Supporting information

IR820 Functionalized Melanin Nanoplates for Dual-Modal Imaging and Photothermal Tumor Eradication

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Experiment section

Materials

Melanin was purchased from Sigma-Aldrich. Sodium hydroxide (NaOH) and hydrochloric acid (37 wt% HCl) were acquired from Sinopharm Chemical Reagent Beijing Co., Ltd. Amine-PEG_{5000}-thiol (NH_2-PEG_{5000}-SH, 5 kDa) was obtained from Xi'an rulxl Biological Technology Co., Ltd. New Indocyanine Green (IR820) was acquired from Shanghai Yuanye Bio-Technology Co., Ltd. Dimethyl sulfoxide (DMSO) and potassium bromide were purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. Cell Counting Kit-8 and Calcein-AM/PI Double Stain Kit were acquired from Shanghai Yeasen Biotechnology Co., Ltd. Annexin V-FITC/PI apoptosis detection kit was bought from Nanjing KeyGEN biosciences company. Fluorescein Isothiocyanate (FITC) was purchased from Energy Chemical Co., Ltd. 4′,6-diamidino-2-phenylindole (DAPI) was purchased from Boster biological Technology Co., Ltd. All reagents were used without further purification.

Synthesis of IR820-PEG

The MPI nanoplates were synthesized by NH_2-PEG_{5000}-SH coupling of IR820 and MNP. Typically, IR820 (17 mg) was dissolved in DMSO solution (2 mL). Then a DMSO solution

containing NH_2-PEG_{5000}-SH (5.5 mg) was added into the above solution, accompanying with stirring in the dark under bubbling of argon flow for 48 h. The excess organic solvent was removed by a rotary evaporator, then deionized water (10 mL) was added to mix well. Finally, the IR820-PEG solution was centrifuged and washed several times for subsequent use.

Preparation of MNP-PEG-IR820（MPI）

Melanin nanoparticles (MNP) were obtained by an approach previously reported [1]. Next, MNP (5 mg) was dissolved in deionized water (5 mL) under sonication. The IR820-PEG solution (1 mL) was dropped to melanin solution under vigorous stirring overnight. The sample was centrifuged and washed for three times. Finally, the powered MPI was obtained by freeze-drying.

Characterization of MNP-PEG-IR820（MPI）

A Nano-Zetasizer (Malvern Instruments Ltd.) was applied to analyze the hydrodynamic diameter and zeta potential of MPI. Transmission electron microscopy (TEM) images of MPI were performed by a JEM-2100F microscope. The absorption spectra of MNP, IR820 and MPI solution were obtained with a UV-Vis-NIR spectrometer (UV-6100, MAPADA). The chemical property of the as-prepared MPI was tested by an TENSOR Ⅱ Fourier transform-infrared (FT-IR) spectrometer. Fluorescence spectra was acquired using RF-6000 fluorescence spectrophotometer (Shimadzu).

In vitro PA and FL imaging

For in vitro PA imaging, MPI solution with different concentrations (0, 12.5, 25, 50, 100 and 200 μg/mL) were performed by a real-time multispectral optoacoustic tomographic (MSOT) imaging system (in Vision 128, iThera Medical GmbH, Neuherberg, Germany). For in vitro FL imaging, MPI solution with different concentrations (0, 15.625, 32.25, 62.5, 125, 250, 500 and 1000 μg/mL) were added into 200 μL tubes respectively, which were performed by a fluorescence imaging instrument (Series III 900/1700)[2].

In vitro photothermal imaging

To analyze the photothermal conversion capability, MPI solution with different concentrations (0, 50, 100, 200 and 400 μg/mL) were measured followed by illumination with 808 nm laser for 5 min at a power density of 0.4 W/cm^2. The dependency between temperature and power was evaluated under illumination with different power densities at 0.2, 0.4, 0.6, 0.8 and 1.0 W/cm^2. After the samples were naturally cooled down to room temperature, five cycles of heating-cooling process were repeated.

