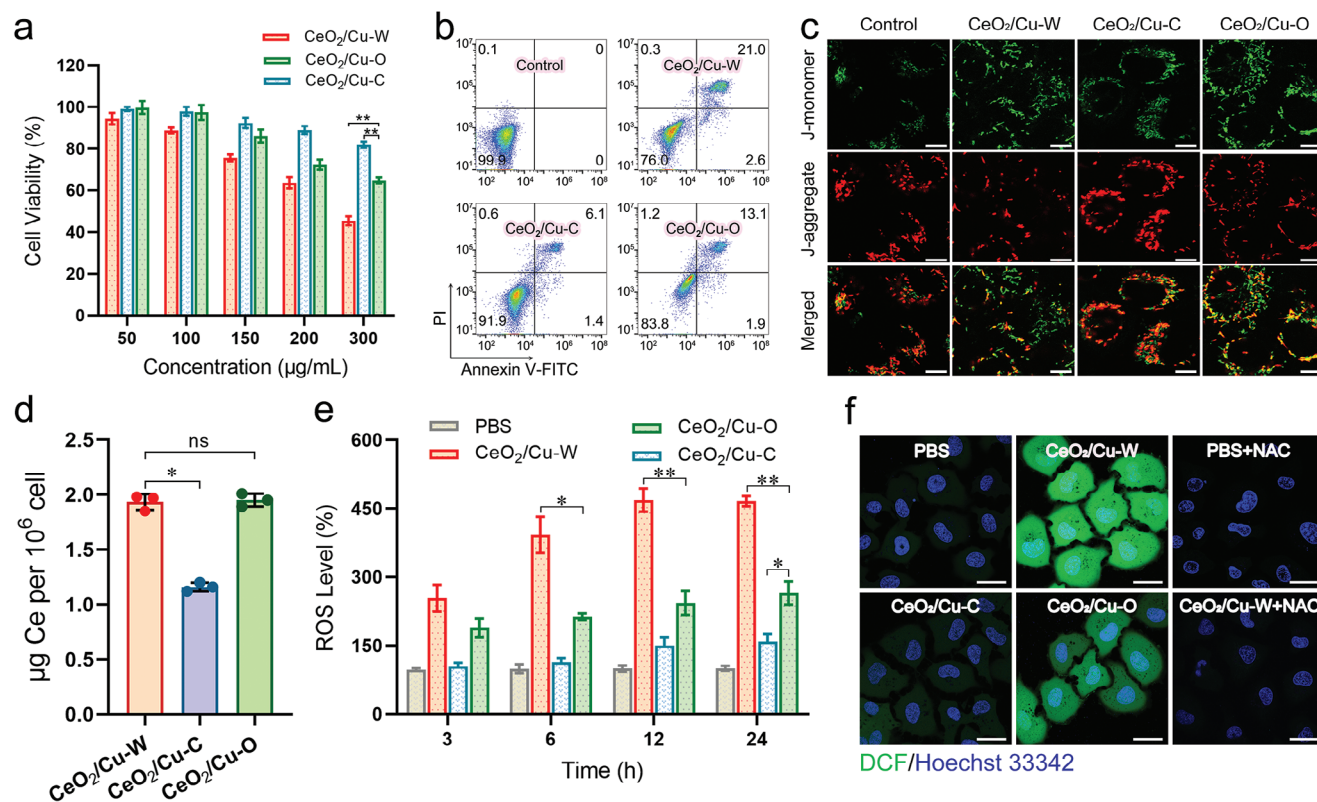


Figure 6. a) Cell viability of MDA-MB-231 cells after incubation with different CeO₂/Cu nanoparticles for 24 h ($n = 5$, $^{**}p < 0.001$). b) cell apoptosis and c) mitochondrial membrane potential changes of MDA-MB-231 cells after various treatments for 24 h. (Scale bar: 10 μm). d) Intracellular Ce in MDA-MB-231 cells after co-incubation with CeO₂/Cu-W, CeO₂/Cu-C or CeO₂/Cu-O (50 μg mL⁻¹) for 6 h ($n = 3$, $^{*}p < 0.01$, ns nonsignificant). e) Quantitative analysis of intracellular ROS after exposure to CeO₂/Cu nanoparticles for different durations ($n = 3$). PBS-treated cells served as a control. $^{**}p < 0.01$, $^{*}p < 0.05$. f) CLSM images of intracellular ROS in cells after incubation with CeO₂/Cu nanoparticles for 12 h. DCF fluorescence (green) for intracellular ROS and Hoechst 33342 (blue) for cell nuclei. (Scale bar: 20 μm). Data was expressed as mean ± SD. The P values were calculated using a one-way ANOVA with Tukey's post-hoc test a,d,e).

In view of the efficient catalytic properties of CeO₂/Cu-W nanoparticles $O_2^{\cdot-} \rightarrow H_2O_2 \rightarrow \cdot OH$, we studied whether they have a potential cytotoxic effect on tumor cells. The cytotoxicity of the CeO₂/Cu nanoparticles was determined by CCK-8 assays. As the concentration of CeO₂/Cu nanoparticles increases, the viabilities of human breast carcinoma MDA-MB-231 cells showed a general decrease in a dose-dependent manner (Figure 6a). At a concentration of 300 μg mL⁻¹, CeO₂/Cu-W treatment results in more than 50% decrease in the cell viability, which is much higher than CeO₂/Cu-C (18%) and CeO₂/Cu-O (35%). The similar killing effect of CeO₂/Cu-W nanoparticles is identified in 4T1 and A549 cell lines (Figures S10 and S11, Supporting Information). Consistent with the results of cell viabilities, the employment of CeO₂/Cu-W nanoparticles leads to the highest ratio of cell apoptosis (Figure 6b) and significant loss of the mitochondria membrane potential (MMP, Figure 6c).

