Electronic Supplementary Material (ESI) for Journal of Materials Chemistry B.

Cell membrane camouflaged cerium oxide nanocubes for targeting enhanced tumor-selective therapy

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Abstract: Anticancer therapies with profound efficacy but negligible toxicity is a fundamental pursuit, which has been made humanly possible so far through either targeting or tumor-selective therapeutic (TST) approach. Herein, we developed a targeting enhanced tumor-selective cancer therapy aiming at integrating the two approaches by preparing cerium oxide (CeO_2) nanocubes with glucose oxidase (GOx) modified on the cube surface and cancer cell membrane (CCM) camouflaged outside. The immune escape and homotypic binding of camouflaged CCM enables a targeting delivery of the resultant CeO_2-GOx@CCM nanoparticles mostly into cancer tissue while its acid environment (pH<6.6) activated a cascade reaction, in which the glucose was first catalyzed by GOx into H_2O_2 and then by CeO_2 into highly cytotoxic ·OH killing cancer cells. In case of off-targeting when very few CeO_2-GOx@CCM nanoparticles were accidentally delivered into normal tissue, its neutral pH environment (pH=7.4) triggered a protective reaction, in which the H_2O_2 generated was catalyzed by CeO_2 into non-toxic H_2O and O_2. Both *in vitro* and *in vivo* results demonstrated that this targeting enhanced TST achieved the most remarkable antitumor performance with negligible toxic side-effects.

Experimental Procedures

2.1 Materials

Cerium nitrate hexahydrate ($Ce(NO_3)_3 \cdot 6H_2O$), Poly(acrylic acid) (PAA), N-hydroxysuccinimide (NHS), L-ascorbic acid, 3,3′,5,5′-tetramethyl-benzidine (TMB), 1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride (EDC•HCl), and dimethyl pyrroline *N*-oxide (DMPO) were purchased from Aladdin Corporation. Glucose oxidase (GOX), Hoechst 33342, 2′,7′-dichlorofluorescein diacetate (DCF-DA) were obtained from Sigma-Aldrich Corporation. Ammonium molybdate tetrahydrate was purchased from Meryer Chemical Technology Co., Ltd. Protein detection reagent (Coomassie Brilliant Blue) was purchased from Nanjing Jiancheng Bioengineering Institute. Propidium iodide, calcein-AM, penicillin−streptomycin, Dulbecco's modified Eagle's medium (DMEM), and fetal bovine serum (FBS) were obtained from Beyotime Biotechnology Company. IR 780 iodide dye was obtained from Tianjin Heowns Chemical Company. Cell Counting Kit-8 (CCK-8 assay) was obtained from Dojindo Molecular Technologies. All chemicals were used as received without any further purification.

2.2 Characterization

JEOL-1400 Transmission electron microscopy (TEM) working at 100 KV and JEM-2100F scanning electron microscopy (SEM) operating at 200 kV were applied to characterize nanoparticles. TEM samples were prepared by drop-casting dispersion onto copper grids covered by carbon film and energy dispersive X-ray spectroscopy (EDS) were adopted for elemental distribution analysis. A BRUKER D8-ADVANCE operating at 40 mA and 40 kV with Cu Kα radiation (λ=0.15406 nm) was used to acquire the XRD patterns. X-ray photoelectron spectroscopy (XPS) with the excitation source of AlKa was adopted to analysis the oxidation state of nanoceria. An Cary 50 UV/Vis spectrometer (Varian) was used to take the UV/Vis absorption spectra. The hydrodynamic particle size determination and Zeta potentials were measured by Malvern Zetasizer Nanoseries (Nano ZS90); The weight loss of different nanoparticles were measured by NETZSCH TG 209F3 thermogravimetric analyzer (PerkinElmer) with the heating rate of 10 °C /min from 25 to 600 °C under N_2 atmosphere. FT-IR experiments were performed using vacuum dried samples of nanoceria and spectra were recorded on Nicolet 380 FT-IR spectrometer. Electron spin resonance (ESR) spectra were measured at room temperature with a BRUKER A200 EPR spectrometer. The cell viabilities were accessed with an infinite 200 microplate reader (Tecan). Confocal laser scanning microscope (CLSM) and fluorescence microscopy images was taken on a Nikon C2 confocal fluorescence microscopy and a Olympus IX71 fluorescence microscopy, respectively. Photoluminescence was detected with a Cary Eclipse Fluorometer (Varian, USA) at the excitation of 740 nm and Kodak *In-Vivo* FX professional imaging system. Statistical analysis was carried out by using student's t test: $p < 0.05$, $^{**}p < 0.01$.

