Supporting information

Self-quenched Metal-organic Particles as Dual-mode Therapeutic Agents for Photoacoustic Imaging-Guided Second Near-Infrared Window Photo-Chemotherapy

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MATERIALS AND METHODS

Materials

1, 4, 5, 8-tetrahydroxyanthraquinone (organic dye, THQ) was purchased from Shenzhen Regent Biochemistry Technology CO., Ltd. Copper chloride dihydrate were purchased from J&K Scientific. DCFH-DA, Cell Counting Kit (CCK-8) and LIVE / EDAD Viability / Cytotoxicity Kit was obtained from Dojindo China Co., Ltd. (Shanghai, China). PEG-$(NH_2)_2$ (2000) were purchased from Xi'an Ruixi Biochemistry Technology CO., Ltd. DI-water with a resistivity of 18.2 M Ωcm was obtained from a Milli-Q Gradient System (Millipore, Bedford, MA, U.S.) and was used for all experiments. Unless specified, all other chemicals were commercially available and used as received.

Cell Culture and animals

The hepatocellular carcinoma cells (HepG2) were purchased from ATCC (Manassas, VA) and cultured in RPMI 1640 medium which was supplemented with 10% FBS and penicillin-streptomycin (100 IU/mL, Cellgro, Manassas, VA) at 37^{o}C in a humidified incubator containing 5% CO_2. Male BALB/c nude mice (22g) and Male SD mice (200g) were purchased from China Wushi, Inc. (Shanghai, China). All animal procedures were approved by the Animal Ethics Committee of Fujian Medical University.

Cu-THQNPs synthesis

First, 1 mg THQ was mixed in DI-water / ethanol solution at the volume ratio of 2:1, and then 0.05 mg/mL $CuCl_2$ (pH 7.4) was added to the mixture, afterwards stirring for 1hr. The result products were centrifuged and washed 2 times with ethanol. After suspending in DI-water, 20 mg/mL PEG-$(NH_2)_2$ (2000) was added and then stirring overnight at room temperature. After that, the result products were collected and washed 3 times by DI-water. The Cu-THQNPs were

then dispersed in DI-water or PBS buffer (pH 7.4) for further usage. The amount of Cu(II) in Cu-THQNPs was determined to be 10.96 μg/mL by XSERIES 2 inductively coupled plasma mass spectrometry (ICP-MS) (Thermo, USA). The amounts of THQ in Cu-THQNPs were calculated by measuring the corresponding absorbance at 510 nm for THQ in THF solution. Briefly, 100 μL Cu-THQNPs were added into the tetrahydrofuran (THF) organic solution, and then measured the absorption at 510 nm. The amount of THQ from Cu-THQNPs was calculated by subtracting the absorbance value of THQ from its standard curve for the concentrations ranging from 1-50 μg/mL ($Y = 1.802x+0.102$, $R^2 = 0.996$).

Characterization of the Cu-THQNPs

Transmission electron microscope image (TEM) was conducted by a JEM-2010 electron microscope (JEOL, Japan), and the chemical compositions of the Cu-THQNPs were performed by EDS and XPS spectra. The FT-IR spectra of Cu-THQNPs were collected on a FT-IR spectrometer (Perkin Elmer, USA). The vis-NIR I / II absorption spectra of Cu-THQNPs were measured by An UH4150 Spectrophotometer (Hitachi Co., Ltd., Japan). The temperature elevation and photothermal stability were performed according to our previous works[44-47]. Briefly, 1 mL aqueous solutions of Cu-THQNPs with different concentrations were irradiated by the 808 nm or 1064 nm laser with a laser power density of 1 W/cm^2. The temperature of the solution was monitored by a thermocouple microprobe (ϕ= 0.5 mm) (STPC-510P, Xiamen Baidewo Technology Co., China) that was submerged in the solution every 10s and IR thermal camera. The photothermal conversion efficiency, η, was calculated using Equation 1 described by the previous reports, where h is the heat transfer coefficient, A is the surface area of the container, T_{max} is the equilibrium temperature, T_{Surr} is ambient temperature of the surroundings, $\Delta T_{max} = T_{max} - T_{Surr}$, I is incident laser power (1 W cm^{-2}), and A_λ is the absorbance of Cu-THQNPs

at 1064 nm. Q_s is the heat associated with the light absorbance of the solvent, which is measured independently to be 25.2 mW by using deionized water without nanoparticles as the solvent.

$$\eta = \frac{hA\Delta T_{max} - Q_s}{I(1-10^{-A_\lambda})} \qquad (1)$$

The value of h_A is derived according to Equation 2:

$$\tau_s = \frac{m_D C_D}{hA} \qquad (2)$$

Where τ_s is the time constant of sample system, m_D and C_D are the mass (1 g) and heat capacity (4.2 J g^{-1}) of deionized water when used as the solvent, respectively.