In order to investigate whether the difference in the level of cell apoptosis induced by these nanoparticles is attributed to the number of internalized nanoparticles, the intracellular Ce concentration was determined by ICP-MS. The intracellular content of Ce in CeO₂/Cu-W-treated cells is similar to that in the CeO₂/Cu-O-treated cells but slightly higher than that in the CeO₂/Cu-C-treated cells (Figure 6d), indicating that the shape of

CeO₂/Cu-W and CeO₂/Cu-O nanoparticles have limited effect on their cellular internalization, and the stronger antitumor effect of CeO₂/Cu-W than CeO₂/Cu-O is not due to the level of cellular internalization.

Then, the intracellular level of ROS (including $O_2^{\cdot-}$, H_2O_2 and $\cdot OH$) was examined by a microplate reader using DCFH-DA. After 12 h incubation, the relative ROS level in the CeO₂/Cu-W-treated cells is three times of that in the CeO₂/Cu-C-treated cells ($^{**}p < 0.01$) and two times of that in the CeO₂/Cu-O-treated cells ($^{**}p < 0.01$), respectively (Figure 6e). Furthermore, the cells exposed to CeO₂/Cu-W display the strongest green fluorescence in the images captured by confocal laser scanning microscopy (CLSM) (Figure 6f), indicating that CeO₂/Cu-W has the strongest ROS production ability, in agreement with the results shown in Figures 4,5. However, after the cells are pretreated with N-acetyl-L-cysteine (NAC), a ROS scavenger, the intracellular green fluorescence intensity drastically decreases (Figure 6f; Figure S12, Supporting Information). ROS plays important roles in diverse biological processes, but a high level of ROS usually causes cell damage and induces autophagy.