Photothermal conversion efficiency (η) calculation

In order to evaluate the photothermal conversion efficiency, the temperature change of 0.2 mL MPI solution was recorded under 808nm laser irradiation [3]. After laser irradiation for 5 minutes, the laser was turned off and solution was cooled to initial temperature. The value η of photothermal conversion efficiency was calculated by the formula (1). T_{max} is the maximum temperature induced by MPI. $T_{max,water}$ is the highest temperature induced by pure water. I is laser power, A_{808} is the absorbance of MPI at the wavelength of 808 nm. The value of hS can be obtained by (2) (3). Where m_i is the mass and $C_{p,i}$ is the heat capacity of measured solution (the heat capacity of water is 4.2 J/g). τ_s is the system time constant of MPI, which was determined by the linear fit slope of the experimental data plotted by equation (3) (τ_s=232.615 for MPI). T_{sur} represents the temperature of the surrounding. hS can be determined by applying the linear time data from the cooling period – lnθ. Thus, substituting hS value into equation (1), the photothermal conversion efficiency (η) of MPI can be calculated. The maximum steady temperature (T_{Max}) of MPI solution was 69.1℃ and The maximum water temperature ($T_{max,water}$) was 29.8℃. So, the temperature change (T_{Max}-$T_{max,water}$) of MPI solution was 39.3℃. The laser power I is 0.8 W. The absorbance of MPI at 808 nm A_{808} is 1.499. In addition, m_i is 0.206 g and $C_{p,i}$ is 4.2 J/g. τ_s was calculated as 232.61s. Thus, according to Eq. 2, hS can be calculated. According to Equation 1, the η value of MPI is determined to be 24.7%.

$$\eta = \frac{hS(T_{max} - T_{maxwater})}{I(1 - 10^{-A808})} \quad (1)$$

$$hs = \frac{\sum_i m_i C_{p,i}}{\tau_s} \quad (2)$$

$$\tau_s = -\ln\left(\frac{T(t) - T_{sur}}{T_{max} - T_{sur}}\right) \quad (3)$$

Cell culture

The human laryngeal cancer Hep-2 cell line was purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and cultured in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO) containing 10% fetal bovine serum (FBS), 1% glutamine and 1%

penicillin/streptomycin at 37 ℃ under a 5% CO_2-containing atmosphere.

Cell cytotoxicity assay

In vitro cytotoxicity of MPI was measured by cell counting kit-8 (CCK-8) assay. The Hep-2 cells were seeded into 96 well plates at a density of 9000 cells per well and incubated for 24 h. Then, dead cells were washed with PBS, and live cells continued to incubate with a series of concentrations of MPI (0, 6.25, 12.5, 25, 50 and 100 μg/mL) for another 24 h. After that, the culture medium was discarded and cells were incubated with fresh medium containing 10% CCK-8 for 4 h. Finally, the absorbance of each well at 450 nm was measured using a microplate absorbance reader (SpectraMax Plus384).

Cellular-uptake behavior

Cellular uptake of MPI was investigated by confocal laser scanning microscopy (CLSM) and flow cytometry. Hep-2 cells were seeded into the small glass dishes for adherence. The mixture of fluorescein isothiocyanate (FITC) and MPI was obtained by stirring in the dark. Then, the Hep-2 cells were incubated with MPI-FITC (200 μg/mL) at 37 ℃ for 4 h. After the subsequent staining and washing, CLSM images were obtained by a SP8 confocal laser scanning microscope and flow cytometry analysis was recorded by using a FACS-Calibur flow cytometry.

In vitro antitumor performance

Both calcein AM/PI assay and Annexin V-FITC/PI apoptosis assay were performed to evaluate the in vitro antitumor efficiency and mechanism. As for calcein AM/PI assay, Hep-2 cells were seeded into 96 well plate at a density of 8000 cells per well. After incubation with MPI (200 μg/mL), Hep-2 cells were stained with Calcein AM (for live cells) and PI (for dead cells) with or without laser irradiation (808 nm, 1 W/cm^2) for 5 min. Fluorescence images of cells were obtained under an inverted fluorescence microscope (SeriesI400/1700-C). As for Annexin V-FITC/PI assay, Hep-2 cells were incubated with different formulations in the presence or absence of NIR irradiation as described above. Finally, the cells were stained and the percentage of apoptosis was analyzed by a flow cytometer (FACS-Calibur, BD).