2.3 Synthesis of CeO_2

CeO_2 nanocubes were synthesized according to a reported method[1]. Typically, cerium nitrate（0.868 g) and NaOH (9.8 g) was dissolved in 5 and 35 mL of ultra-pure water, respectively. Then l ml Poly (acrylic acid) (PAA, 50%) was added into the solution of cerium nitrate under continuous stirring. After aging for 30 min, the mixture was removed to a hydrothermal reactor and kept at 180 ℃ for 24 h. Then the mixture was centrifuged and washed with ultra-pure water to collect the CeO_2 nanocubes.

2.4 Synthesis of CeO_2-GOx

The aqueous solution of CeO_2 (10 mg/mL; 20 mL) was added with 10 mg EDC and NHS, and reacted for 2 h under magnetic stirring. After that, the activated CeO_2 was added into the aqueous solution of GOx (5 mg/mL; 2 mL), and kept stirring for another 4 h. Then the mixture was centrifuged and washed with ultra-pure water to obtained the CeO_2-GOx nanocubes.

2.5 Synthesis of CeO_2-GOx@CCM

The HeLa cell membranes were obtained according to a reported method. Typically, membrane protein extraction buffer solution was added into the collected cancer cells, and kept for 20 min in an ice bath. Subsequently, these cancer cells were treated with freezing-thawing process for three time. After that, the resulting mixture was subjected to centrifugation at 1000 g for 8 min. Finally, the supernatant was centrifuged at 14 000 g for 30 min to obtained the HeLa cell membranes. Then, 10 mg CeO_2-GOx were vigorously mixed in the 5 mL as-prepared HeLa cell membrane solutions, and then were treated in ultrasonic bath for 10 min to achieve the HeLa cell membrane coating.

2.6 Protein detection using coomassie brilliant blue method

The amount of GOx onto the surfaces of CeO_2 was determinated by Protein detection reagent (Coomassie Brilliant Blue) according to the Bradford assay. The coomassie brilliant blue G250 solution can be prepared by dilute the coomassie brilliant blue stock solution acquired from Protein detection reagent (Coomassie Brilliant Blue) seven times with deionized water. Then 2 ml coomassie brilliant blue G250 solution were mixed with 200 μL deionized water, 200 μL CeO_2-GOx (2 mg/mL) solution and the mixture of 100 μL standard protein solution (0.563g/μL) and 100 μl deionized water respectively for ten minutes. The protein content can be reflected by the absorbance values at 595 nm of three groups measured by UV/Vis spectrophotometer, which is calculated by formula：Protein Content $=[OD_{sample} - OD_{control}]/[OD_{standard} - OD_{control}]/2/2*0.563*100\%$.[2]

2.7 Catalytic cctivity measurements of CeO_2-GOx

To investigate the pH change of CeO_2-GOx with the addition of glucose, CeO_2-GOx was added into 5 ml glucose (10 mg/mL) solution or 5 ml distilled ultra-pure water. At a predetermined time, the pH of each mixture was detected by using a pH meter. Then, the increased H_2O_2 in the different concentrations of CeO_2-GOx (0, 50 and 250 μg/mL)

solutions were detected at the glucose concentration of 25 mg/mL using ammonium molybdate spectrophotometric method[3]. Before determination, a calibration curve was recorded by measuring the absorbance values at the absorbance of 2 ml 2.4 mmol/L molybdate solution with the addition of 1 ml H_2O_2 solution with different concentration (0,1,1.25, 2.5, 5 and 10 mM) at 350 nm.

2.8 Catalase-like Activity of CeO_2-GOx

The ability of CeO_2-GOx to decompose H_2O_2 was evaluated from two aspects: the increase of dissolved O_2 in 20 mL CeO_2-GOx solutions with different concentration (0, 25 and 50 μg/ml in PBS buffer) in the presence of H_2O_2 (10 mM) and the decrease of H_2O_2 in 25ml CeO_2-GOx solutions with different concentration (0, 100 and 200 μg/ml in PBS buffer with a pH of 7.4) in the presence of H_2O_2 (5 mM). On one hand, the generated O_2 was detected using a dissolved oxygen meter. On the other hand, the amount of the residual H_2O_2 was measured by using ammonium molybdate spectrophotometric method[3].

