Self-Assembled Au_4Cu_4/Au_{25} NCs@liposome tumor nanotheranostics with PT/fluorescence imaging-guided synergetic PTT/PDT

Xinyu Liu,‡[a] Ying Yang,‡[a] Xueyan Wang,[a] Xuan Liu,[a] Hanlong Cheng,[a] Peisan Wang,[b] Yuhua Shen,*[a] Anjian Xie[a] and Manzhou Zhu*[a]

[a] *School of Chemistry and Chemical Engineering, Institute of Physical Science and Information Technology, Anhui University, Hefei 230601, P R China*

[b] *School of Biomedical Engineering, Anhui Medical University, Hefei Anhui 230032, P R China*

*Corresponding authors.

E-mail addresses: s_yuhua@163.com (Y. Shen), zmz@ahu.edu.cn (M. Zhu).

Experimental section

Main Chemicals

Cholesterol, lecithin, gold chloride trihydrate ($HAuCl_4 \cdot 3H_2O$), methanol (MeOH) and sodium borohydride ($NaBH_4$) were obtained from Aladdin Reagent Co., Ltd. (Shanghai, P. R. China). N-hexane (C_6H_{14}), chloroform ($CHCl_3$, TCM), 1, 3-diphenylisobenzofuran (DPBF), 2, 2, 6, 6-tetramethylpiperidine (TEMP) dichloromethane (CH_2Cl_2, DCM), tetraoctylammonium bromide (TOAB), captopril, copper(II) chloride ($CuCl_2$), bis(diphenylphosphino)methane (Dppm), 1-adamantanethiol ($C_{10}H_{16}S$, HAdmS, 99.99%), N, N-dimethylformamide (DMF), dimethyl sulfoxide (DMSO), 2', 7'-dichlorodihydrofluorescein diacetate (DCFH-DA) and phosphate buffered solution (PBS) were purchased from Aladdin biochemical technology Co., Ltd. Dulbecco's Modified Eagle's Medium (DMEM), fetal calf serum, trypsin-EDTA solution, 3-[4, 5-dimethylthialzol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT), Hoechst 33342 and propidium iodide (PI) were purchased from Tianjin Chemical Co., Ltd. (P. R. China). Penicillin and streptomycin were got from Sigma-Aldrich Reagent Co., Ltd., USA. HeLa cells (human cervical cancer cells) and H22 cells (mouse hepatoma cell) were obtained from the Key Laboratory of Ecological Engineering and Biotechnology of Anhui Province, School of Life Science, Anhui University (Hefei, P. R. China). Female institute of cancer research (ICR) mice were purchased from the Laboratory Animal Center of Anhui Medical University. Deionized (DI) water was obtained from Millipore Milli-Q system (resistivity: 18.2 MΩ.cm). All chemical reagents used in the experiments were of analytical grade without further purification.

Characterization

Transmission electron microscopy (TEM) and scanning electron microscopy

(SEM) images were obtained using JEM-100SX and Hitachi S-4800 instruments, respectively. Ultraviolet visible-NIR (UV-Vis-NIR) measurements were performed by an UV-3900 spectrometer (Hitachi Co., Ltd., Japan). X-ray photoelectron spectroscopy (XPS) was acquired using ESCALAB-250Xi apparatus. The size distributions and zeta potentials of all samples were collected by dynamic light scattering (DLS) and laser doppler electrophoretic methods of Malvern zetasizernano (UK). The zeta potential values were measured in water. Fluorescence spectra were measured using a spectrofluorometer (FL-7000, Hitachi, P. R. China). The photothermal effects of all products were studied by a Fluke Ti32 infrared thermography camera. A CW diode laser (LSR808NL-2000) with a wavelength of 808 nm was used for the laser irradiation experiments. The amount of dissolved O_2 in the phosphate buffer solution (PBS) was determined by an intelligent portable instrument (Heng Xin AZ8403). The electron spin resonance (ESR) spectra of singlet oxygen ($^1O_2$) was measured by the Nano X-band system (Bruker, German). Room-temperature ESR measurement was performed at School of Chemistry and Chemical Engineering, Anhui University. The optical density (OD) values in the MTT assays were measured with a spectrophotometric microplate reader (RT-2100C, Rayto, P. R. China). Cell fluorescence images were achieved with an inverted fluorescence microscope (DMI3000B, Leica, German). Confocal laser scanning microscope (CLSM) images were captured using an CLSM (TCS SP8X, Leica, German). The biodistributions of nanoplatforms *in vivo* were measured by inductively coupled plasma mass spectrometer (ICP MS). The fluorescence images of the mice were captured by small animal imaging system (IVIS Lumina system).