Accordingly, the ability of CeO₂/Cu-W to induce autophagy in MDA-MB-231 cells was evaluated by acridine orange (AO), an autophagy probe, which appears red in acidic organelles

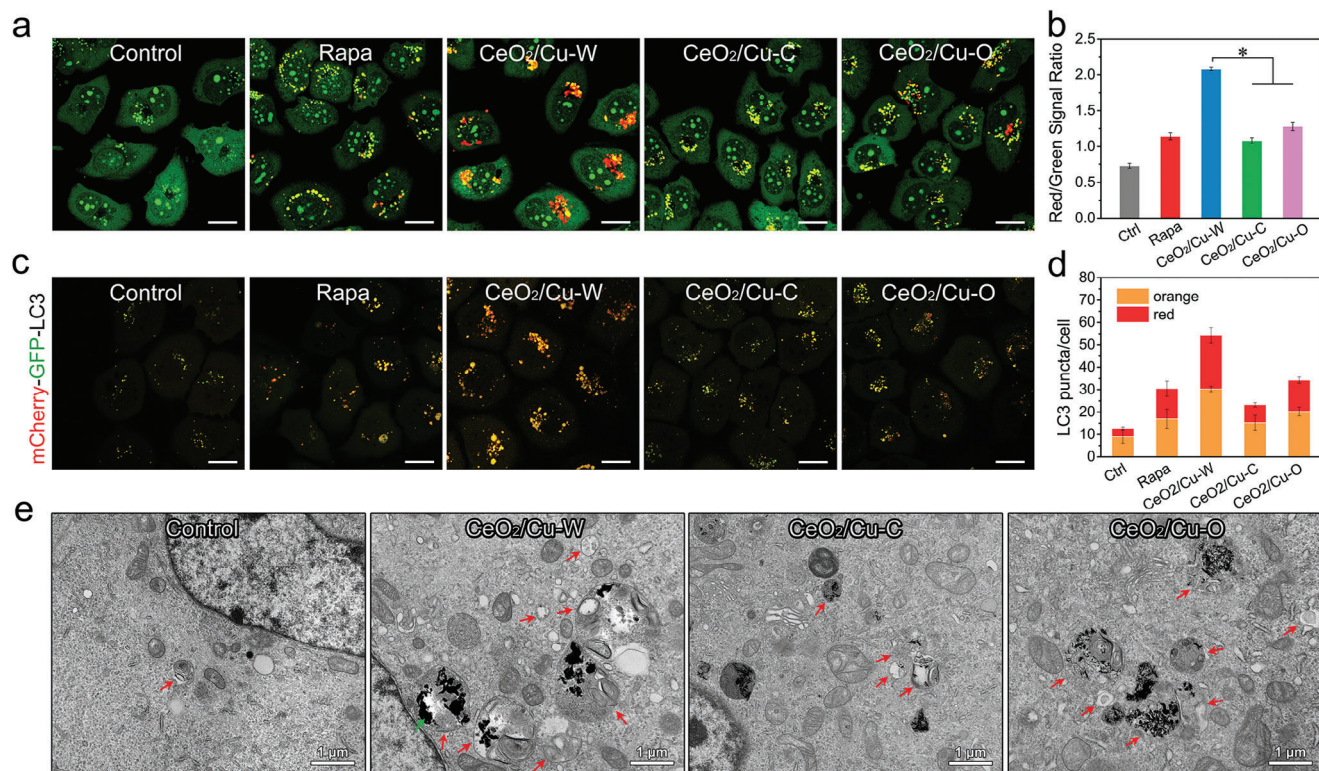


Figure 7. Analysis of intracellular autophagic levels activated by nanoparticles. a) CLSM images and b) FACS analysis of AO-stained MDA-MB-231 cells after exposure to Rapa (50 nM), CeO₂/Cu-W, CeO₂/Cu-C or CeO₂/Cu-O (100 $\mu\text{g mL}^{-1}$) for 24 h ($n = 3$, $*p < 0.01$). c) Representative CLSM images of mCherry-GFP-LC3-expression cells and d) Quantification of LC3 puncta per cell after incubation with different formulations for 24 h. e) TEM images for ultrastructural analysis of cells receiving treatment with PBS, CeO₂/Cu-W, CeO₂/Cu-C, or CeO₂/Cu-O (100 $\mu\text{g mL}^{-1}$) for 24 h (red arrows: typical autophagic vacuoles; green arrow: CeO₂/Cu nanoparticles).

