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A Multimodal strategy of Fe_3O_4@ZIF-8/GOx@MnO_2 hybrid nanozyme via TME modulation for tumor therapy

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Characterization

The microstructure and morphology were observed by transmission electron microscopy (TEM, JEM2010, JEOL, Japan) at 200 kV. The crystal phase analysis was investigated by X-ray powder diffraction with Cu Kα radiation (XRD; Bruker D8 Advance X-ray diffractometer). Dynamic light scattering (DLS) measurement was gained by Malvern ZEN3700 zetasizer. The valence state of various elements was analyzed by X-ray photoelectron spectra (XPS) on a Thermo ESCALAB 250 Xi using Al Kα (1486.6 eV) as the excitation source. Thermal gravimetric analysis was performed by SDT 650. Electron spin resonance (ESR) signal was obtained on a Bruker E-500 spectrometer. The generated oxygen concentration was monitored by dissolved oxygen meter (LH-D9, Hangzhou). Confocal laser scanning microscopy (CLSM) images were taken with a LEICA TCS SP5 confocal microscope (LEICA, Germany). Temperature changes were recorded by a near-infrared imager (RX-500). Fourier Transform Infrared (FT-IR) spectroscopy spectra were carried out by a Nicolet iS5 FT-IR spectrometer. CCK8 assay was carried out to examine the cell viability using a microplate reader at 490 nm (Spectra Max M2/M2e). Flow cytometic analysis was carried out from FACSCelesta™.

Synthesis of Fe_3O_4/5-AF@ZIF-8/GOx@MnO_2

In brief, 5-Aminofluorescein (5-AF) (10 mg) was dissolved in deionized water at room temperature, after which EDC (20 mg, 0.104 mmol), NHS (10 mg,0.087 mmol) and Fe_3O_4 (10 mg) were added and stirred for 24h. Then, the resulting mixture was collected by centrifugation at 4000 rpm for 10 min followed by being washed three times using deionized water and dried in a vacuum freeze drying oven for 24 h. The above solution dispersion liquid (4 ml, 0.25 $mg \cdot ml^{-1}$) was added in methanol solution to 2-MeIm (26 ml, 0.82 g). The mixture was treated with ultrasound for 30 min, and supplemented with 10 ml of $Zn(NO_3)_2 \cdot 6H_2O$ in methanol (30 $mmol \cdot l^{-1}$) and kept at room temperature

for 1 h. Finally, the product was separated and washed with methanol. The remaining steps are the same as the preparation of FZGM.

In vitro photothermal effect of FZGM composites

Upon 808 nm laser irradiation for 14 min, the temperature of Fe_3O_4 and FZGM aqueous solution (0.5 mg·ml^{-1}, 1 ml) in a centrifuge cube (1.5 ml) was measured by an infrared thermal imager (RX-500). The temperature of FZGM with different concentrations (0, 0.2, 0.5 mg·ml^{-1}, 1 ml) were recorded in the same way and the pictures were taken from the infrared thermal imager. 500 μg·ml^{-1} FZGM (1 ml) solution was exposed to 808 nm laser for 10 min and then the laser was shut off, and the solution was cooled naturally. The same process was repeated for three times and the temperature was monitored using the infrared thermal imager. The power density of 808 nm laser is 1 W·cm^{-2}.

In vivo photothermal properties of FZGM nanozymes

For *in vivo* photothermal imaging, 4T1 cells were subcutaneously inoculated into the left armpit of Kunming mice. When the tumor size reached about 200 mm^3, FZGM (3.0 mg·kg^{-1}) were injected into the mice. Then the tumor was exposed to 808 nm laser irradiation (1.0 W·cm^{-2}) for 5 min, and the photothermal images of the mice were captured by infrared thermal imager.

Test of enzymatic activity of GOx

To study the influence of temperature on enzymatic activity of GOx, it (25 μg·ml^{-1}) was dispersed in 20 ml DI water containing 0.1 M glucose. The catalytic oxidation of

glucose occurred at different temperatures (37 °C, 43 °C and 50 °C) for 6 min. The pH of the solution was measured by a microprocessor pH-meter (Mettler-Toledo LE438).