Preparation of Au_4Cu_4 nanoclusters (NCs)

The modified method of preparing Au_4Cu_4 NCs was based on our previous work.[1] $HAuCl_4 \cdot 3H_2O$ (80 mg, 0.20 mmol) was dissolved in 20 mL of DCM containing 100 mg of TOAB under vigorous stirring. After 15 min, the color of solution slowly turned red, followed by the addition of $CuCl_2$ (34mg, 0.20mmol). Then, Dppm (100 mg, 0.26 mmol) and HAdmS (100mg, 0.60mmol) were added and the solution changed to colorless. After 30 min, a freshly prepared solution of $NaBH_4$ (60 mg, dissolved in 5 mL H_2O) was mixed with above solution and the color immediately turned purple. This reaction lasted for 12 h at room temperature. The crude product was obtained by centrifugation. The precipitate was washed with excess n-hexane for three times. $Au_4Cu_4(Dppm)_2(SAdm)_5Br$ (Au_4Cu_4 NCs) were crystallized from CH_2Cl_2/hexane at room temperature after 2 days.

Preparation of Au_4Cu_4/Au_{25}@Lip nanoplatform

The preparation process of Au_{25} NCs referred to the reported method.[2] Liposome (Lip) was prepared via film hydration method.[3,4] Specifically, 30 mg of lecithin and 10 mg of cholesterol were dissolved in 20 mL of mixed MeOH/ TCM solvent (V_{MeOH}:V_{TCM} = 1:4) with ultrasound for 10 min, and then the solvent in the mixture was

removed by rotary evaporation process. Thus, a lecithin/cholesterol composite film formed after being dried in vacuum for 45 min. Then, 15 mL of Au_{25} NCs (0.6mg/mL) aqueous dispersion and 1 mL of Au_4Cu_4 NCs (4 mg/mL) methanol dispersion were added to the round bottom flask containing the lecithin/cholesterol composite membrane. The mixed system was rotated and hydrated for 2 h under normal temperature and pressure, and then placed overnight to remove MeOH, and then filtered by polycarbonate membrane (0.22μm) for 3 times.

As control, the preparation processes of Au_4Cu_4@Lip nanoplatform were similar to that of Au_4Cu_4/Au_{25}@Lip, except that Au_4Cu_4/Au_{25} was replaced by Au_4Cu_4 NCs.

Photothermal effect of the samples

1 mL of PBS dispersions containing Lip, Au_4Cu_4@Lip, Au_{25} NCs, Au_4Cu_4/Au_{25}@Lip (1 mg/mL) were added in a small bottle and exposed to the laser irradiation (808 nm, 1 W/cm^2), respectively. 1 mL of PBS dispersion was as the control group. All groups were irradiated for 12 minutes at one minute intervals. The changes of temperature were measured by a Fluke Ti32 thermal infrared camera. And the photothermal conversion efficiencies of Au_{25} NCs and Au_4Cu_4/Au_{25}@Lip were calculated by the method and formulas reported in the literatures, respectively.[5-7]

Catalytic activity towards H_2O_2 for producing O_2

1 mL of PBS dispersions containing Lip, Au_4Cu_4@Lip, Au_4Cu_4/Au_{25}@Lip (1 mg/mL) were mixed with 25 mL PBS or PBS+H_2O_2 (10^{-4} M), respectively. The amount of dissolved O_2 in PBS dispersions within 20 min was determined by an intelligent portable instrument.

Detection of the generation of $^1O_2$

DPBF, as a molecular probe, was used to detect the production of $^1O_2$. DPBF (5 μg/mL) was mixed with different DMF dispersions containing Lip, Au_4Cu_4@Lip, Au_4Cu_4@Lip+H_2O_2, Au_4Cu_4/Au_{25}@Lip, Au_4Cu_4/Au_{25}@Lip+H_2O_2 (2mL, 200μg/mL), respectively. The content of H_2O_2 in the dispersions was 10^{-4} M. Then, the mixtures were irradiated using an 808 nm laser, and the absorbance of DPBF at 410 nm was recorded at different irradiation time by a UV-3900 spectrophotometer, respectively. All experiments were performed for 3 times.

