Supporting information for

A multifunctional metal-based nanozyme for CT/PTI-guided photothermal/starvation/chemodynamic therapy against colon tumor

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Materials and Methods

1 Materials

Cerium (III) Nitrate Hexahydrate ($CeN_3O_9·6H_2O$), inositol hexaphosphate (IP6), and 3,3',5,5'-tetramethylbenzidine (TMB) were obtained from Macklin Ltd. Chloroauric acid ($HAuCl_4$) and sodium citrate ($C_6H_5O_7Na_3$) were sourced from Aladdin Reagents Ltd. Hoechst 33258 staining solution was purchased from Shanghai Beyotime Bio-reagent Co. The CM-H_2DCFDA reactive oxygen species detection kit was obtained from Invitrogen, USA, while the CCK-8 kit was acquired from MedChemExpress. Calcein-AM/PI live-dead cell staining reagent was sourced from Beijing Solarbio Technology Co. All reagents were used as received without further purification.

2 Characterization

Transmission electron microscopy (TEM) images were obtained using a Tecnai G20 (FEI, USA). Field emission scanning electron microscopy (FESEM) images and energy dispersive spectroscopy (EDS) data were collected with a SIGMA500 (Carl Zeiss, Germany). X-ray photoelectron spectroscopy (XPS) spectra were measured using an Escalab 250Xi (TF, USA). The crystalline structure was analyzed with an X-ray diffractometer (D8 Advance, Bruker, Germany). Compositional structure and chemical bonding information were obtained from a UV-visible (UV-vis) spectrophotometer (UV 1800 PC, Mapada, China) and an FTIR spectrometer (Nicolet iS50, TF, USA). Average hydrated particle size, distribution, and zeta potential were measured using a dynamic light scattering scanner (ZS90, Malvern, UK). Elemental species and their contents were detected by ICP-MS (NexlON 2000, PerkinElmer, USA), while thermal images were acquired using an IR thermal imager (PC210, Guide, CN).

3 Preparation of CeO_2@Au@IP6 nanoparticles

CeO_2 Nanoparticles (CeO_2 NPs): 30 mL of ethylene glycol, 1 g of $Ce(NO_3)_3·6H_2O$, and 400 mg of PVP were combined and dispersed using ultrasound. Following this, 2 mL of deionized water was added, and the mixture was stirred for an additional 30 minutes. The resulting solution was then transferred to a PTFE-lined autoclave (50 mL) and heated at 160℃ for 8 hours. After cooling to room temperature, the product was collected and washed three times with deionized water and anhydrous ethanol (centrifuged at 8000 rpm/min for 10 min). The purified solids were then dried and calcined in a muffle furnace at 300℃ for 1 hour. The final CeO_2 NPs were collected and stored in a refrigerator at 4℃.

CeO_2@IP6 Nanoparticles (CeIP): CeO_2 NPs (30 mg) were dispersed in a 4 mL aqueous solution of IP6 (70%) and subjected to ultrasound at room temperature for 1 hour. The mixture was then stirred vigorously for 12 hours. After this, the product was centrifuged and washed three times with deionized water (at 10,000 rpm/min for 10 min) and subsequently freeze-dried to obtain CeIP.

CeO_2@Au Nanoparticles (CeA): CeO_2 NPs (30 mg) were added to 10 mL of deionized water and dispersed using ultrasound for 30 minutes, followed by vigorous stirring on a magnetic stirrer. $HAuCl_4$ solution (250 mM, 0.06 mL) was added dropwise while stirring. After 1 hour, the temperature was raised to 90℃, and sodium citrate (0.1 M, 1 mL) was added. The reaction was maintained at 90℃ for 6 hours, then cooled, centrifuged (10,000 rpm, 10 min), and freeze-dried to obtain CeA.