The detection for GSH consumption

A (5,5'-Dithiobis)-(2-nitrobenzoic acid) (DTNB) PBS solution (2.0 $mg \cdot ml^{-1}$, 200 μl) and GSH aqueous solution (10 mM, 20 μl) were added into FZGM aqueous solution (0, 25, 50, 100 and 200 $\mu g \cdot ml^{-1}$). After that, the mixtures were maintained at room temperature under stirring for 1 h, then FZGM were removed by centrifugation, and the absorbance at 412 nm of GSH in the supernatant was measured by a microplate reader.

In vitro simulation of H_2O_2 production

FZGM (0.5 $mg \cdot ml^{-1}$, 1ml) were added to a 2.5 ml PBS solution (pH=6.5) containing 0.72 $mg \cdot ml^{-1}$ TMB. Then 200 μl of glucose solution with different concentrations (0, 1, 2, 4, 8 $mg \cdot ml^{-1}$) was added to the mixture. After 10 min, the absorbance of the solution was recorded by UV-Vis spectroscopy.

In vitro penetration

1.0×10^4 4T1 cells per well were seeded in six-well plates and incubated for fifteen days to form MCSs. To demonstrate the deep tumor penetration of our designed hybrid nanozyme, the formed MCSs were treated with FAZM (10 $\mu g \cdot ml^{-1}$) or FAZGM (10 $\mu g \cdot ml^{-1}$) under different conditions (pH 7.4 (+), pH 6.5 (+) or pH 6.5 (-)). After 4 h co-incubation, the fluorescent images of 5-AF (λ_{ex}=488 nm, λ_{em}=515 nm) were detected by CLSM. ((-) means no glucose, (+) indicates that the amount of glucose is 10 mM)

Combined therapy in vitro

To test the anticancer effect of the FZGM nanozymes, 4T1 cells were incubated in 96-well plates for 4 h and then the culture medium was removed. 100 μL fresh medium containing Fe_3O_4, or FZGM with different concentrations of 0.2, 0.5 and 1.0 $mg{\cdot}ml^{-1}$ was added. The control group was treated with PBS. Then, the cell viabilities tests in different groups were evaluated using the CCK-8 Assay Kit.

Intracellular ROS detection

The production of intracellular ROS was monitored by a fluorogenic substrate 2', 7'-dichlorofluorescein diacetate (DCFH-DA). Briefly, 4T1 cells were cultured overnight and then the culture medium was removed and fresh RPMI 1640 medium containing DCFH-DA was added. After incubation for 30 min, the cells were washed with PBS for two times and 1$mg{\cdot}ml^{-1}$ of different samples (PBS, Fe_3O_4, FZG, FZGM and FZGM) was added. After incubation for 10 h, the last group were irradiated for 5 min under 808 nm laser at a power density of 1$W{\cdot}cm^{-2}$ and ROS generation was observed by CLSM.

O_2 generation

To verify O_2 production capacity of FZGM, H_2O_2 (1.0×10^{-4} $mol{\cdot}L^{-1}$) was added into FZGM (0.2 $mg{\cdot}ml^{-1}$) aqueous solution. The concentration of the generated O_2 was measured by a portable dissolved oxygen determining meter.

Calculation of photothermal conversion efficiency

The photothermal conversion efficiency (η) of FZGM could be calculated according to the equation (1):

$$\eta = \frac{hA(\Delta T_{max,mix} - \Delta T_{max,H_2O})}{I(1 - 10^{-A_\lambda})} \quad (1)$$

where h ($W \cdot cm^{-2} \cdot K^{-1}$) represents the heat transfer coefficient and A (cm^2) means the surface area of the container, $\Delta T_{max,mix}$ (K) and $\Delta T_{max,H_2O}$ (K) show the maximum temperature change of the FZGM dispersion and water respectively, I ($W \cdot cm^{-2}$) is the laser power and A_λ means the absorbance of FZGM aqueous solution at 808 nm. Using equation (2), h and A could be calculated:

$$t = -\frac{\sum_i m_i C_{p,i}}{hA} \ln\theta \quad (2)$$

where $\sum_i m_i C_{p,i}$ could be calculated from liner relationship of t versus –ln(θ). Mass of enzyme (2×10^{-7} kg) is too little compared with that of water (1×10^{-3} kg) and the specific heat of other materials is much lower than water. Therefore, the m_{NPs} and $C_{p,NPs}$ were neglected. The value of hA was calculated to be 0.0288.