(autophagosomes and lysosomes) but green in the cytosol and nuclei. The number of red spots counted in CLSM images has positive correlation with the concentration of nanoparticles (Figure S13, Supporting Information). The maximum number of red spots is found in the cells after treatment with CeO₂/Cu-W (100 $\mu\text{g mL}^{-1}$) for 24 h, indicating that CeO₂/Cu-W has the strongest autophagy induction ability (Figure 7a). Accordingly, the red to green signal ratio in the cells treated with CeO₂/Cu-W determined by fluorescence-activated cell sorter (FACS) is the highest among all groups (Figure 7b), and it elevated significantly compared to that in the cells treated with CeO₂/Cu-C, CeO₂/Cu-O and rapamycin (Rapa, a clinical autophagy inducer), demonstrating the strong ability of CeO₂/Cu-W to induce production and accumulation of acidic organelles. But AO is able to differentiate whether the labeled organelles are autophagosomes and autolysosomes. Thus, mCherry-GFP-LC3-expressed MDA-MB-231 cells were utilized to monitor the autophagic process induced by different shaped nanoparticles.

LC3, a specific autophagy-related protein, aggregates on the autophagosome membranes when autophagy occurs. mCherry, an acid-stable protein, shows red fluorescence in both autophagosomes and autolysosomes, while GFP is an acid-unstable protein and its green fluorescence quenches after autophagosomes fuse with lysosomes to form autolysosomes. As shown in Figure 7c,d, CeO₂/Cu-W exposure leads to a significant increase in red and large yellow puncta in comparison with CeO₂/Cu-C and

CeO₂/Cu-O, indicating that CeO₂/Cu-W could effectively stimulate the autophagic flux in cells. Finally, the bio-TEM method was used to directly observe the autophagy level (Figure 7e). Compared to the PBS-treated control cells, cells treated with CeO₂/Cu-W reveal abnormally accumulated autophagic vacuoles, while a small number of autophagic vacuoles is detected in the cells treated with CeO₂/Cu-C and CeO₂/Cu-O. These results are in consistence with the observations by confocal fluorescence imaging, providing support for the conclusion that the ability of eliciting autophagy by CeO₂/Cu nanoparticles is shape-dependent, and CeO₂/Cu-W elicits a high level of autophagy in the MDA-MB-231 cancer cells. As the upstream of autophagy. Indeed, the elimination of ROS by NAC could attenuate the CeO₂/Cu-W-induced autophagy (Figure S14, Supporting Information).

It is worth noting that the effect of activated autophagy on cancer cells could be either pro-survival or pro-death. The cytotoxicity of different treatments was then investigated after adding an autophagy inhibitor, 3-methyladenine (3-MA). Adding 3-MA alone does not induce additional cytotoxicity. However, the cytotoxicity of CeO₂/Cu-W and CeO₂/Cu-O nanoparticles decreases after autophagy is inhibited by 3-MA (Figure S15, Supporting Information). Then, the assessment of whether CeO₂/Cu nanoparticles at a noncytotoxic concentration (100 $\mu\text{g mL}^{-1}$) would impact the cancer cell death induced by doxorubicin (DOX) was performed.^[31] As shown in Figure 8a, the combination of CeO₂/Cu-W and DOX enhances more than 20% of cell death