CeO_2@Au@IP6 Nanoparticles (CeAIP): CeA (15 mg) were dispersed in a 2 mL aqueous solution of IP6 (70 wt%) using ultrasonic dispersion at room temperature for 1 hour, followed by vigorous stirring for 12 hours. The products were collected, centrifuged, and washed three times with deionized water (10,000 rpm/min for 10 min), then freeze-dried to obtain CeAIP.

4 Stability measurement

CeO_2 NPs, CeA, CeIP, and CeAIP (100 μg/mL) dispersions in deionized water were prepared to evaluate dispersibility after 14 days. Additionally, CeAIP were dispersed in deionized water, DMEM, and PBS, and their size was measured by DLS every two days for 14 days to assess stability.

5 Assessment of catalase-like (CAT-like) activity

CeO_2 reacts with hydrogen peroxide to produce oxygen ($2CeO_2 + H_2O_2 + 6H^+ = 2Ce^{3+} + 4H_2O + O_2$). CeO_2 and CeAIP (400 μg/mL, 4 mL) in PBS, plus a PBS control, were treated with hydrogen peroxide (200 μL, 1 M). Oxygen concentration was measured for 15 minutes with data recorded every 20 seconds using a dissolved oxygen meter.

6 Photothermal performance in vitro

Au NPs exhibit photothermal properties in the near-infrared spectrum, prompting the use of an 808 nm

laser to evaluate the in vitro photothermal characteristics of CeAIP. Dispersions of CeIP and CeAIP (400 μg/mL, 1 mL) were prepared, with DI water as the control. Each sample was exposed to an 808 nm laser (1.5 Wcm^2) for 10 minutes, and temperature changes were monitored and recorded using infrared thermography every 20 seconds. Additionally, the correlation between the photothermal properties and either material concentration or laser power was evaluated separately. Finally, after 10 minutes of laser exposure at 400 μg/mL, cooling temperatures were recorded every 20 seconds until recovery to the initial temperature, generating temperature difference curves post-irradiation. The photo-thermal conversion efficiency (η) was determined utilizing the below equation:

$$\eta = \frac{hS(\Delta T_{max} - \Delta T_{water})}{I(1 - 10^{-A_{808}})}$$

hS is calculated using the following equation:

$$hS = \frac{mc}{\tau}$$

τ is obtained by the following equation:

$$t = -\tau \ln\frac{\Delta T}{\Delta T_m}$$

Here, h is the heat transfer coefficient, and S denotes the area receiving laser, I is the laser power, A_{808} is the UV absorbance at 808 nm, m refers to the mass of the solution, c is the specific heat capacity of water, and τ represents the thermal constant of the system, and ΔT is the difference between the real-time temperature and room temperature.

7 Evaluation of peroxidase-like (POD-like) performance

The POD-like activity of CeAIP nanozymes was assessed in vitro using 3,3',5,5'-tetramethylbenzidine (TMB) to detect reactive oxygen radicals. Six experimental groups were established: (1) TMB, (2) TMB + H_2O_2, (3) TMB + Laser, (4) TMB + CeAIP, (5) TMB + CeAIP + H_2O_2, and (6) TMB + CeAIP + H_2O_2 + Laser. TMB: 20 μL and 1 mM, H_2O_2: 10 μL and 10 mM. CeAIP: 2 mL and 400 μg/mL. The laser parameters: 808 nm, 1.5 W/cm^2 and 2 min. Additionally, investigate the concentration dependent POD-like activity of the nanomedicine. Solutions were measured for changes at 650 nm using UV-vis analysis.

8 Glucose oxidase-like (GOD-like) properties

To evaluate the GOD-like catalytic potential of CeAIP nanozymes, a dispersion of 400 μg/mL was added to a 50 mM glucose solution, monitoring glucose concentration and pH every 2 min with a glucometer and pH meter. The generation of ·OH was confirmed via TMB colorimetric reaction. Five experimental groups were established: (1) TMB, (2) TMB + Glucose, (3) TMB + CeAIP, (4) TMB + CeAIP + Glucose, and (5) TMB + CeAIP + Glucose + Laser. TMB:20 μL and 1 mM. Glucose: 500 μL and 20 mM. CeAIP: 1.5 mL and 100 μg/mL. The laser parameters: 808 nm, 1.5 W/cm^2 and 2 min. Additionally, varying concentrations of CeAIP (50, 100, 200, 300, and 400 μg/mL) and glucose (10, 20, 30, 40, and 50 mM) were tested, with the absorbance measured around 650 nm using UV-vis analysis.

