Electronic Supplementary Information (ESI)

Indocyanine Green-Modified Hollow Mesoporous Prussian Blue Nanoparticles Loading Doxorubicin for Fluorescence-Guided Tri-Modal Combination Therapy of Cancer

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**Supplementary Figures**

**Fig. S1** High-resolution TEM images of (a, b) solid PB NPs, (c, d) HMPB NPs and (e, f) HMPB@PEI NPs under different magnifications.
**Fig. S2** FESEM images of as-prepared HMPB NPs under various magnifications. (a) scale bar: 200 nm; (b) scale bar: 1 μm.

**Fig. S3** Size distribution of (a) solid PB NPs, (b) HMPB NPs, (c) HMPB@PEI NPs, (d) HMPB@PEI/ICG NPs and (e) HPID NPs measured by dynamic light scattering (DLS).
Fig. S4 (a) Size variation of HPID NPs dispersed in various aqueous media measured by DLS over 2 weeks; (b) a high-resolution TEM image showing the surface of HPID NPs (scale bar: 10 nm).

Fig. S5 (a) Pore size distribution of HPID NPs; (b) $N_2$ adsorption–desorption isotherm of HPID NPs.
Fig. S6 Vis-NIR absorbance spectra of HPID NP dispersion at various concentrations.

Fig. S7 Fluorescence spectra of HMPB@PEI NPs and HPID NPs (equivalent DOX concentration: 0.1µg·mL⁻¹): (a) λ<sub>ex</sub> = 488 nm for exciting DOX fluorescence; (b) λ<sub>ex</sub> = 765 nm for exciting ICG fluorescence. NIR laser irradiation (808 nm, 2 W·cm⁻²) was conducted for 5 min where applicable.
Fig. S8 (a) Vis-NIR spectra of fed DOX (0.125 mg·mL⁻¹) and the supernatants after three times of centrifugation. The product (precipitates after 1st centrifugation) was washed twice by mixing with 40 mL of DI water and centrifugation to obtain the supernatants (2nd and 3rd). (b) Vis-NIR spectra of five-fold diluent of fed ICG (0.25 mg·mL⁻¹) and the supernatants after three times of centrifugation. The product (precipitates after 1st centrifugation) was washed twice by mixing with 20 mL of DI water and centrifugation to obtain the supernatants (2nd and 3rd). Optical path length was set as 1 cm and the ambient temperature was constant as 20°C.

Fig. S9 Peak temperature elevation of free ICG and HPID NPs (equivalent ICG concentration: 10 μg·mL⁻¹) during five cycles of NIR laser irradiation.
Fig. S10 Fitting curve of the time data versus -ln(θ) derived from the cooling period. The time constant for heat transfer was calculated to be 352.8 sec.

Fig. S11 Flow cytometry analysis of 4T1 cells incubated with HPID NPs over a range of incubation time (0.5 h, 1 h, 2 h and 4 h).
Fig. S12 Flow cytometry assay of 4T1 cells pretreated with different endocytosis inhibitors, followed by incubation with HPID NPs (equivalent DOX concentration: 10 μg·mL⁻¹) at 37°C for 4 h: (a) fluorescence intensity histogram; (b) cellular uptake efficiency corresponding to (a).
Fig. S13 (a) Decay of the normalized peak absorbance intensity of DPBF at 417 nm as the function of NIR laser irradiation time (808 nm, 2 W·cm\(^{-2}\), equivalent ICG concentration: 10 μg·mL\(^{-1}\)); the optical absorbance spectra of DPBF incubated with (b) HPID NPs, (c) ICG and (d) HMPB NPs under various laser irradiation time.
**Fig. S14** Detection of intracellular ROS generation using DCFH-DA probe (FITC channel) after various treatments, where “L” denotes the application of NIR laser irradiation (scale bars: 20 µm).
**Fig. S15** Change of mitochondrial membrane potential (MMP) in 4T1 cells characterized by JC-1 staining (aggregates: Cy3 channel; monomers: FITC channel) after various treatments, where “L” denotes the application of laser irradiation (scale bars: 50 µm).
**Fig. S16** Left: a regular image showing the vials containing HMPB NPs, DOX, ICG and HPID NPs; Right: an infrared image showing the fluorescence (near 830 nm) of ICG and HPID NPs in aqueous solution under NIR excitation (780 nm). Equivalent DOX concentration: 10 μg·mL⁻¹.
**Fig. S17** Representative fluorescence images of BALB/c mice bearing 4T1 tumors after intravenous injection of free ICG over 36 h.
Fig. S18 \textit{In vivo} pharmacokinetic curves over 48 h after intravenous injection of HPID NPs.

\begin{equation*}
y = 0.768 \exp(-x/1.7299) + 12.5 \exp(-x/3.253) + 6.7697
\end{equation*}

Fig. S19 Biodistribution of HPID NPs at 2, 12, 24, 48 and 72 h post-injection in BALB/c mice bearing 4T1 tumors.
Fig. S20 Survival rates of tumor bearing BALB/c mice subject to various treatments.

