Supporting Information

Gold Nanocluster as a GSH Activated Mitochondrial Targeting Photosensitizer for Efficient Treatment of Malignant Tumor

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Chemicals:

HAuCl$_4$ 3H$_2$O, Tetra-n-octylammonium bromide (TOAB), sodium borohydride (NaBH$_4$) and 1,3-Diphenylisobenzofuran(DPBF) were purchased from Aladdin Reagent, Ltd. (Shanghai, China). 2,7-Dichlorodihydrofluorescein diacetate (DCFH-DA), Calcein-AM and 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) were obtained from Sigma Aldrich (St. Louis, MO, USA). The rest of the chemical reagents were supplied by the Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). All chemical reagents were analytical grade and used without further purification. All aqueous solutions were prepared by ultrapure water (Mill-Q, Millipore, 18.25 MΩ cm). Mice were purchased from Wuhan Goodbio technology Co. Ltd. (Wuhan, China). All animal studies were performed according to the Guidelines for the Care and Use of Laboratory Animals of the Chinese Animal Welfare Committee and approved by the Institutional Animal Care and Use Committee, Wuhan University Center for Animal Experiment, Wuhan, China.
**Instruments:**

Absorption spectra were measured on a Shimadzu UV 2550 UV-Vis spectrophotometer. The fluorescence spectra were tested on a Shimadzu RF-6000 fluorophotometer. CCK-8 assay was conducted on Thermo Scientific Multiskan Mk3 microplate reader. The confocal laser scanning microscope images were acquired by Andor Revolution XD microscopy. Fourier transform infrared spectroscopy (FT-IR) tests were recorded on a NICOLET 5700 FTIR Spectrometer (Thermoelectron, USA). X-ray photoelectron spectroscopy (XPS) spectra were performed on an ESCALAB 250xi X-ray Photoelectron Spectrometer (Thermo Scientific, US). Atomic force microscopy (AFM) image was acquired by a Park AFM NX10. Field emission scanning electron microscope (FESEM) images were obtained from Zeiss Merlin Compact (Oxford Instruments, UK).
Experimental section

1. Synthesis of \( \text{Au}_{25} \) (capt)\(_{18}^- \) nanoclusters.

\( \text{Au}_{25} \) (capt)\(_{18}^- \) nanoclusters (noted as \( \text{Au}_{25} \) NCs) were prepared in a typical method at room temperature and under air.\(^1\) In briefly, \( \text{HAuCl}_4 \cdot 3\text{H}_2\text{O} \) (0.20 mM, 78.7 mg) and Tetra-n-octylammonium (TOAB, 0.23 mM, 126.8 mg) were first dissolved in 10 mL methanol and vigorously stirred for 20 min, with the solution color changing from yellow to deep red gradually. Then, captopril (1 mM, 217.2 mg) was dissolved in 5 mL methanol and rapidly added into the reaction mixture under stirring, and the color of the mixture turned to white quickly. After stirring for 30 min, \( \text{NaBH}_4 \) (2 mM, 75.6 mg) was dissolved in 5 mL of ice-cold water and injected to the reaction system with vigorous stirring. The color of the solution turned to brown-black immediately. The reaction was supposed to proceed for 8 h, and then the reaction mixture was centrifuged to remove unreacted and insoluble \( \text{Au(I)} \) polymers. The supernatant was collected and concentrated by rotary evaporation. The \( \text{Au}_{25} \) (capt)\(_{18}^- \) nanoclusters were precipitated by adding ethanol to the solution. Then, the precipitate was extracted with minimum amounts of methanol several times. Finally, the nanoclusters were obtained by adding ethanol to precipitated and dried under vacuum.

2. Synthesis of \( \text{MnO}_2 \) nanosheets.

\( \text{MnO}_2 \) nanosheets were synthesized according to previous literature report.\(^2\) Typically, 18 mL of 0.66 M tetramethylammonium hydroxide (TMAH) aqueous solution and 2 mL of \( \text{H}_2\text{O}_2 \) (30 wt\%) solution were mixed thoroughly in a round-bottom flask with constant stirring. Then, the mixture solution was injected to 10 mL of 0.3 M \( \text{MnCl}_2 \) aqueous solution within 15 s. The color of the mixture turned to dark-brown immediately and the reaction proceeded overnight at room temperature and under air. The bulk \( \text{MnO}_2 \) was obtained after centrifugation and washed with water and methanol alternatively several times. Furthermore, the as-prepared bulk \( \text{MnO}_2 \) was dried at 40 °C in vacuum. In order to obtain the \( \text{MnO}_2 \) nanosheets, 10 mg of bulk \( \text{MnO}_2 \) was dispersed in 20 mL of water. The suspension was ultrasonicated for 10 h to form layered \( \text{MnO}_2 \) nanosheets.