9 Cell culture

Mouse colon cancer cells (MC38) and normal human intestinal epithelial cells (HIEC) from ATCC were utilized in experiments. The complete medium consisted of 500 mL DMEM, 50 mL serum, and 10 mL double antibiotics. Cells were cultured in a thermostatic incubator at 37℃ with 5% CO^2.

10 Intracellular ROS generation

The intracellular ROS-generating efficacy of CeAIP was tested in MC38 cells (1×10^4 cells/well, 12-well plates, 24 h incubation). Six groups were set up: PBS (pH 7.4), Laser, H_2O_2, CeAIP, CeAIP + H_2O_2, and CeAIP + H_2O_2 + Laser in DMEM. After replacing the medium, incubation continued for 24 hours, followed by light treatment. Cells were stained with CM-H2DCFDA (1 μM) and Hoechst 33258 (1 μM), and observed under a fluorescence microscope after 15 minutes. On the other hand, the GOD-like properties of CeAIP were also tested in MC38 cells cultured with or without glucose for 24 h. Cells were incubated with PBS (pH 7.4), IP6, CeIP, and CeAIP for 24 h. After CM-H2DCFDA/Hoechst 33258 dual staining for 15

min, cells were observed under a fluorescence microscope. CeIP and CeAIP: 100 μg/mL. IP6: 50 μg/mL. Glucose: 4.5 g/L. The laser parameters: 808 nm, 1.5 W/cm^2 and 2 min.

11 Cytotoxicity test

CCK-8 assay was used to assess cell viability and the effects of various treatments. MC38 and HIEC cells were seeded in 96-well plates (8 × 10^3 cells/well) and incubated for 24 h. The medium was replaced with CeAIP diluted in DMEM at concentrations of 12.5, 25, 50, 100, and 200 μg/mL, and cells were incubated for another 24 h. After adding 10 μL of CCK-8 reagent per well, cells were incubated for 1 hour in the dark. Absorbance was measured at 450 nm to determine relative cell viability (OD).

To evaluate the cytotoxicity of nanomedicines, MC38 cells were treated with gradient concentrations of IP6 (3.125, 6.25, 12.5, 25, and 50 μg/mL), CeIP, and CeAIP (12.5, 25, 50, 100, and 200 μg/mL). PBS (pH 7.4) served as the control. Cell viability (%) = ($OD_{450\ experimental\ group}$ - $OD_{450\ blank\ group}$) / ($OD_{450\ control\ group}$ - $OD_{450\ blank\ group}$) × 100%.

Tumor cell survival and mortality were assessed qualitatively via calcein-AM/PI co-staining. MC38 cells were seeded in 24-well plates (2 × 10^4 cells/well) and incubated for 24 h before treatment with PBS (pH 7.4), laser (808 nm, 1.5 W/cm^2, 2 min), IP6 (50 μg/mL), CeIP, CeAIP (200 μg/mL), and CeAIP + laser. After 15 minutes of incubation with 2 μL of Calcein-AM (1 mg/mL in DMSO) and 3 μL of PI (1 mg/mL in PBS), cells were washed three times, fixed, and observed under an inverted fluorescence microscope.

12 Animal model establishment

Subcutaneous injections of 2 × 10^6 MC38 cells were performed in five-week-old female C57BL/6 mice (purchased from Wuhan Mobili Biotechnology Co.). Experiments began when tumor volumes reached approximately 200 mm^3, calculated using the formula: tumor volume (mm^3) = (tumor length × tumor width^2)/2. All procedures were approved by the Animal Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology.