Cell PA and FL imaging

Hep-2 cells were treated with a series concentration of MPI (12.5, 25, 50, 100 and 200 μg/mL). As for PA imaging, the images of Hep-2 cells were recorded by a MSOT imaging system [4]. As for FL imaging, an NIR fluorescence inverted microscope (SeriesI400/1700-C) was used to observe

cell fluorescence at 880 nm excitation wavelength.

Animal tumor model

The six-week-old female nude mice (16-18g) were purchased from Beijing Vital River Experimental Animal Company. After 2 weeks of acclimatization, tumor models were established by subcutaneous injection of 1×10^6 Hep-2 cells into the flank region of the nude mice. When the tumor volumes reached 100 mm^3, the mice were used for imaging and treatment in vivo.

In vivo PAI and FL study

For in vivo PAI, the tumor-bearing mice were injected with MPI (2 mg/mL, 200 μL) via tail vein and then monitored by a real-time MSOT imaging system with a laser wavelength of 730 nm at 2, 4, 6, 12, 24, 48 and 72 h postinjection. For in vivo FL, the images of the tumor location were collected by the fluorescence imaging instrument at indicated time points after injection.

In vivo PTT study

To investigate the PTT effect of MPI in vivo, Hep-2 tumor-bearing mice were divided into four groups and given following treatments: Ⅰ, PBS; Ⅱ, Laser; Ⅲ, MPI; Ⅳ, MPI + Laser. After 48 h tail vein injection, the mice were irradiated with 808 nm NIR laser at a power density of 2 W/cm^2 for 5 min. Then an infrared thermal camera (Fluke Ti400) was applied to record the temperature of tumor site. After treatment, the tumor volume and body weight of each mice were inspected in the following 16 days.

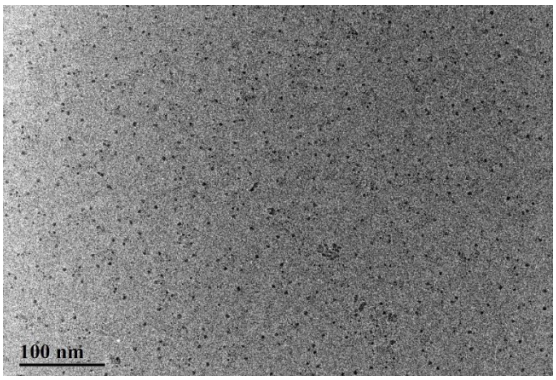

Figure S1 TEM images of melanin NPs

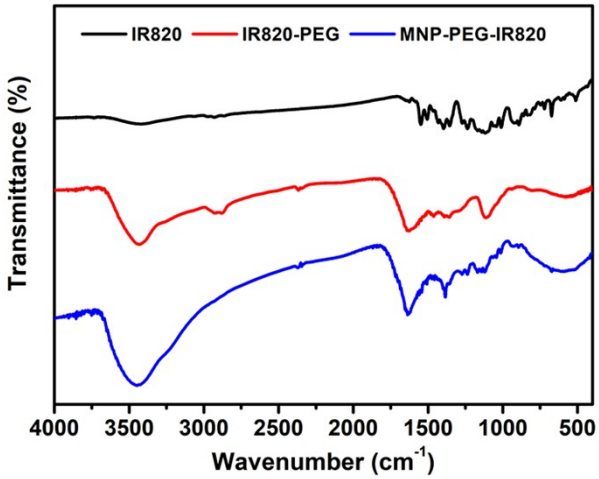

Figure S2 FT-IR spectra of IR820, IR820-PEG and MNP-PEG-IR820.