In order to obtain the h_A, herein introduce θ, which is defined as the ratio of ΔT to ΔT_{max}:

$$\theta = \frac{\Delta T}{\Delta T_{max}} \qquad (3)$$

h_A can be determined by applying the linear time data from the cooling period versus -lnθ. Substituting h_A value into Equation 1, the photothermal conversion efficiency (η) of Cu-THQNPs can be calculated.

$h_A = m_D C_D/\tau_s = 4.2$ J/214.61709; $A_\lambda = 0.905$; $\tau_s = 214.61709$; $I = 1$ W cm^{-2}; $\Delta T_{max} = 21.7$ °C

$\eta = ((4.2/214.61709) \times 21.7\text{-}0.0252) / (1 \times (1\text{-}10^{-0.905})) = 51.9\%$

In vitro cytotoxicity analysis and the photochemotherapy of cancer cells

Localized photo-killing effects of Cu-THQNPs were also evaluated on HepG2 cells according to our pervious published protocols [47-50]. Briefly, the HepG2 cells were first seeded into a plate at a density of 1×10^6 cells at 37°C in a 5% CO_2 atmosphere for 24hrs. Then, the cells were washed with PBS to remove dead cells, followed by incubation with Cu-THQNPs with or without 808 and 1064 nm laser irradiation with the laser power intensity of 1W/cm^2 for 5 min. After laser irradiation, the cells were incubated with fresh culture medium at 37°C for 1hrs. Then, the cells

were stained with a LIVE / EDAD Viability / Cytotoxicity Kit for the visualization of live and dead cells. Subsequently, the cytotoxicity of Cu-THQNPs was evaluated on HepG2 cells using a CCK8. The cells were first seeded in a 96-well plate at a density of 1×10^5 per well and incubated under a humid atmosphere (with 5% CO_2) for 24hrs. The original medium was replaced with a fresh culture medium containing different concentration of Cu-THQNPs from 6 to 48 μg/mL and incubated for 4hrs. Then, the cells treated with Cu-THQNPs were irradiated by 808 nm or 1064 nm laser for 5 min. Subsequently, 100 mL of culture medium and 10 mL of CCK8 solution were added to the wells. After incubation for 4hrs at 37°C, the absorbance of the solution in each well at 450 nm was measured by a microplate reader (Spectra Max M5e, Germany). Cell viability was expressed as follows:

$$\text{Cell viability (\%)} = (OD_{sample}-OD_{blank}) / (OD_{control}-OD_{blank}) * 100. \quad (4)$$

The OD_{sample} and $OD_{control}$ are the absorbance values of treated cells and the untreated control cells, respectively. The OD_{blank} was the absorbance of CCK8 agent itself at 450 nm. All experiments were performed in five independent experiments.

To investigate the cell uptake of our Cu-THQNPs, the ICP-MS was performed. Briefly, the HepG2 cells were seeded into a 6-well plate at a density of 5×10^5 cells at 37°C in a 5% CO_2 atmosphere for 24 hrs. Then, the cells were incubated with Cu-THQNPs (0.048 and 0.096 mg/mL) for 4hrs. Afterwards, the cells were washed with PBS to remove the non-uptaken Cu-THQNPs and further incubated for 20 hrs. Subsequently, the incubation medium was collected to determine the Cu content through XSERIES 2 inductively coupled plasma mass spectrometry (ICP-MS) (Thermo, USA). Afterwards, the cells were also detached from the dish by scraping method. After centrifugation of the detached cells at 300 g for 5min, the cell pellet was washed with PBS buffer and “Cell Wash Solution” supplied by Men-PERTM Plus Membrane Protein

Extraction Kit respectively, followed by suspending the cells in the Permeabilization Buffer at 4^{o}C with constant mixing for 10 min. Then, this cell suspension was centrifuged at 700 g for 5 min to separate the plasma membrane (in supernatant) and cytoplasm (in precipitates). The precipitates were collected to determine the Cu(II) amount in the cytoplasm through ICP-MS. On the other hand, the cell membrane in supernatant was further collected by high-speed centrifugation at 16000 g for 15 min to measure its Cu(II) content.

Thermal imaging and Photoacoustic imaging in vitro and in vivo.