13 Haemolysis test

Mouse erythrocytes were isolated by centrifugation (1000 rpm, 5 min) and diluted in PBS (pH 7.4) to a concentration of approximately 5 × 10^7 cells/mL. A 500 μL aliquot of the erythrocyte solution was co-incubated with gradient concentrations of CeAIP (6.25, 12.5, 25, 50, 100, 200, 300, and 400 μg/mL) for 4 hours at 37℃. After incubation, the solution was centrifuged (1000 rpm, 10 min), and the supernatant was collected for analysis in a 96-well plate using an enzyme-linked immunosorbent assay (ELISA) reader. Relative haemolysis (%Hem) = (As-Aneg)/(Apos-Aneg) × 100%, where As, Aneg, and Apos represent the OD_{492} values of the treated, negative control, and positive control groups, respectively. The negative control consisted of untreated erythrocytes in PBS, and the positive control was erythrocytes treated with 1% Triton X-100.

14 Circulation and tissue distribution

Nanomedicine distribution in mice was assessed by measuring cerium ion levels in blood and tissues at various time points. Mice (n=3) with confirmed tumors were intravenously injected with CeAIP dispersion (250 μL, 3 mg/mL) via the tail vein. Blood samples (10 μL) were collected at designated intervals, dissolved in aqua regia, and cerium ion content was analyzed using ICP-MS. The half-life of cerium ions in blood was calculated using a two-compartment model. For tissue distribution, a separate group of mice (n=6) with tumors received the same CeAIP injection. After 12 and 24 hours, three mice per time point were sacrificed, and their hearts, livers, spleens, lungs, kidneys, and tumors were dissected and dissolved in aqua regia for cerium ion analysis by ICP-MS.

15 CT assessment

In vitro CT imaging of CeAIP was performed at various concentrations (0.3, 0.6, 1.2, 2.5, 5, and 10 mg/mL) in a 96-well plate format, using a CT detector (Aquilion TSX-101A). For in vivo CT imaging, CeAIP (250 μL, 3 mg/mL) were intravenously injected into tumor-bearing mice via the tail vein. CT images were captured at 6, 12, 24, and 48 hours post-injection. The imaging parameters were: HP = 41.0, PF = 0.641, Voltage = 120 kV, Current Time Product = 40 mAs, Layer Thickness = 0.5 mm, Reconfiguration Layer Thickness = 1.0 mm, and Layer Spacing = 0.5 mm.

16 In vivo antitumor efficacy

CeAIP (250 μL, 3 mg/mL) were intravenously injected into tumor-bearing mice via the tail vein. Twenty-four hours later, laser irradiation (808 nm, 1.5 W/cm^2, 10 min) was applied to the tumor area, with real-time tumor temperature monitored using an IR thermographic camera to evaluate the in vivo photothermal efficacy of the nanomedicine. To assess the anti-tumor effects of the treatment, six

experimental groups were established based on preclinical tests: PBS, Laser, IP6, CeIP, CeAIP, and CeAIP + Laser. Laser irradiation was applied 24 hours post-injection to mice in the laser group. Body weight and tumor volume were monitored in each experimental group over a 14-day treatment period. After 14 days, the mice were euthanized, and the major organs and tumor masses were weighed and preserved in paraformaldehyde. The in vivo anti-tumor efficacy of the nanoparticles was further evaluated by H&E staining analysis of the tissue sections.

17 Statistical analysis

Statistical analysis was performed on experimental data utilizing SPSS 18.0 in mean ± standard deviation format. Values from diverse experimental groups were compared employing one-way variance analysis (ANOVA). Statistical significance was denoted with asterisks (*), as follows: $P < 0.05$, $*P < 0.01$, and $***P < 0.001$.