2.9 Detection of Hydroxyl Radical (•OH) production by CeO_2-GOx

The 1 ml 3,3′,5,5′-tetramethyl-benzidine (TMB, 3 mM) was dissolved in PBS buffer (2 mL) with the addition of 1 mg CeO_2-GOx and 13.5 mg glucose under different pH condition (pH= 6.2,6.6 and 7.4) at 37, respectively. The amount of hydroxyl radical (•OH) in measured liquid can be detected by measured the absorbance at 652 nm using a UV/Vis spectrophotometer at different time points.[4]

2.10 Cell Culture

HeLa cells (the Human cervical cancer cells), U87MG cells (the human primary glioblastoma cells) and HUVEC cells (Human Umbilical Vein Endothelial Cells) were incubated in DMEM medium containing 10% FBS and 1% antibiotics (penicillin–streptomycin, 10 000 U/mL) at 37 °C and 5 % CO_2 incubator.

2.11 Endocytosis and Intracellular Distributions of Nanoparticles.

The targeting and endocytosis of IR-806 labelled CeO_2-GOx and CeO_2 were observed using a CLSM. The HeLa and HUVEC cells were cultured in confocal dish for 24 h. Then, the cells were treated with IR-806 labelled CeO_2-GOx and CeO_2 (0.1 mg/mL) in DMEM culture medium and maintained at 37 °C in 5% CO2 for 4 h. After that, the cells were washed twice with fresh medium, stained with Hoechst 33342 for 15 mins, and imaged with CLSM. The confocal images were captured with 405 nm excitation for Hoechst 33342 and 806 nm excitation for IR-806. Moreover, the collected cells were analyzed by flow cytometry(Cytomics FC500, Beckman Coulter, USA).

2.12 Intracellular ROS Levels Measurement

HeLa cells were incubated with CeO_2-GOx (50 μg/ml) and CeO_2 (50 μg/ml) under different pH condition (pH=6.2, 6.6 and 7.4) for 12 h at 37 °C. And the normal cells (HUVEC cells) were also incubated with CeO_2-GOx (50 μg/ml) and CeO_2 (50 μg/ml) in neutral (pH = 7.4) culture mediums for 12 h at 37°C. To measure the level of the intracellular ROS, cultured cells were then washed with PBS twice, and incubated with ROS probe DCF-DA (20 μM) for 30 min. The intracellular fluorescence can be observed by fluorescence microscope.

2.13 Cell Cytotoxicity of CeO_2-GOx

CCK-8 assay was applied to evaluate the cytotoxicity of CeO_2-GOx nanoparticles over various cancer cells (HeLa, U87MG and HUVEC cells). First, HeLa, U87MG and HUVEC cells were cultured on 96-well plates for 24 h. Then, HeLa and U87MG cells were incubated with CeO_2-GOx at varied concentrations (50, 25, 12.5 and 6.25μg ml^{-1}) under different pH condition (pH=6.2, 6.6 and 7.4) with a Glucose concentration of 2 mg/ml for 24 h at 37 °C. And the normal cells (HUVEC cells) were also incubated with the gradient concentrations of CeO_2-GOx in neutral (pH = 7.4) culture mediums with the same conditions. The cells grown without any particles were used as a control. After the treatment, cells were washed with fresh culture medium to remove the residual NPs, incubated with 100 ul fresh culture medium after the addition of 10 μl CCK-8 solution for 2 h. Finally, the absorbance was monitored using a microplate reader at the wavelength of 450 nm for analyzing the cell viability.

To visually observe the cell cytotoxicity of nanoparticles, calcein-AM and PI solution were used to show the viable and dead cell distributions after the treatment of CeO_2-GOx. After HeLa and U87MG cells incubated with 50 μg ml^{-1} CeO_2-GOx under different pH condition (pH=6.2 , 6.6 and 7.4) for 24 h and HUVEC cells incubated with 50 μg ml^{-1} CeO_2-GOx in neutral (pH = 7.4) culture mediums for 24 h, these cells were then stained with PI and calcein-AM solution for another 30 mins, and observed using a fluorescence microscope.