Furthermore, η of FZGM nanoenzyme was calculated from the known parameters. $\Delta T_{max,mix}$ and $\Delta T_{max,H_2O}$ was equal to 39.8 ºC and 3.4 ºC, respectively, and I was 2.46 W because the area of the light spot was 2.46 cm^2. A_λ was calculated to be 0.4026 by multiplying the light path of FZGM nanoenzyme dispersion (in a well of 96-well culture plate, about 0.81 cm) and extinction coefficient of FZGM (0.497 per cm) at the same concentration. The heat conversion efficiency (η) of the samples and the corresponding parameters are listed in Table 1.

Table 1. The related parameters for the calculation of photothermal conversion efficiency (η) of FZGM.

Sample	$T_{max,mix}$-$T_{max,H2O}$	A_{808}	hA	η
FZGM	36.4	0.4026	0.0288	65.71%

Test of hydroxyl radical

The detection of ·OH was performed through the degradation of methylene blue

(MB). Briefly, MB (100 μg·ml^{-1}) and FZGM (0.5 mg·ml^{-1}) were mixed in the solution containing 50 ml PBS (pH = 6.5) and glucose solution with different concentrations (1, 2, 4 and 8 mg·ml^{-1}). The absorbance of the mixedsolution was assayed by UV-Vis spectroscopy. Additionally, ESR spectroscopy was also used to monitor the generation of ·OH with 5, 5 -Dimethyl-1-pyrroline N-oxide (DMPO) as a spin trapping agent.

Pathological analysis

After in vivo treatment, the mice were sacrificed and the major organs (heart, liver, spleen, lung and kidney) were harvested and fixed in 4% paraformaldehyde for hematoxylin and eosin (H&E) staining. The tumors were harvested and stained by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) immunofluorescence and H&E, and the images were obtained from a microscope (BX51, Japan).

Western blotting analysis

Western Blotting (WB) assay of caspase-3 was detected from the tumor tissue slices in different groups.

Cytokine detection

The tumors in different groups were collected after treatment and analyzed by enzyme-linked immunosorbent assay (ELISA) to determine the content of tumor necrosis factor α (TNF-α), interferon-γ (IFN-γ), interleukin-12 (IL-12), interleukin-10 (IL-10), transforming growth factor-β (TGF-β), and Granzyme B (GZMB).

Immunofluorescence staining

Tumor-bearing mice were sacrificed after treatment, and the tumor tissues were extracted, sliced and labeled with anti-CD4 (A0363), anti-CD8 (A02236-1), anti-Foxp3 (BF0630), anti-CD86 and anti-CD206. Finally, BX51 was used to observe the images of stained tumor sections.

Statistical Analysis

All data were given as mean ± standard deviation. Statistical analysis was carried out by Graphpad Prism software (version 6.0) using Student's T-test. Value of $p < 0.05$ was considered as significant, and $p < 0.01$ was considered as highly significant.

Supplementary figures

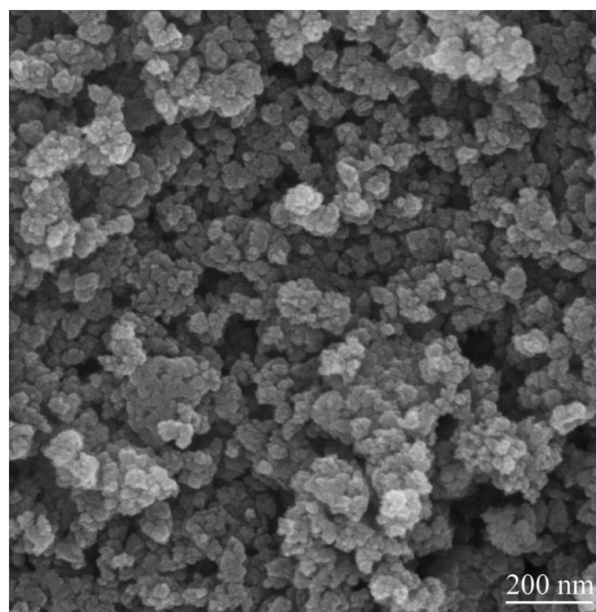

Fig. S1. SEM image of FZGM.