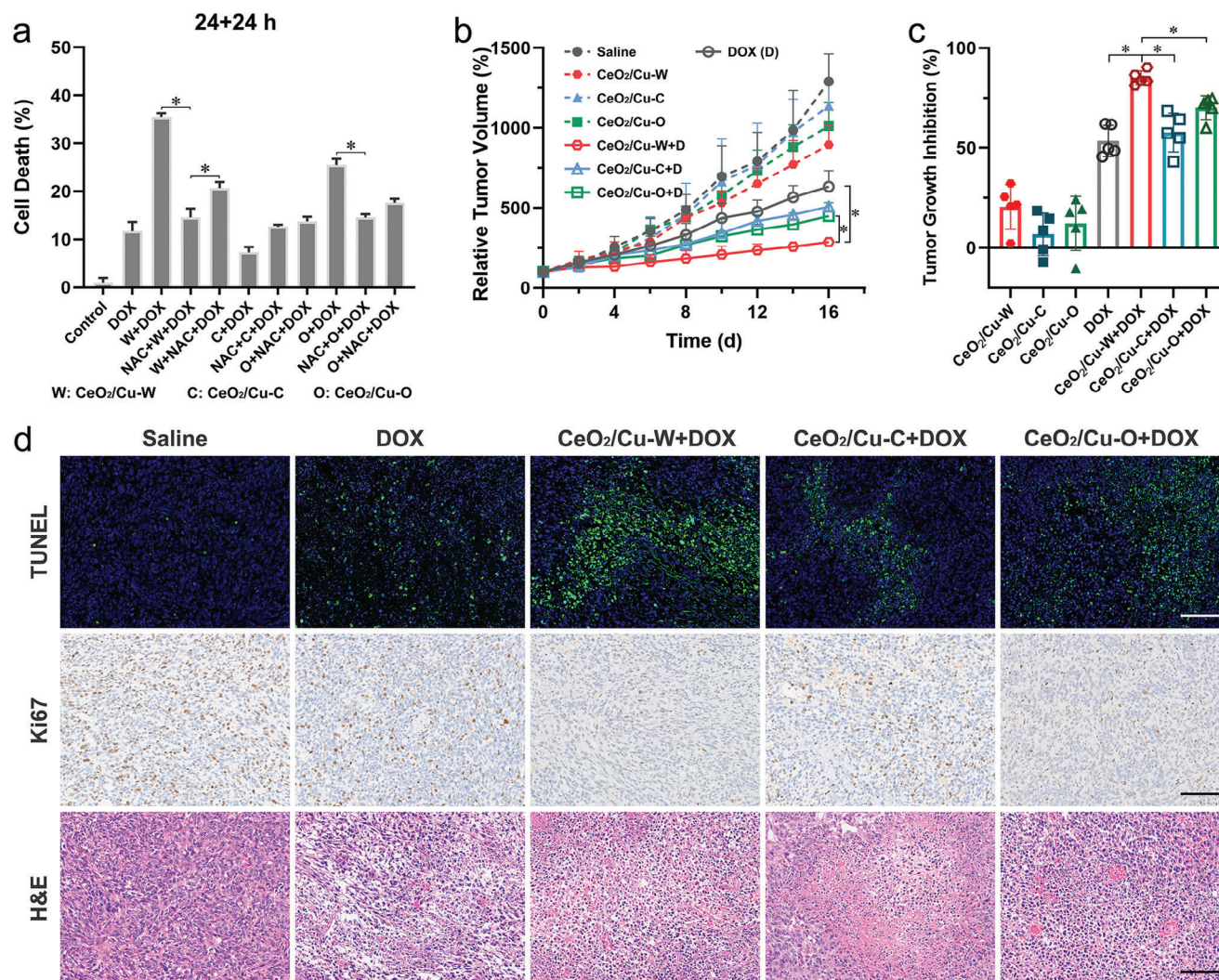


Figure 8. Antitumor efficiency of the nanoparticles. a) Cell mortality of MDA-MB-231 cells treated with different combinations for 48 h ($n = 5$, $^{**}p < 0.01$, $^{*}p < 0.05$). CeO₂/Cu nanoparticles: 100 $\mu\text{g mL}^{-1}$; DOX.HCl: 1.0 $\mu\text{g mL}^{-1}$; NAC: 1 mM. b) Relative tumor volume and c) tumor growth inhibition (TGI) treated with different groups ($n = 5$, $^{**}p < 0.01$, $^{*}p < 0.05$). d) Histological and immunohistochemical images of tumor tissue were detected by H&E, Ki-67, and TUNEL assays (brown: Ki-67-positive cells, blue: TUNEL-positive cells. Scale bar: 100 μm).

compared to the treatment with DOX, probably due to the autophagic death elicited by CeO₂/Cu-W. Furthermore, when NAC is added before the addition of CeO₂/Cu-W nanoparticles, the cell death rate is pronouncedly decreased, supporting the chemo-sensitization effect caused by autophagy. However, when NAC is added after the addition of CeO₂/Cu-W nanoparticles, a negligible reduction in the chemo-sensitization effect is observed since autophagy is induced by the nanoparticles.

Finally, the in vivo synergistic antitumor effect of CeO₂/Cu nanoparticles and DOX was assessed in the Balb/c nude mice bearing MDA-MB-231 tumors through i.v. injection of DOX with a dose of 5 mg kg^{-1} and intratumoral injection of CeO₂/Cu nanoparticles (5 mg kg^{-1}) every 4 days for four times. As presented in Figure 8b, the treatment with CeO₂/Cu-W nanoparticles slightly inhibits tumor growth. The tumor volumes in the mice increase by 6 times after DOX treatment. Encouragingly, the treatment with CeO₂/Cu-W nanoparticles in combination

with DOX has a synergistic antitumor effect, resulting in 3 times smaller of the tumor volume and the tumor inhibit rate (TGI) reaches up to 81.6% (Figure 8c). Furthermore, immunohistochemical studies reveal the fewest Ki67-positive proliferative cells as well as the most TUNEL-positive apoptotic cells in the tumor tissue with the treatment