The generation of $^1O_2$ also could be tested by an ESR method using TEMP as the spin-trapping adducts. In briefly, 10.0 μL of TEMP (500 mM) was mixed with 100μL, 0.2 mg/mL Lip, Au_4Cu_4/Au_{25}@Lip or Au_4Cu_4/Au_{25}@Lip + H_2O_2 (10^{-3} M) dispersions in PBS, respectively. After 5 min irradiation (808nm, 1W/cm^2), the $^1O_2$ signals of samples were immediately detected by the ESR.[8]

Detection of intracellular reactive oxygen species (ROS)

To study intracellular ROS of cells, DCFH-DA was used as the probe to detect ROS. HeLa cells were seeded on 6-well plate for 24 h to allow the cells at a density of

5×10^4 cells per well followed by incubation with 300 μL DMEM solution containing different samples (1mg/mL) for 6 h. The cells were washed with PBS for three times. And 100 μL of H_2O_2 (100 μmol/L) was supplemented into the control group. All groups were incubated with DCFH-DA (300 μL, 10 μmol/L) at 37 °C, 5% CO_2 for 50 min. Then, Hela cells were further washed with PBS for three times and the laser groups were irradiated by laser (808 nm, 1 W/cm^2) for 3 min. Therefore, intracellular ROS in cells could be detected accurately and imaged using an inverted fluorescence microscope. All experiments were repeated in triplicate.

Cell cytotoxicity experiments

The *in vitro* biocompatibility and tumor cell killing ability of samples were detected by cell cytotoxicity experiments. Lip, Au_4Cu_4@Lip, Au_4Cu_4/Au_{25}@Lip were dispersed in DMEM solution, then diluted to different concentrations (0.2, 0.4, 0.6, 0.8, 1 mg/mL). Hela cells were seeded in 96-well plates with a density of 5×10^4 cells per well and cultured for 24 h (5% CO_2, 37℃). After removing the incubate medium, 100 μL of fresh culture medium containing different concentrations of prepared samples were added into the wells, which were incubated for 4 h. For the control group, H_2O_2 aqueous solution (100 μmol/L, 100 μL) was added. Then, the irradiation groups were exposed to the NIR laser (808 nm, 1 W/cm^2) for 10 min with the same culture condition. After all groups were incubated for another 20 h, the culture medium was removed and the culture dishes were washed with PBS. The cell viability of samples was measured by MTT assay.[8]

For further investigating the cytotoxicity of samples, Hoechst 33342 and PI were used to observe the killing effect for cells. In brief, HeLa cells were seeded in 6-well plates at a concentration of 5×10^4 cells per well at 37 ℃, 5% CO_2. After adhesion, 300 μL of DMEM dispersion containing Lip, Au_4Cu_4@Lip, Au_4Cu_4/Au_{25}@Lip (1mg/mL) were added to each well respectively for about 4 h incubation. And 100 μL of H_2O_2 (100 μmol/L) was supplemented into the control group. For phototherapy, corresponding culture dishes were further cultured for 1 h, and then exposed to NIR laser (808 nm, 1 W/cm^2) for 10 min. After irradiation, HeLa cells were further incubated for 12 h, then washed with PBS for removing the culture medium. The cytotoxicities of the samples were evaluated by using Hoechst 33342/PI method.[9]

***In vitro* imaging**

HeLa cells were seeded in confocal sterile dish (20 mm diameter) for 12 h to allow the cells to attach to the surface of the wells with a density of 5×10^4 cells per well, and then washed with PBS solution for three times. Subsequently, the cells were incubated with Au_4Cu_4/Au_{25}@Lip (100μg/mL) which dissolved in DMEM without phenol red for 2 h and 4 h, respectively. And the cells cultured with PBS and Lip were used as control groups for 2h. After cultivation, all mediums were washed with PBS solution. In order

to obtain the results of cell imaging, CLSM was used to observe the cell imaging and phagocytosis effect of different groups at an excitation wavelength of 420 nm.