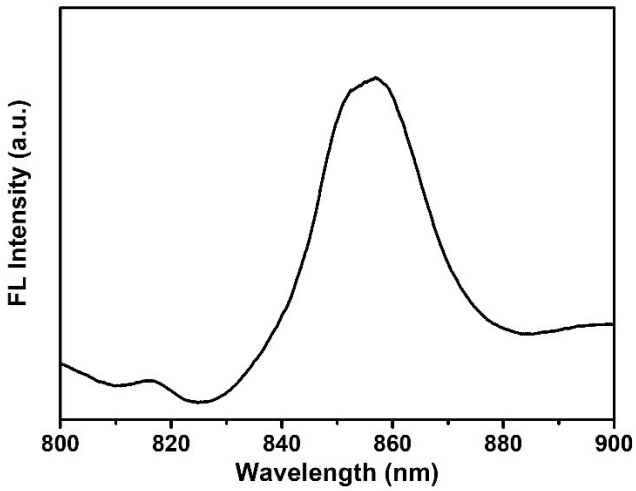

Figure S3 Fluorescence spectra of MPI

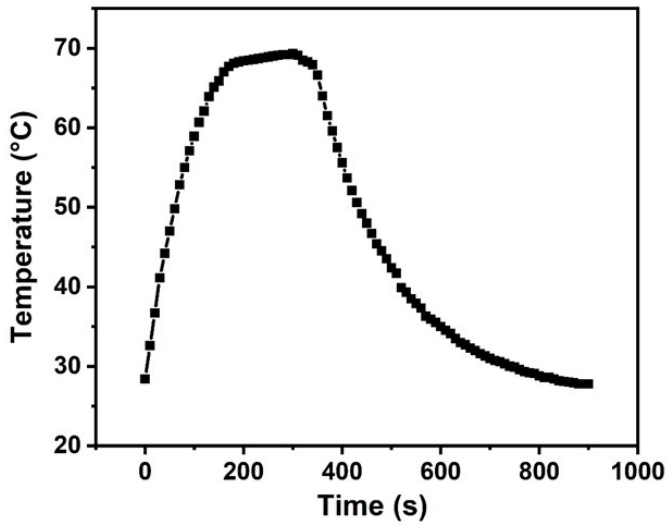

Figure S4 The photothermal response of the MPI solution (200 μg mL^{-1}) for 500 s with an NIR laser (808 nm, 2 W cm^{-2}).

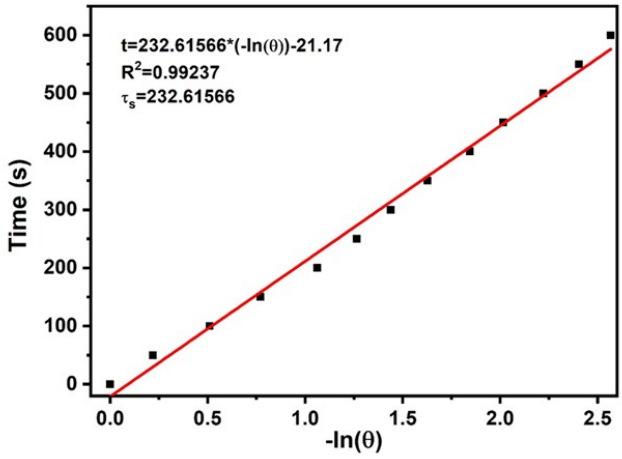

Figure S5 Plot and linear fit of time versus negative natural logarithm of the temperature increment for the cooling rate of MPI solution.

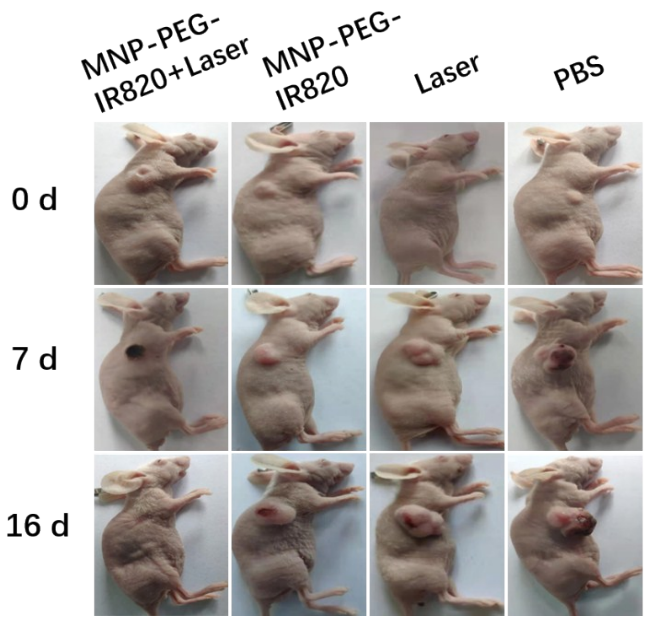

Figure S6 Representative photographs of tumor-bearing mice after treatment for 0–16 days (I. PBS; II. Laser; III. MNP-PEG-IR820; IV. MNP-PEG-IR820+Laser).