3. Synthesis of Mito-Au\(_{25} \).
The captopril-stabilized Au$_{25}$NCs were conjugated to a mitochondrial targeted peptide (Fr-(Cha)-K-NH$_2$)$_3$ as follow: 1 mg of Au$_{25}$(capt)$_{18}$ nanoclusters were dissolved in 1 mL of 50 mM 2-(Nmorpholino) ethanesulfonic acid (MES) buffer solution (pH=6.0), and 100 μL of peptide (0.1 mg) aqueous solution was added into it slowly. The solution was mixed thoroughly. Then 1 mg of EDC was added into the mixture solution and incubated at room temperature with constant stirring for 4 h. Then excess EDC and peptide were removed by ultrafiltration at 7500 rpm for 5 min and washed with ultrapure water at least three times. The resulting Mito-Au$_{25}$ conjugates were concentrated and stored at 4 °C for future use.

4. Preparation of Mito-Au$_{25}$@MnO$_2$ nanocomposites.

The 2.5 mg of Mito-Au$_{25}$ conjugates were adhered to 0.08 mg of MnO$_2$ nanosheets by electrostatic interaction. The Mito-Au$_{25}$@MnO$_2$ composites were obtained after centrifuging and washing with ultra-pure water for several times.

5. Detection of singlet oxygen in solution.

DPBF, a kind of $^1$O$_2$ probe, which absorbance at 412 nm will decline after react with singlet oxygen was used to monitor the generation of $^1$O$_2$. Briefly, a stock solution of DPBF in DMF (10 mM) was mixed with Au$_{25}$NCs, Mito-Au$_{25}$ or Mito-Au$_{25}$@MnO$_2$ solution to achieve final concentration of DPBF and nanocomposites was 200 μM and 200 μg/mL, respectively. The mixture was then irradiated with 808 nm laser (0.8 W/cm$^2$), and the absorption spectrum of DPBF was recorded every 30 seconds. The $^1$O$_2$ quantum yield of Au$_{25}$NCs was calculated through Equation 1.

$$\Phi_x=\Phi_s(m_s/m_x)(F_s/F_x)$$

Equation 1

Where $\Phi$ represents the $^1$O$_2$ quantum yield, $m$ is the slope of fitting line of the absorption changes of the probe (at 412 nm) with irradiation time and $F$ is the absorption correction factor calculated from $F=1-10^{-OD}$ (OD at the irradiation wavelength). $S$ and $X$ refer to standard and unknown, respectively.

6. Testing thermal stability of Mito-Au$_{25}$@MnO$_2$ nanocomposites.

The Mito-Au$_{25}$@MnO$_2$ nanocomposites were incubated at 25, 37 and 45°C, respectively. To examine the thermal stability, the UV-Vis absorption spectrum of the
nanocomposites was collected every 5 min intervals during 30 min. Absorbance value at 370 nm was chosen to draw line chart.

7. **Cellular uptake of Au\textsubscript{25}NCs and Mito-Au\textsubscript{25}@MnO\textsubscript{2}.**

The fluorescence of Au\textsubscript{25}NCs was used to investigate the cellular uptake process by confocal laser scanning microscope (CLSM). HepG2 cells were implanted into dishes and incubated for 24 h. Then Au\textsubscript{25}NCs (200 µg/mL) were co-cultured with the cells for 0.5, 1, 2 and 4h, respectively. After that, the cells were rinsed with PBS three times, and fixed with 4% paraformaldehyde for 15 min. Ultimately, the cells were rinsed with PBS three times and used for CLSM imaging. The cellular uptake procedure of Mito-Au\textsubscript{25}@MnO\textsubscript{2} was as same as Au\textsubscript{25}NCs but with different incubation time (1, 2, 4 and 6h).

8. **In vitro PDT efficiency.**

The cellular cytotoxicity was performed using CCK-8 assay. Typically, HepG2 cells were seeded in 96-well plate and incubated at 37°C in 5% CO\textsubscript{2} for 24 h. Subsequently, Au\textsubscript{25}NCs or Mito-Au\textsubscript{25}@MnO\textsubscript{2} with different final concentration (40, 80, 120 and 160 µg/mL) was added to the wells. In addition, the blank control was treated with DMEM medium only. After cellular uptake, the cells were treated with 808 nm laser irradiation (0 or 2.5 W/cm\textsuperscript{2}) for 8 min (with a 4 min interval after each 2 min of irradiation to prevent overheating). After another 20 h of incubation, 10 µL of CCK-8 stock solution was added to each well and further incubated 4 h at 37 °C. Finally, the absorbance at 450 nm of each well was measured by Thermo Scientific Multiskan Mk3 microplate reader and converted to cell viability compared with the blank control (100%).