Fig. S21 Evaluation of hemocompatibility: (a) hemolytic rate of RBCs incubated with HPID NPs at various concentrations; (b) images of RBC suspensions incubated with HPID NPs under various concentrations (positive control: RBCs in DI water; negative control: RBCs in 1×PBS).
Fig. S22 Complete blood count of the mice intravenously injected with saline or HPID NPs. The orange hatched areas denote the reference ranges of hematology data of healthy female KM mice obtained from Chongqing Tengxin biotechnology Co. LTD.
Supplementary Methods

Calculation of the photothermal conversion efficiency of HPID NPs

The total energy balance between the input and dissipation for the system is presented as:

\[ \sum_i M_i C_i \frac{dT}{dt} = Q_{NP} + Q_{sys} - Q_{out} \] \hspace{1cm} (1)

where \( M \) and \( C \) denotes the mass and heat capacity of water, respectively; \( T \) represents the medium temperature; \( Q_{NP} \) is the energy absorbed by NPs; \( Q_{sys} \) denotes the energy from the pure water system; \( Q_{out} \) is heat dissipation from the system.

The heat absorbed by HPID NPs can be calculated as:

\[ Q_{NP} = I \left(1 - 10^{-A_{808}}\right) \eta \] \hspace{1cm} (2)

where \( I \) is the power of NIR laser, \( \eta \) indicates the photothermal conversion efficiency, and \( A_{808} \) denotes the absorbance of HPID NPs at 808 nm.

Heat dissipation is linear to the system temperature, defined as:

\[ Q_{out} = hS(T - T_{surr}) \] \hspace{1cm} (3)

where \( h \) is the heat transfer coefficient, \( S \) is surface area of the container, and \( T_{surr} \) is the ambient temperature.

After reaching a steady state temperature \((T_{max})\), the input and output of heat are in equilibrium.

\[ Q_{NP} + Q_{sys} = Q_{out} = hS(T_{max} - T_{surr}) \] \hspace{1cm} (4)

Upon removal of laser, \( Q_{NP} + Q_{sys} = 0 \), Eq. (1) can be converted to:

\[ \sum_i M_i C_i \frac{dT}{dt} = -Q_{out} = -hS(T - T_{surr}) \] \hspace{1cm} (5)
\[
dt = \sum_i M_i C_i \frac{dT}{hS \left( T - T_{\text{sur}} \right)} \\
t = -\sum_i M_i C_i \frac{T - T_{\text{sur}}}{hS \ln \left( \frac{T_{\text{max}} - T_{\text{sur}}}{T_{\text{max}} - T_{\text{sur}}} \right)}
\] (6)

(7)

A system time constant \( \tau_s \) can be defined as

\[
\tau_s = -\sum_i M_i C_i \frac{1}{hS}
\] (8)

and \( \theta \) is introduced for substitution,

\[
\theta = \frac{T - T_{\text{sur}}}{(T_{\text{max}} - T_{\text{sur}})}
\] (9)

which transforms Eq.(8) and Eq (9) into:

\[
t = -\tau_s \ln \theta
\] (10)

Since \( Q_{\text{sys}} \) can be calculated based on

\[
Q_{\text{sys}} = hS \left( T_{\text{max, } H_2 O} - T_{\text{sur}} \right)
\] (11)

Eq. (4) can be expressed as

\[
Q_{NP} = I \left( 1 - 10^{-A_{\text{808}}^g} \right) \eta = hS \left( T_{\text{max}} - T_{\text{max, } H_2 O} \right)
\] (12)

\[
hS = -\frac{\sum_i M_i C_i}{\tau_s}
\] (13)

where \( \tau_s \) is equal to 352.76 s, \( m \) is 3.0 g and \( c \) is 4.2 J/g, \( h_s \) can be calculated as 0.03572 W/°C. Substituting \( I = 2.0 \) W, \( A_{808} = 2.485 \), \( T_{\text{max}} - T_{\text{sur}} = 25.4 \) °C into Eq. (12), the photothermal conversion efficiency of HPID NPs can be determined as 45.51%.
Reference

Synthesis of HMPB NPs
Firstly, 3 g of PVP and 131.7 mg of K₃[Fe(CN)₆] were dissolved in 40 mL of diluted HCl solution (0.01 M) under magnetic stirring. After 30 min of stirring at room temperature in a round-bottom flask, a clear yellow solution was obtained. Then, the flask containing the product was placed into an electric oven and heated at 80°C without stirring for 20 h. To obtain the solid PB NPs with the protection of PVP layers, precipitates were collected by centrifugation and washed with DI water thrice, followed by drying in a vacuum oven at 50 °C for 12 h.