2.14 Biocompatibility of CeO_2

HeLa and U87MG cells were seeded in 96-well plate at cell densities of 10000 cell per well, and incubated for 24 h. After that, different concentrations of CeO_2 (100, 50, 25, 12.5 and 6.25 μg/ml) was added into each well and co-incubated for another 24 h. Then, the cell viabilities of U87MG and HeLa cells were measured via the aforementioned CCK-8 assay.

2.15 *In Vivo* imaging.

The *in vivo* imaging of nanocubes was performed on HeLa tumor-bearing nude mices, which intravenously injected with 200 μL CeO_2-GOx or CeO_2-GOx@CCM at the concentration of 1 mg/mL. After that, the fluorescence images were recorded via an IVIS imaging systems at 0, 24, 48, and 96 h. At 96 h after injection, the nude mice were sacrificed and the major organs of heart, liver, spleen, lung, kidney, and tumor were collected, and analyzed by using *In Vivo* imaging systems.

2.16 *In Vivo* anticancer experiments

The animal study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the "Rules for Experimental Animals" published by the Chinese Government (http://www.lascn.net/Item/952.aspx). The animal study was approved and conducted in accordance with the policies set by the committee on the Ethics of Animal Experiments of the University of the Harbin Institute of Technology (Permit Number: IACUC2021067). HeLa cancer cells (5×10^6) were collected in PBS and injected in nude mice (male) to construct the tumor model. When tumor volume grows to 100 mm^3, these mice were randomly divided into five groups ($n = 6$) and received treatment. The tumor-bearing mice were intravenously (i.v.) injected with 200 μL PBS or PBS containing various nanoparticles (i.e., CeO_2, GOx or CeO_2-GOx) at the concentration of 5 mg mL^{-1}, respectively. During 14 days after the corresponding treatments, their body weights and tumor volume were measured every other day to evaluate the therapeutic effects. To further evaluate the treatment efficiency, all these mice were sacrificed after 14-day treatments, and major organs of spleen, liver, heart, kidney, lung, and tumor were collected and fixed in formalin. After embedded within paraffin, these fixed tissues were then stained with hematoxylin and eosin, and observed with a microscopy for histopathological analysis.

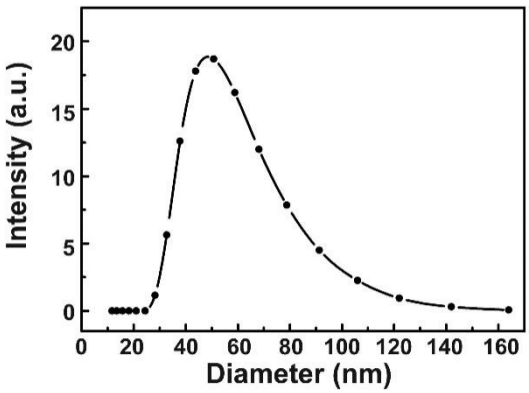

Figure S1. The DLS spectrum of CeO_2 nanocubes. DLS spectrum shows the as-synthesized CeO_2 particles have a a monodispersed size distribution centering at 50 nm.

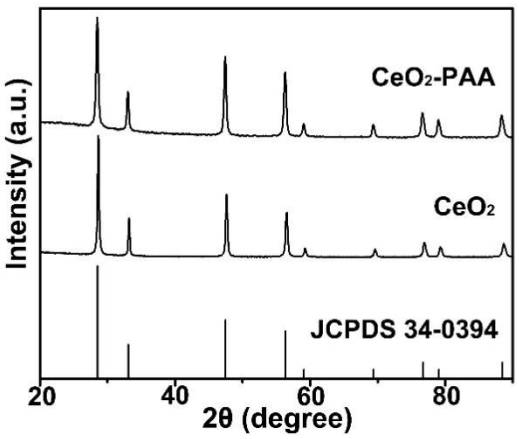

Figure S2. The XRD spectra of CeO_2 and PAA modified CeO_2. And them were in a utterly comported with the diffraction pattern of CeO_2 in JCPDS card 34-0394, which shows that the CeO_2 core has a cubic lattice[6] and the addition of PAA didn‘t change the crystal structure of CeO_2.