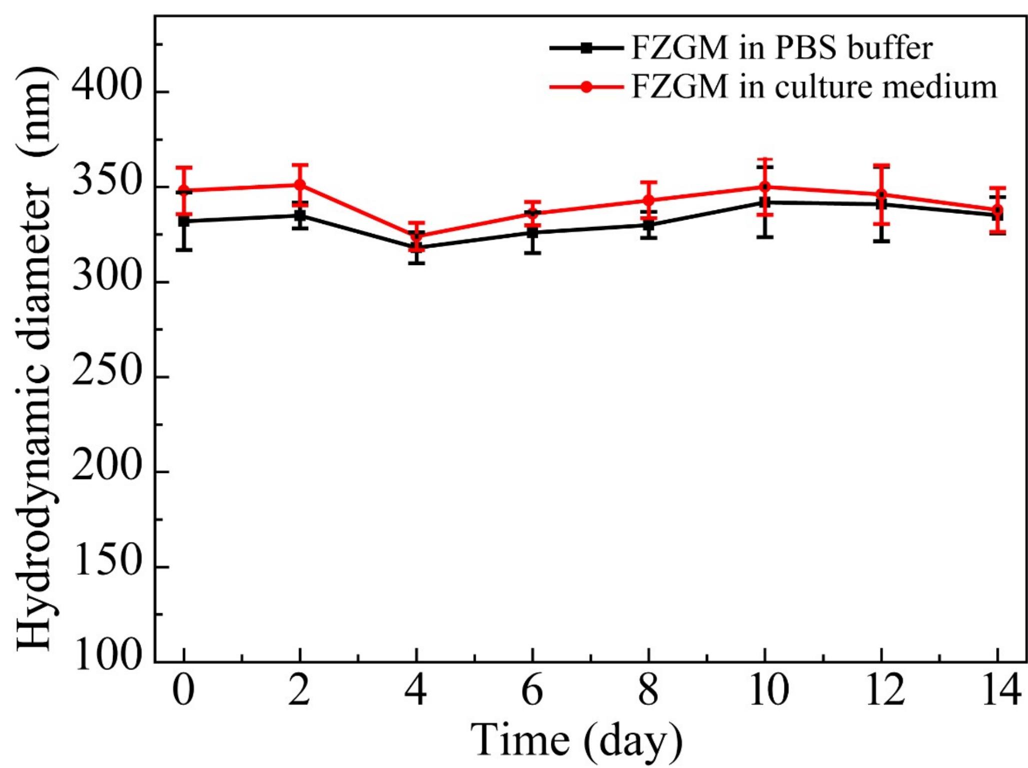

Fig. S2. The stability of FZGM in PBS buffer and culture medium.

Fig. S3. Photographs O_2 generation by FZGM with water (left) or H_2O_2 (right) addition.

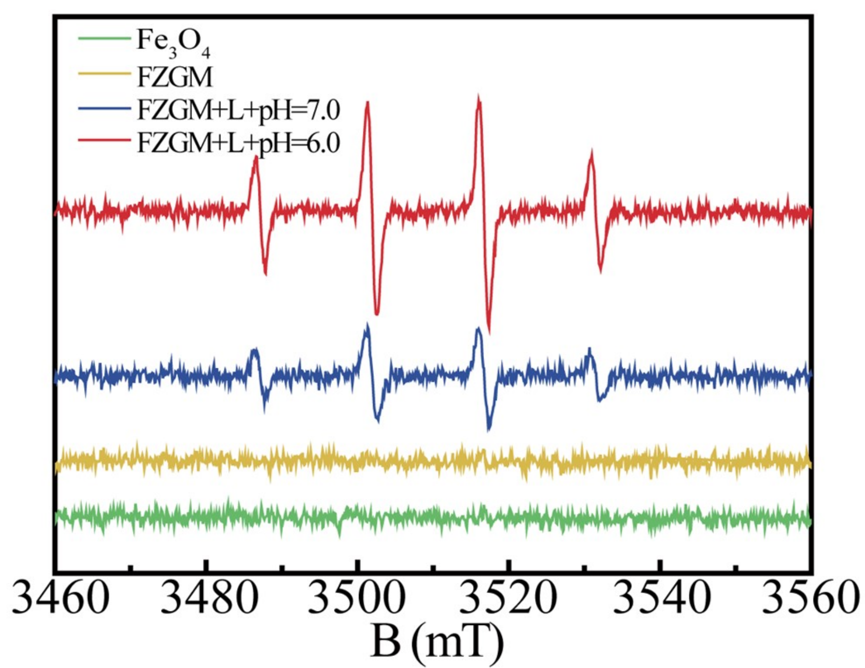

Fig. S4. ESR spectra of Fe_3O_4 and FZGM in the presence of H_2O_2 with different treatments (“L” means 808 nm laser irradiation, 1.0 $W \cdot cm^{-2}$).