For thermal imaging of Cu-THQNPs in agarose gel *in vitro*, we first prepared 5% agarose gel, and then the Cu-THQNPs were added into 5% agarose gel and stored at 4^{o}C for 10 min. After that, the agarose gel contained Cu-THQNPs were obtained, and the size of 3D agarose gel was measured by vernier caliper. The length of agarose gel was 3.5 cm (X), the width was 1.2 cm (Y) and the high was 3.5 cm (Z). The agarose gel containing Cu-THQNPs was then irradiated by 670 nm, 808 nm and 1064 nm laser with the laser power intensity of 2 W/cm^2, respectively. The thermal image and 3D-IR^{TM} image of agarose gel were recorded by IR image camera. For *in vivo* thermal imaging, tumor-bearing nude mice were prepared by subcutaneously injecting a suspension of the HepG2 cells (10^7 cells) in sterilized 1 × PBS buffer. When the tumor size reached about 100 mm^3, the tumor-bearing nude mice were intravenously injected with Cu-THQNPs. After 4hrs of injection, the mice was irradiated by 1064 nm lasers for 5 min, and the thermal images were collected by IR thermal camera.

For photoacoustic imaging *in vitro and in vivo*, the agar gel cylinders that contained different concentration of Cu-THQNPs were obtained according to above methods[51]. For the phantom experiments *in vitro*, we placed a 3.5 cm diameter solid cylindrical phantom. The phantom

materials included Intralipid as the scatter and India ink as the absorber with agar powder (1%–2%) for solidification. The different concentration of Cu-THQNPs was used as the absorber of the target, and the size of the target was 3 mm in diameter. Then, triple-wavelength PAI was performed at 750 and 808 nm of the pulsed laser, and the incident optical fluence was controlled at ~3 mJ/cm^2. For tracking the Cu-THQNPs by PAI system *in vivo*, the tumor-bearing nude mice were intravenously injected with Cu-THQNPs and then imaged by photoacoustic tomography (PAT) system according to our co-author previously published work[51]. Briefly, an optical parametric oscillator (OPO) (Surelite II-20, Continuum, Santa Clara, CA, USA) with a pulse duration of 4-6 ns and pulse repetition rate of 20Hz was used as the light source. The OPO operated at 680 or 1100 nm was used to irradiate the samples for generating photoacoustic signals. The incident fluence levels were maintained below 10 mJ/cm^2. The laser beam was expanded by a concave lens and then homogenized with a ground glass. The light then irradiated onto the agar phantom. Water was used as the coupling of PA signal. A 3.5 MHz ultrasound transducer (V381-SU, Olympus) was controlled by a precise rotator to scan circularly around the sample in the horizontal plane. The whole imaging interface was mounted on a rotator and scanned 120 steps with a constant interval of 3° to cover a 360° receiving angle. The PA signals acquired from the ultrasound transducer were amplified (5073PR, Olympus) and then recorded with a digital oscilloscope (MDO3012, Tektronix) at a sampling rate of 500 Msamples·s^{-1}. At each sampling position, the PA signal was averaged for 32 traces. Finally, the computer acquired all of the amplified and digitized data and reconstructed the distribution of absorbed optical energy density in the imaging plane. The ultrasound speed is assumed to be exactly 1,500 m·s^{-1} for every PA reconstructed image. The spatial resolution of our PAT system is ~ 400 μm.

Synergistic Antitumor Efficacy in vivo of Photothermal/chemotherapy

Male BALB/c immune deficiency nude mice with body weight of ~ 22g from China Wushi, Inc. (Shanghai, China) were used for evaluating the photo / chemotherapeutic efficiency. All animal procedures were approved by the Animal Ethics Committee of Fujian Medical University. Tumor-bearing nude mice were prepared by subcutaneously injecting a suspension of the HepG2 cells (10^7 cells) in sterilized 1 × PBS buffer. When the tumor size reached about 100 mm^3, 0.8 mg/mL of Cu-THQNPs (100 μL injection of each mouse) were intravenously injected into each mouse (n = 4). One group of mice treated with the same volume of sterilized PBS was taken as the control. The mice were segregated into following four groups:

(1) PBS treated group without laser irradiation; (n = 4);

(2) PBS treated group with laser irradiation (1064 nm, 1 W/cm^2) for 5min; (n = 4);

(3) Cu-THQNPs treated group without laser irradiation; (n = 4);

(4) Cu-THQNPs treated group with laser irradiation (1064 nm, 1 W/cm^2) for 5min; (n = 4);

The irradiation was conducted after 4hrs of intravenous injection, and then the therapeutic effects of photothermal / chemotherapy was evaluated through monitoring the tumor volume and body weight change in each group every 2 days. The size of tumors was calculated by calipers, and the volume of tumor (V) was calculated by the following equation:

$V=A*B^2 / 2$ (5)

A and B are the longer and shorter diameter (mm) of tumor.