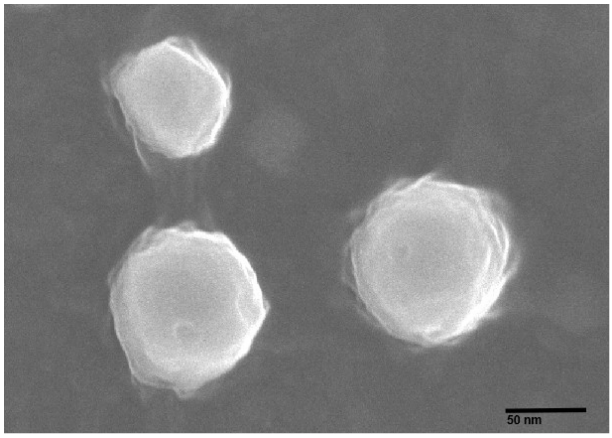

Fig. S1. FE SEM image of CeO_2 NPs.

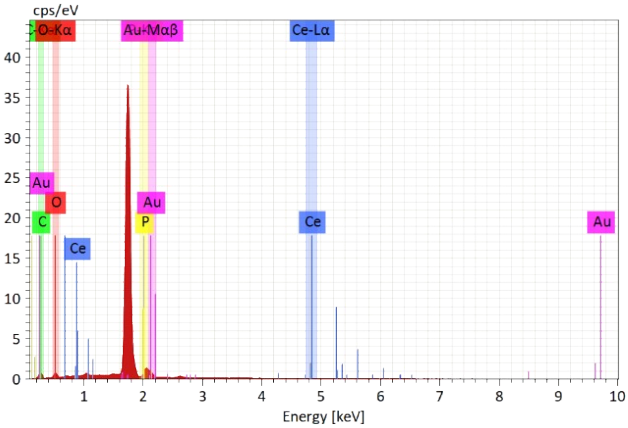

Fig. S2. EDS energy spectrum of CeAIP.

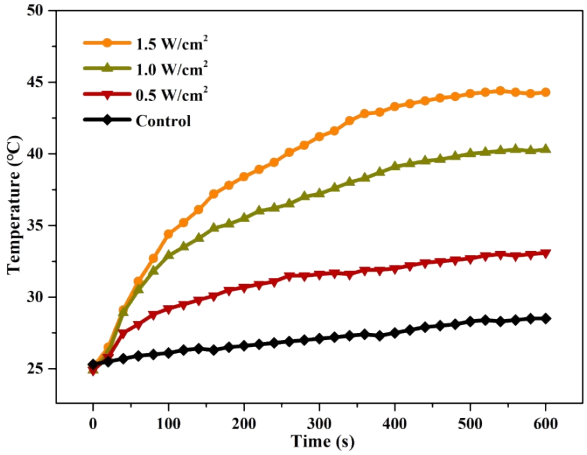

Fig. S3. Temperature change curves of CeAIP (400 μg/ml) under 808 nm laser irradiation at different powers (0.5, 1.0, 1.5 W cm^{-2}) for ten minutes.

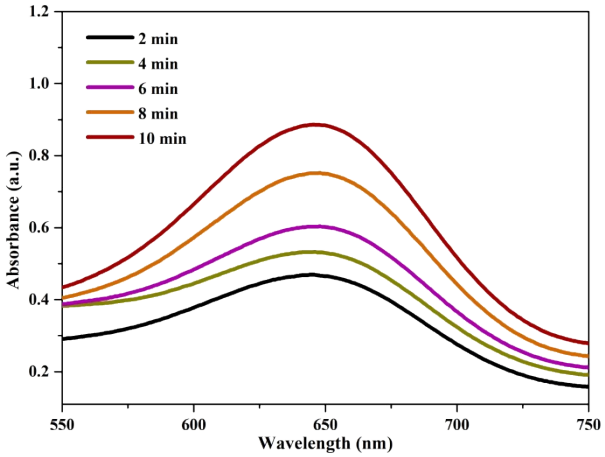

Fig. S4. Uv absorption spectra of TMB + CeAIP + glucose at different time intervals.