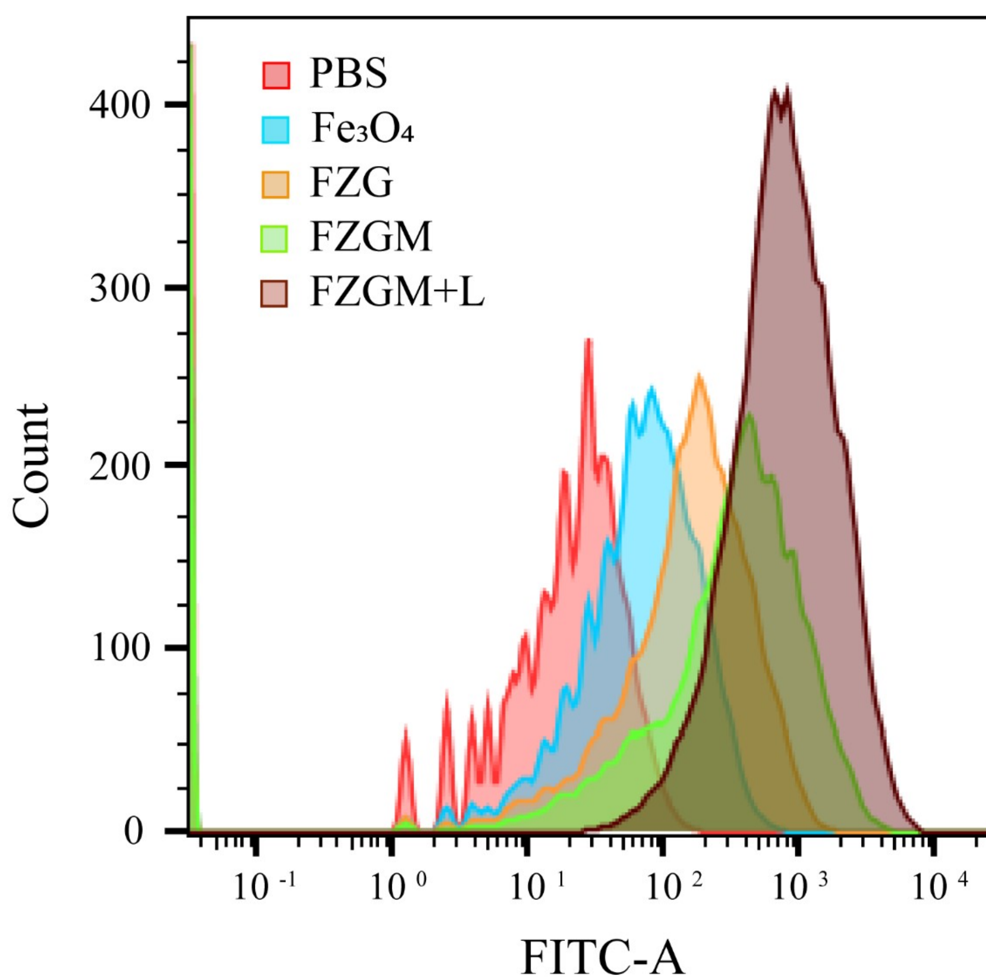

Fig. S5. Flow cytometric analysis of DCFH-DA treated 4T1 cells in different groups.

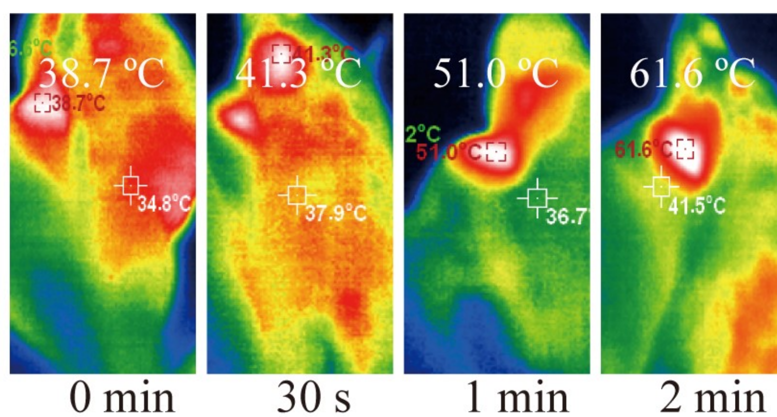

Fig. S6. Infrared thermal images of mice administrated with FZGM under laser irradiation.