Histological Examination and Long-term Toxicity Assessment

To examine the histological changes of tumors, one tumor-bearing mouse in each group was sacrificed after 24hrs of laser irradiation, and the tumors were collected, and then stained with hematoxylin and eosin (H&E) for histopathology evaluation and Ki67 antibody for

immunohistochemical analysis. To assess the long-term systematic toxicities of Cu-THQNPs, the tumor-bearing mouse were sacrificed 14d after treatment, and the major organs (heart, liver, spleen, lungs, and kidneys) of the mice were collected, fixed in 4% neutral formaldehyde, embedded in paraffin, stained with H&E, and observed under a Zeiss microscope (Axio Lab.A1). To assess the long-term systematic toxicities after treatment, the Cu-THQNPs (4.03 mg/kg) treated SD mice in different time points were sacrificed, and the blood was collected for analysis.

Statistical analysis: Statistical analysis of data was performed using one-way of variance (ANOVA) method or the two-tailed paired Student's T-test, *$p<0.05$, ****$p<0.0001$. All the data were shown as means ± SD through at least three experiments.

Table S1. The reference ranges of blood biochemical analysis in female SD rats according to perviously reported works[52].

Parameters	**Control**
ALT (IU/L)	33.4 ~98.0
AST(μ/L)	63.0~154.0
ALP (IU/L)	227.1 ~454.1
CREA(mmoI/L)	35.4~53
GLU(mmoI/L)	6.2~8.2
TCHOL(mmoI/L)	1.7~3.7
WBC(10^9/L)	3.3~21.3
MCV(fL)	54.0~70.0
RBC(10^{12}/L)	4.5 ~8.7
HGB (g/L)	95~184
HCT(%)	27.4~57.4
PLT(10^9/L)	875.8~ 1181.8

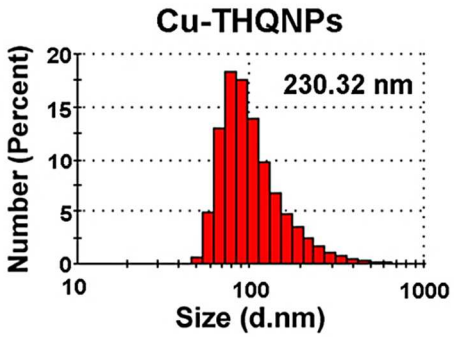

Figure S1. DLS result of Cu-THQNPs in PBS buffer.

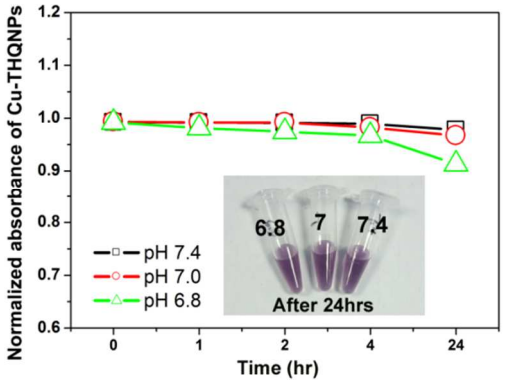

Figure S2. Water-stability of Cu-THQNPs in different pH conditions, the insert picture is Cu-THQNPs in different pHs after 24hrs.

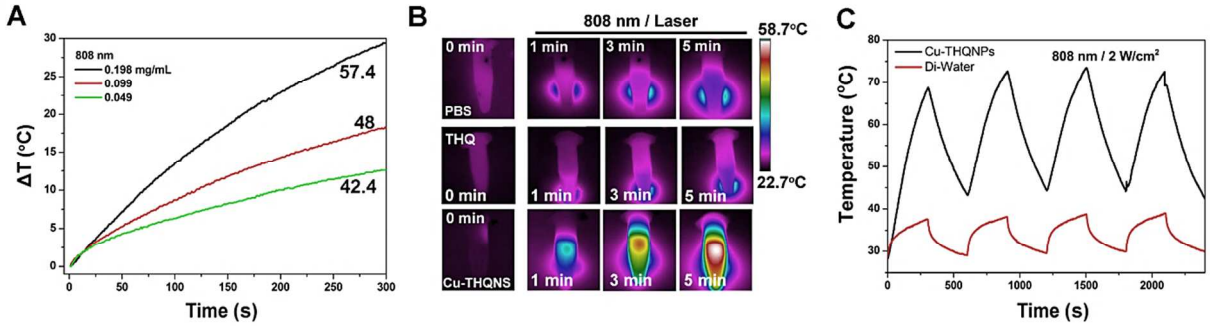

Figure S3. (A) Temperature elevation curves of Cu(II)-THQNPs with different concentrations. (B) IR thermal image of PBS, THQ and Cu(II)-THQNPs upon the 808 nm laser irradiation. (C)Temperature elevation curves of Cu(II)-THQNPs over four rounds of on/off cycling in 500s intervals under the high power conditions, DI-water acts as a control.