of CeO₂/Cu-W nanoparticles plus DOX (Figure 8d). Hematoxylin and eosin (H&E) results show remarkable damage to the tumor tissues after the treatment with CeO₂/Cu-W nanoparticles plus DOX, compared with compact and superabundant tumor cells in the saline-treated group. Our results mutually confirm that CeO₂/Cu nanoparticles combined with DOX could have a potent synergistic effect on the eradication of cancer cells. Consistent with previous data in vitro, the synergistic antitumor effect in vivo is not simply due to an additive effect of two treatments, which provides a promising strategy for combination therapy of multiple types of cancer by promoting the autophagy level. Moreover, the body weight of the mice

treated with CeO₂/Cu nanoparticles gathered during the treatment period displays a slight increase (Figure S16, Supporting Information), sharing a similar trend with that of the saline-treated group, which indicates that intratumoral injection of CeO₂/Cu nanoparticles does not induce distinct systemic toxicity.

3. Conclusion

In this study, CeO₂/Cu are reported as a biomimetic SOD to transform O₂^{•−} → H₂O₂ and peroxidase (POD) to realize H₂O₂ → •OH. Importantly, the artificial enzyme of CeO₂/Cu with a wire shape promotes direct alteration of endogenous ROS to the target molecule (•OH). Particularly, it can be applied to the intervention of intracellular ROS in cancer cells. With superior SOD-like and POD-like catalytic efficiencies, the wire-shaped CeO₂/Cu nanoparticles exhibit the best antitumor effect and improve the antitumor drug efficiency. The work offers a promising strategy for manipulating oxygen vacancy formation and the structural shape of catalytic inorganic nanoparticles to regulate the level of intracellular ROS.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

Research data are not shared.

Keywords

cerium oxide/copper, nanocatalytic therapy, nanozymes, oxygen vacancies, reactive oxygen species

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