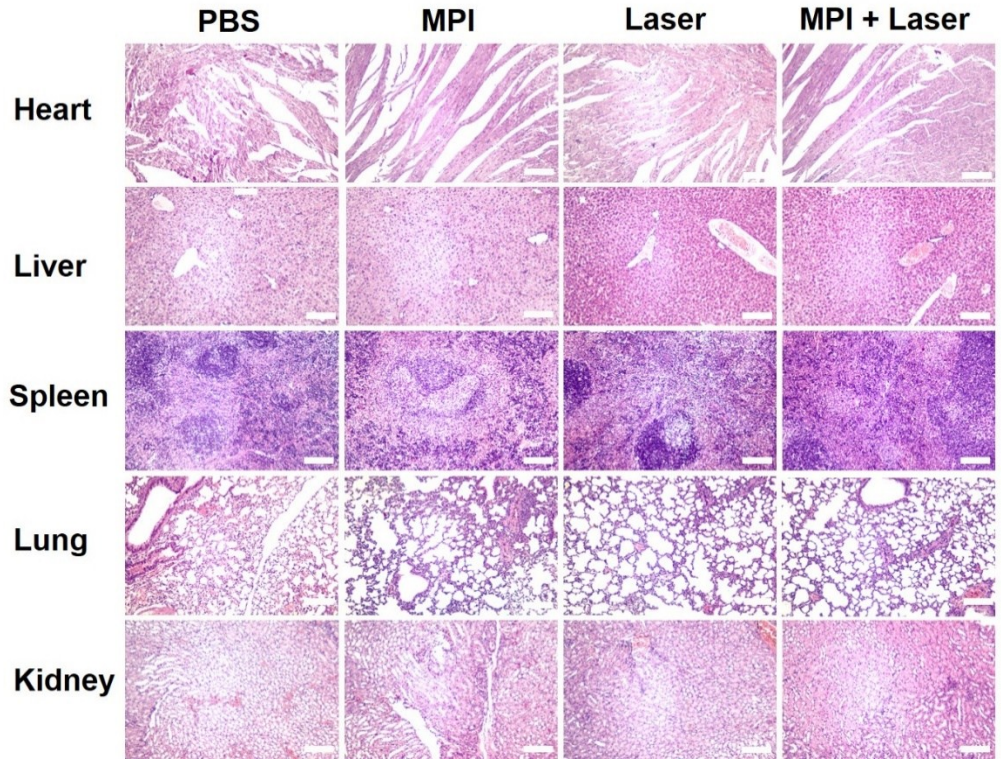

Figure S7 Histological analyses of the major tissues after therapy for 16 days. Scale bar: 200 μm.

Reference

1. R. Zhang, Q. Fan, M. Yang, K. Cheng, X. Lu, L. Zhang, W. Huang, Z. Cheng, *Adv. Mater.,* 2015, **27**, 5063-5069.
2. Q. Wang, Y. Dai, J. Xu, J. Cai, X. Niu, L. Zhang, R. Chen, Q. Shen, W. Huang, Q. Fan, *Adv. Funct. Mater.,* 2019, **29**, 1901480.
3. Y. Cheng, Y. Chang, Y. Feng, H. Jian, Z. Tang, H. Zhang, *Angew. Chem. Int. Ed.,* 2018, **57**, 246-251.
4. J. Sun, W. Xu, L. Li, B. Fan, X. Peng, B. Qu, L. Wang, T. Li, S. Li, R. Zhang, *Nanoscale,* 2018, **10**, 10584-10595.