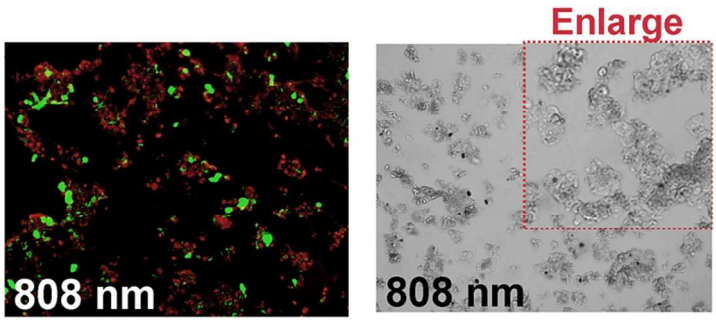

Figure S4. Confocal image of Cu(II)-THQNPs treated HepG2 cells upon the 808 nm laser irradiation with the laser power intensity of 1W/cm^2. The morphological change of HepG2 cells was recoded after Cu(II)-THQNPs treatment then upon the 808 nm laser irradiation with the laser power intensity of 1W/cm^2.

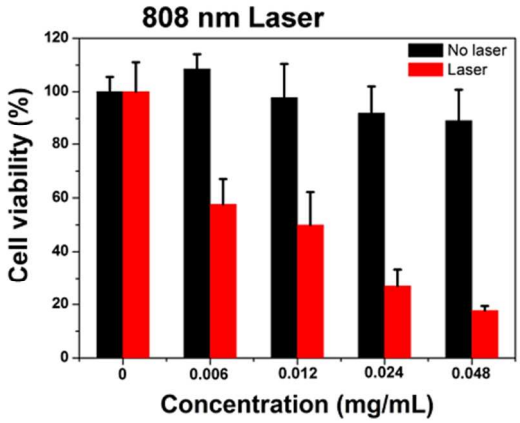

Figure S5. The cell viability of the Cu-THQNPs treated HepG2 cells with or without 808 nm laser irradiation (n=5).

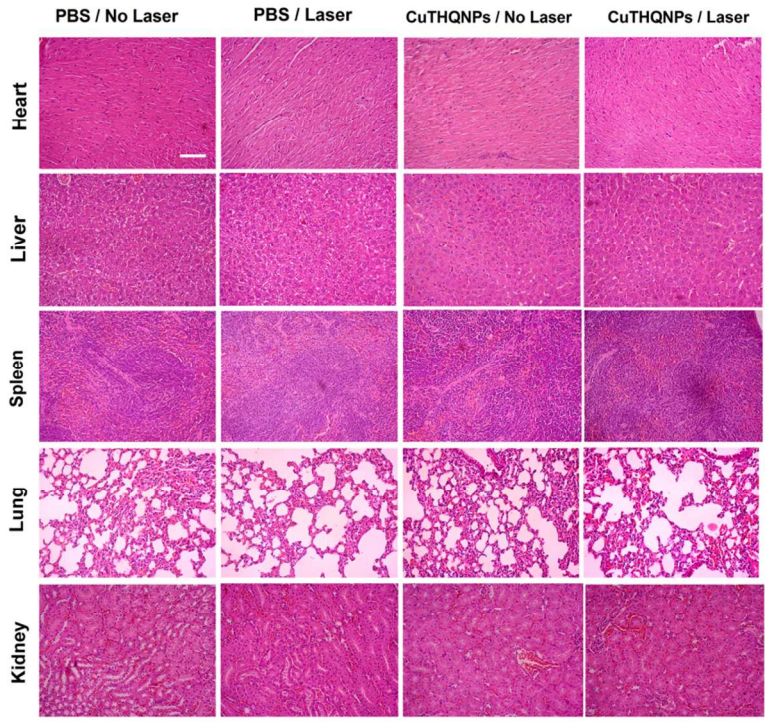

Figure S6. Representative images and H&E results from the main organs after indicated treatment. (Scale bar = 50 μm).