9. **Mitochondrial targeting ability of Mito-Au\textsubscript{25}.**

Mito Tracker Green (MTG) was used to analyze colocalization efficiency. HepG2 cells underwent trypsin digestion were prepared into cell suspension and planted in confocal dishes with a proper density. After incubating for 24 h, 160 µg/mL of Mito-Au\textsubscript{25} or Au\textsubscript{25}NCs was added into the dishes, respectively, and incubated for another 2 h. Subsequently, the cells were rinsed with PBS three times and stained with
MTG (200 nM) for 30 min at 37 °C in dark. The fluorescence images were collected by the confocal laser scanning microscope.

10. Detection of $^{1}O_2$ generated in HepG2 cells.

DCFH-DA, a kind of $^{1}O_2$ probe, was utilized to detect $^{1}O_2$ generated from nanomaterials in HepG2 cells. DCFH-DA is lipophilic and easy to accumulate in cells, which is prone to be hydrolyzed by esterase in cells and produce DCFH. When oxidized by $^{1}O_2$, DCFH will turn into green fluorescence emission molecular called DCF. The concentration of $^{1}O_2$ is connected to fluorescence intensity of DCF.

HepG2 cells were seeded in glass cover dishes and incubated for 24 h to proper density. Mito-Au$_{25}$@MnO$_2$ (160 µg/mL) was incubated with cells for 4 h. After that, the cells were irradiated under 808 nm laser (0 or 2.5W/cm$^2$) for 8 min (4 min interval after 2 min irradiation to prohibit overheating). Besides, cells treated with only 808 nm laser or Mito-Au$_{25}$@MnO$_2$ (160 µg/mL) was added to exclude the influence from light and materials. After the incubation of another 10 min, 10 µM of DCFH-DA solution diluted with Opti-MEM was added and incubated for another 30 min. Finally, cells were rinsed with PBS and used for CLSM imaging.

11. Live/dead cell staining.

HepG2 cells were seeded in confocal dishes and grown overnight. Then the dishes were divided into five groups including blank control, 808 nm laser irradiation (2.5 W/cm$^2$, 8 min in total with a 4 min interval after each 2 min of irradiation to prevent overheating), Au$_{25}$ NCs (160 µg/mL) with 808 nm laser irradiation, and Mito-Au$_{25}$@MnO$_2$ (160 µg/mL) with 808 nm laser irradiation. After cultured another 24 h, the cells were stained by a mixture solution of Calcein-AM (2 µM) and PI (4.5 µM) at 37 °C for 30 min. The fluorescence images were collected by the confocal laser scanning microscope.

12. In Vivo Systematic Toxicity.

Mito-Au$_{25}$@MnO$_2$ (0, 5 and 10 mg/kg) were tail intravenously injected into the healthy female Balb/c mice. Each group included five mice. The body weight was recorded every 2 days. At the 15th day, blood of mice was used for biochemical
analysis. Meanwhile, the major organs (heart, liver, spleen, lung, and kidney) were removed for H&E staining.

13. Animal Experiments.

4T1 cells were injected subcutaneously into female Balb/c mice. When the tumor volume reached 100-150 mm³, the mice were divided into five groups randomly (five mice each group) and were treated with (1) PBS; (2) 808 nm light irradiation; (3) Mito-Au_{25}@MnO_{2}; (4) Au_{25} NCs and 808 nm light irradiation; (5) Mito-Au_{25}@MnO_{2} and 808 nm light irradiation. All materials (PBS, Au_{25}NCs and Mito-Au_{25}@MnO_{2}) were directly injected into the tumor at the concentration of 10 mg/kg. After injecting the drug for 1 h, the mice were exposed to 808 nm (1.46 W/cm²) laser for 20 min (10 min interval after 5 min irradiation to prohibit overheating). After all treatments, the tumor size and the body weight were recorded every 2 days and the mice were fed for 14 days. At the 15th day all of them were sacrificed. The major organs and tumor tissues were excised for further H&E and TUNEL staining analysis.
Supporting Figures

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Figure S23. H&E staining images of major organs of 4T1 tumor bearing mice after receiving different treatments. Scale bar: 100 μm.
Reference

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