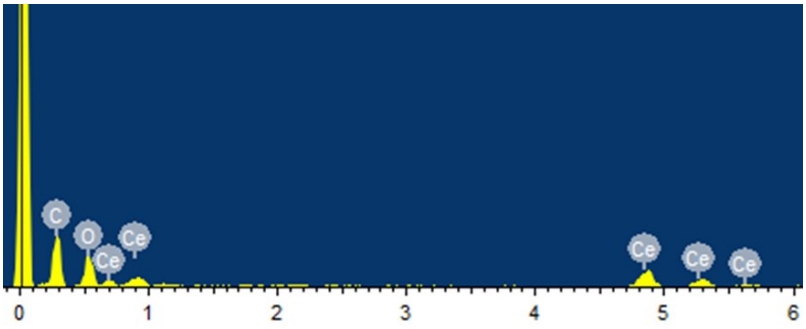

Figure S3. Energy-dispersive X-ray spectroscope (EDS) spectrum of CP (CeO_2). All the major elements (Ce, O, C) are found in the spectrum. Among these elements, the atomic percent of the Ce coming from CeO_2 is 7.10% and the atomic percent of the C coming from PAA is 65.38%, while the same percent of O which not only comes from CeO_2, but also comes from PAA is 27.52%.

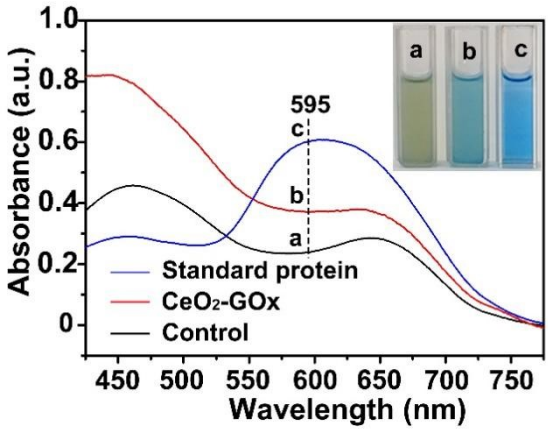

Figure S4. Ultraviolet absorption spectra obtained by protein modification detection of CeO_2-GOx by coomassie brilliant blue. The absorbance measurements at 595 nm is linear versus protein concentration, so that the protein concentration of the measured liquid can be acquired by 0.563 g/ml*[$OD_{(samples)}$-$OD_{(control)}$]/[$OD_{(standard)}$-$OD_{(control)}$]/2. Then the protein percentage of CeO_2-GOx calculated by protein concentration / nanoparticle concentration is 3.5%, which is consistent with the TG values. Insert shows the color change of different samples.

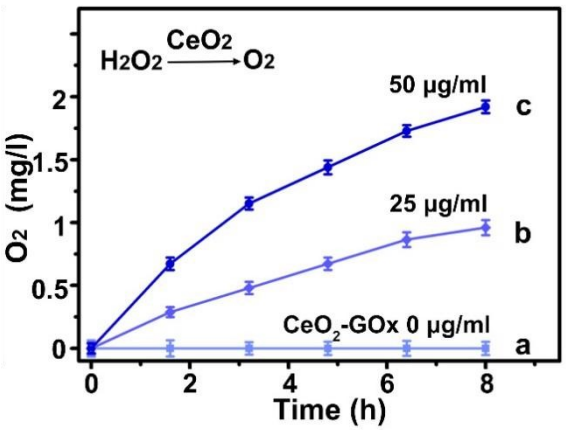

Figure S5. The decomposition of 5mmol/L H_2O_2 solution with the addition of different concentration of CeO_2-GOx (0,100 and 200μg/mL) at pH=7.4. The fact with the production of oxygen could verify the capacity that CeO_2 transform hydrogen peroxide to nontoxic water and oxygen under neutral condition, which is considered as the ability for protecting normal cells.