Animal Models

Kunming white mice were obtained from the Laboratory Animal Center of Anhui Medical University (Certification of quality 34000200000077, 34000200000078). This study was conducted in strict accordance with the recommendations in the Regulations on the Management of Laboratory Animals in China promulgated in 1988. All the animal experiments were in agreement with the guidelines of the Animal Use Committee of Anhui University and approved by the Animal Experiments and Care Regulations of Anhui University.

***In vivo* Fluorescence Imaging**

Around 5×10^6 H22 cells were dispersed in 200 μL of PBS and inoculated into the back of hind leg of ICR mice. The Lip and Au_4Cu_4/Au_{25}@Lip dispersions were injected into the tumor-bearing mice by tail vein in advance at 2h, 4h, 8h, 12h and 24h, respectively. And the mice injected PBS were as control group. Then, the mice were anesthetized. The fluorescence signal and images of the mice were captured by IVIS Lumina system.

***In vivo* Photothermal Imaging**

To assess the photothermal effect of Au_4Cu_4/Au_{25}@Lip, 400 μL of PBS dispersion containing Au_4Cu_4/Au_{25}@Lip (1 mg/mL) was respectively injected into the tumor-bearing mice by intravenous injection. After 4 h post-injection, the tumor sites of mice were irradiated by 808 nm laser at different time intervals (4, 8, and 12 min). Fluke Ti32 thermal infrared camera was used to capture the photothermal images.

***In vivo* synergistic antitumor effect of samples**

To establish the animal models, the tumor-bearing mice were divided into seven groups randomly and treated with 200 μL of different samples which were injected via tail vein at a dose of 5-6 mg/Kg (sample/ mouse weight) under external conditions. Seven groups were treated as follows: (i) PBS (control group), (ii) Lip, (iii) Lip + laser, (iv) Au_4Cu_4@Lip; (v) Au_4Cu_4@Lip + laser, (vi) Au_4Cu_4/Au_{25}@Lip, (vii) Au_4Cu_4/Au_{25}@Lip + laser. Four hours after injection, groups (iii), (v) and (vii) were irradiated with 808 nm laser (1 W/cm^2) for 10 min. Subsequently, the mice were weighted and the tumor sizes were measured by a caliper until the completion of treatment every day. After 10 days of consecutive treatment, all the mice were euthanized, the tumor of each mouse was collected, cleaned and weighted. The digital photographs of the collected tumors were obtained by camera.

For investigating *in vivo* synergistic antitumor effect, the obtained tumors were sliced for further staining by hematoxylin and eosin (H&E) to observe the structure and status of cells.

Biodistribution of the Au_4Cu_4/Au_{25}@Lip nanoplatform *in vivo*

To study the biodistribution of Au_4Cu_4/Au_{25}@Lip nanoplatform *in vivo*, ICP MS was used to measure the content of Au in main organs (heart, liver, spleen, lung, kidney and tumor) of mice. In brief, the H22 cells were suspended in 100 μL of PBS and injected subcutaneously into the back of hind leg of mice. After 7 days, the Au_4Cu_4/Au_{25}@Lip dispersion was injected into ICR mice via tail vein. After 12 h injection, the mice were euthanized and main organs were obtained, and the Au content in each main organ was measured by ICP MS.[10]

Statistical analysis

Data was expressed as mean ± SD. The statistical significance of the data was compared by Student's t-test. Analysis of variance (ANOVA) was used to analyze the differences among the different groups. $P < 0.05$ was considered to statistically significant.

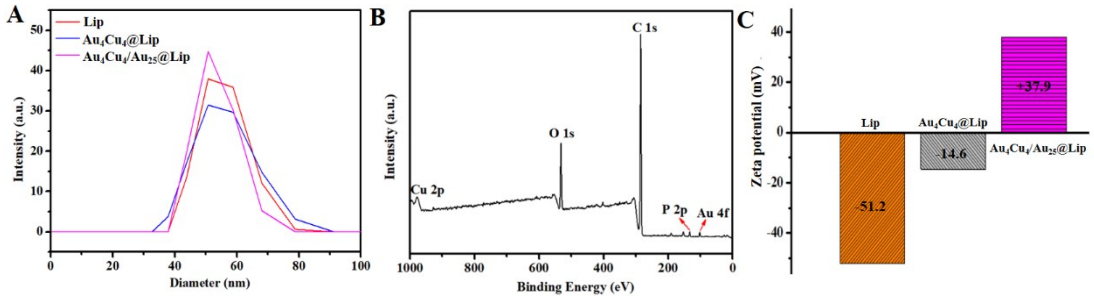

Fig. S1 (A) The size distributions of Lip, Au_4Cu_4@Lip and Au_4Cu_4/Au_{25}@Lip in PBS. (B) XPS spectrum of Au_4Cu_4/Au_{25}@Lip. (C) Zeta potentials of different samples.