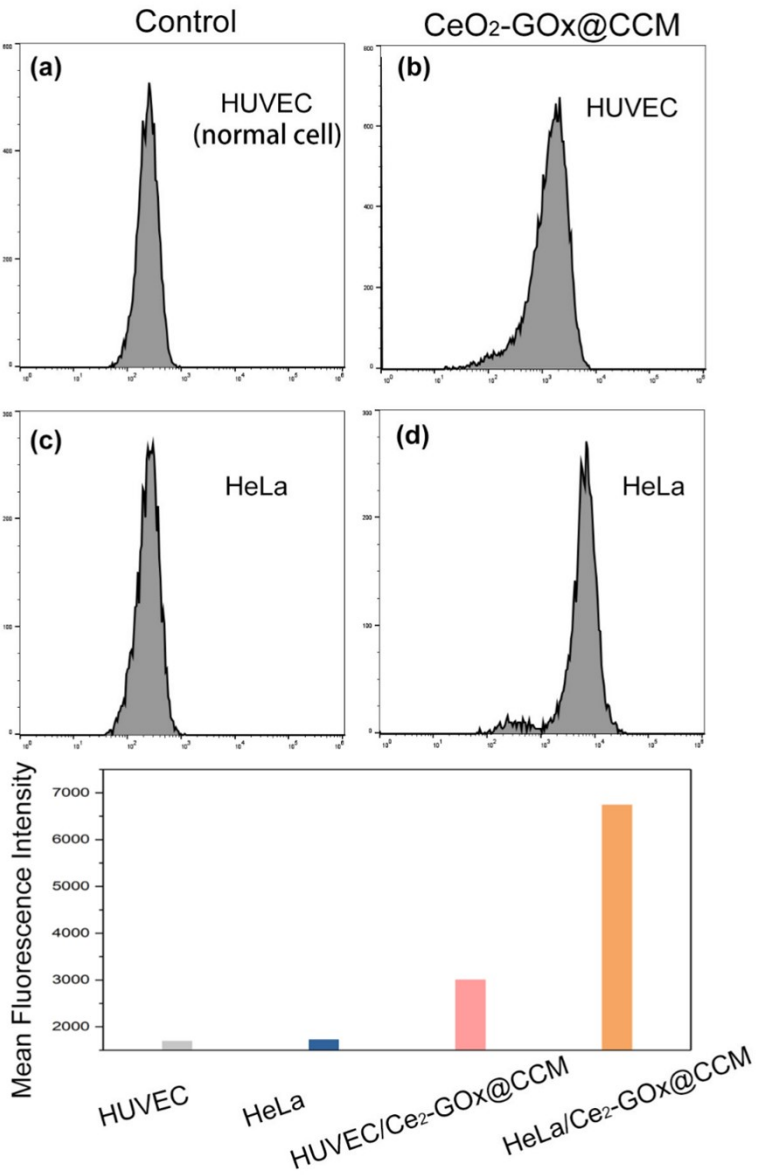

Figure S6. Flow cytometry analysis of HUVEC cells and HeLa cells after treatment with CeO_2-Gox@CCM using HUVEC cells and HeLa cells without any treatment as controls, and their corresponding mean fluorescence intensity (MFI) analysis.

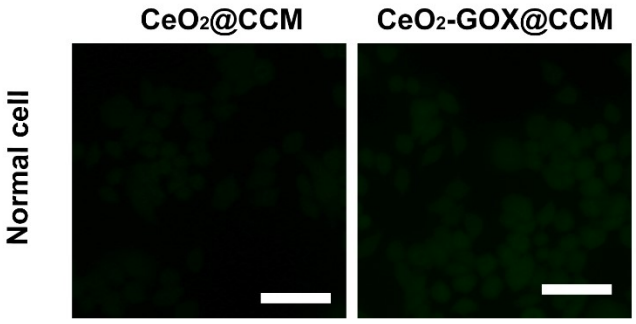

Figure S7. CLSM images of normal cells (HUVEC) stained with fluorescence probe DCFH-DA for ·OH detection after co-incubated with CeO_2@CCM and CeO_2-GOx@CCM at pH 7.4; Scale bar = 100 μm.

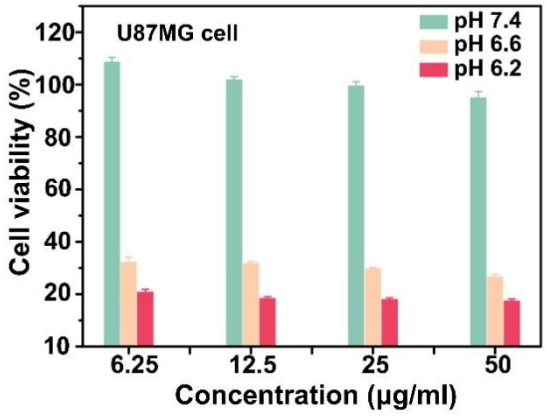

Figure S8. Cytotoxicity of different concentrations (50, 25, 12.5and 6.25μg ml^{-1}) of CeO_2-GOx against U87MG cells under acidic (pH = 6.2), weak acidic (pH=6.6) and neutral (pH = 7.4) conditions.

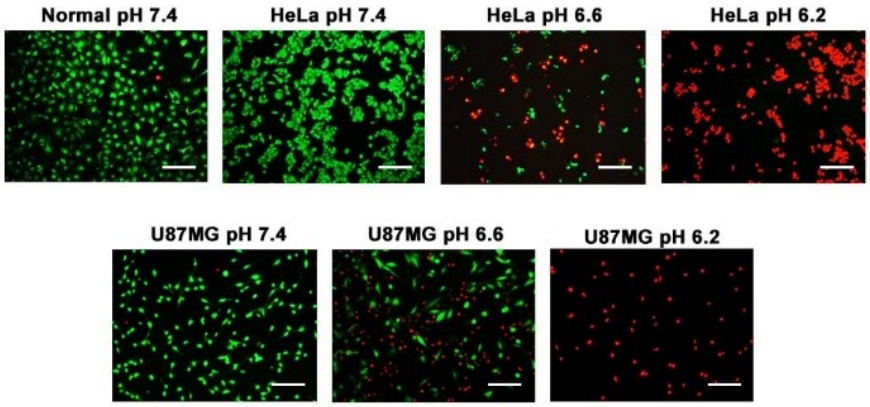

Figure S9. Fluorescence microscope images of viable and dead cell distributions of HeLa, U87MG cells in acidic (pH = 6.2), weak acidic (pH=6.6) and neutral (pH = 7.4) culture mediums and normal cells in neutral (pH = 7.4) culture mediums stained with calcein-AM/PI solutions, after the addition of CeO_2-GOx for 24h. Scale bar = 500μm. Obviously, A majority of dead HeLa and U87mg cells are observed under the acidic condition and partial death of them are observed under the weak acidic condition, while no significant of cells are damaged under neutral condition,no matter what cells. These observations match well with the cytotoxicity profiles of CeO_2-GOx-IR-FA, showed the capacity to selectively kill tumor cells while leaving normal cells undemaged, which is the tumor selectivity.