The potential value of Lip was -52.15 mV, proving that the Lip carrier had excellent stability. After loading positive charged Au_4Cu_4 or Au_4Cu_4/Au_{25}, the zeta potential values of Au_4Cu_4@Lip and Au_4Cu_4/Au_{25}@Lip were -14.6 mV and +37.9 mV respectively, demonstrating the successful preparation carried positive charge and stability of the Au_4Cu_4/Au_{25}@Lip nanoplatform that was beneficial to the application in antitumor.

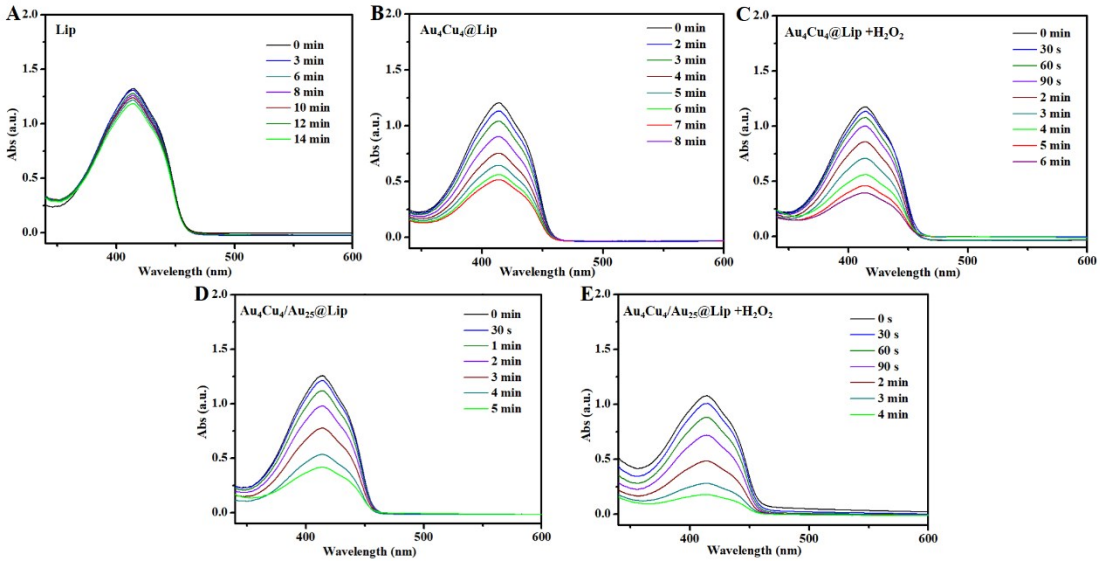

Fig. S2 Ultraviolet visible spectra of DPBF and (A) Lip, (B) Au_4Cu_4@Lip, (C) Au_4Cu_4@Lip+H_2O_2, (D) Au_4Cu_4/Au_{25}@Lip, (E) Au_4Cu_4/Au_{25}@Lip+H_2O_2 mixture under laser irradiation.

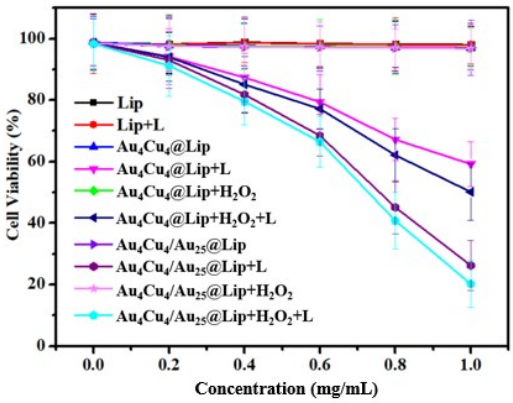

Fig. S3 Relationship between the cell viability and the sample concentration.

Notes and References

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