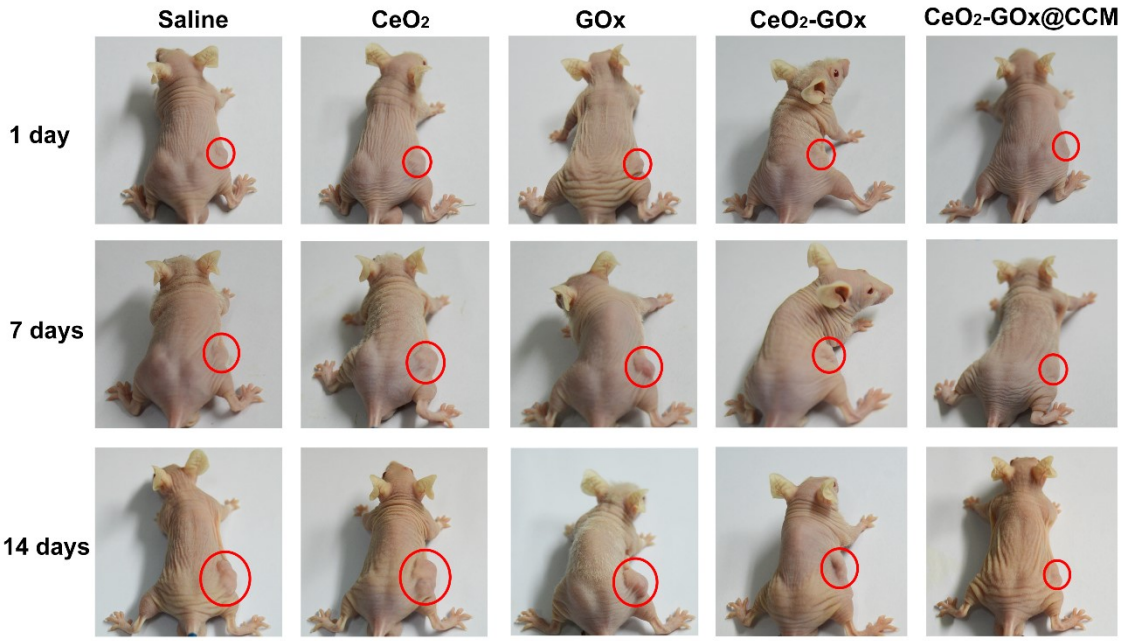

Figure S10. Digital photos of tumor-bearing mice with different treatments at day 1, 7 and 14. As shown in Figure S9, the tumor was completely suppressed with the treatment of CeO_2-GOx@CCM for 14 days, while both CeO_2 and GOx exhibited a rather low anticancer effect, demonstrating that the cascade reactions of CeO_2-GOx@CCM amplify the ROS-mediated anti-cancer effects through the cancer microenvironment.

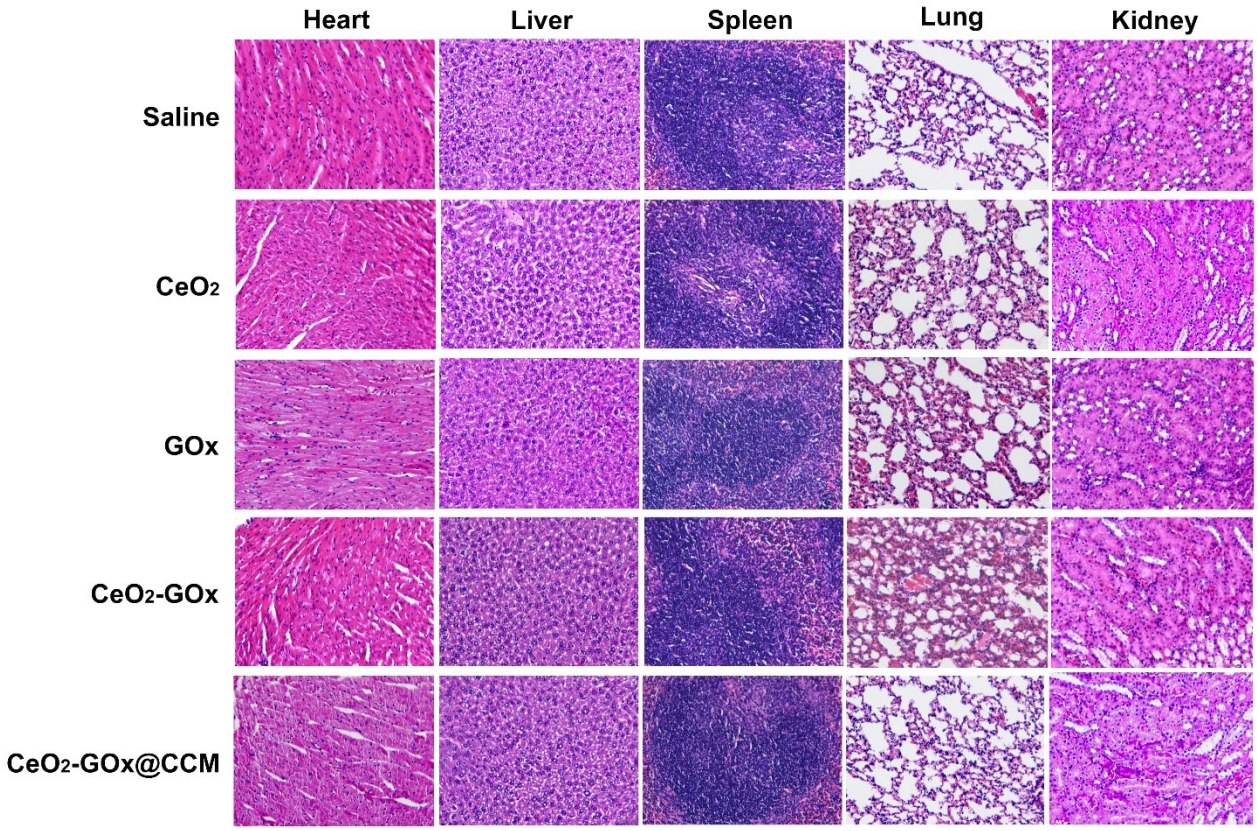

Figure S11. Hematoxylin and eosin (H&E) staining of heart, liver, spleen, lung and kidney in the mice from various groups at the end of treatments.

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References

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