Secondly, 20 mg of solid PB NPs and 100 mg of PVP were added into hydrochloric acid solution (1.0 M, 20 mL) in a Teflon vessel under magnetic stirring for 2 h. Then, the vessel was sealed in a stainless steel autoclave and heated at 140 °C for 4 h in an electric oven. Afterwards, precipitates containing hollow mesoporous Prussian blue (HMPB) NPs were collected by centrifugation, washed with DI water and dried in vacuum oven. During the etching process, a small portion of K₃[Fe(CN)₆] could potentially react with HCl and yield toxic HCN. Thus, the liquid waste needed to be disposed properly after the autoclave was cooled down to room temperature.
Photothermal properties of HPID NPs

A fiber-coupled semiconductor diode NIR laser (wavelength: 808 nm, output power: 2 W·cm\(^{-2}\), SintecOptronics Technology, Singapore) was used as the NIR light source throughout the photothermal property test. 3 mL of aqueous dispersion of HPID NPs at gradient concentrations (50, 100, 150, 200, 250 μg·mL\(^{-1}\)) were respectively loaded into transparent quartz vials, followed by immersing a temperature probe into the sample. All the samples were irradiated by the NIR laser (808 nm, 2 W·cm\(^{-2}\)) for 10 min, during which the temperature elevation was dynamically monitored with a digital thermometer. For comparison, photothermal property of the intermediate products leading to HPID NPs was also evaluated at the equivalent concentration of 200 μg·mL\(^{-1}\) based on the same method. Additionally, photothermal response of HPID NPs was further investigated by tuning the laser output power in the range of 0–3 W·cm\(^{-2}\). To examine the photothermal stability, aqueous dispersion of HPID NPs (200 μg·mL\(^{-1}\)) was periodically irradiated by a NIR laser for 5 cycles, and the resultant temperature variation was recorded in real-time during the irradiation and cooling process. A thermal imager (TiS55, Fluke, US) was also applied to visualize the temperature variations of HPID NP dispersions at gradient concentrations in color mapping.

Photodynamic property of HPID NPs

*In vitro* photodynamic property of HPID NPs was evaluated using a fluorescence probe, DPBF, which has a specific reactivity with singlet oxygen (\(^1\)O\(_2\)). Briefly, 20 μL of DPBF solution (dissolved in DMSO, 1.5 mg·mL\(^{-1}\)) was added into 3 mL aqueous
dispersions of HPID NPs, HMPB NPs or free ICG (equivalent ICG concentration: 10 μg·mL⁻¹) upon rigorous mixing. Then, the mixture was transferred into a 10 mm cuvette and exposed to a NIR laser (808 nm, 2 W·cm⁻²) for 5 min under magnetic stirring. To evaluate the yield of singlet oxygen, UV-vis absorption spectra of each sample was recorded at different time points.

Afterwards, the intracellular ROS generation was determined by another fluorescence probe (2’, 7’-dichlorofluorescein diacetate, DCFH-DA). Typically, 4T1 tumor cells were seeded in a 12-well plate at a density of 1×10⁵ cells per well and incubated for 12 h. Then the cells were incubated with free ICG (10 μg·mL⁻¹) or HMPB@PEI/ICG NPs (equivalent ICG concentration of 10 μg·mL⁻¹) for 4 h, followed by treatment with DCFH-DA (5 µg·mL⁻¹) for 30 min. Finally, cells were rinsed thoroughly with 1×PBS, and the production of intracellular ROS was evaluated by examining the fluorescence emission through confocal laser scanning microscopy (CLSM, LSM 800, Carl Zeiss, Germany).

**Biocompatibility evaluation in vitro**

Human umbilical vein endothelial cells (HUVECs) and Murine L929 fibrosarcoma cells (L929 cells) were selected to evaluate the biocompatibility of HMPB@PEI/ICG NPs as drug carriers in vitro. Specifically, HUVECs and L929 cells at the initial seeding density of 1×10⁴ per well were cultured in a 96-well cell culture plate at 37 °C for 12 h. Then, the original medium in each well was replaced with fresh medium containing HMPB@PEI/ICG NPs at gradient concentrations (0~240 µg·mL⁻¹). After further
incubation for 24 h, cell viability was determined using MTT colorimetric assay. Briefly, the supernatant solution was aspirated and 200 μL of MTT solution (250 μg·mL⁻¹) was introduced into each well. After 6 h of incubation, 150 μL of DMSO was added to replace the previous supernatant. The well plate was gently shaking for 15 min, and the optical absorbance intensity was measured using a microplate reader (SPARK 10M) at 490 nm and 630 nm. The cell viability can be calculated based on formula (14):

\[
\text{Cell viability (\%)} = \frac{\text{OD}_{490\text{nm sample}} - \text{OD}_{630\text{nm sample}}}{\text{OD}_{490\text{nm control}} - \text{OD}_{630\text{nm control}}} \times 100\%
\]  

(14)

Hemolysis assay was conducted using the blood sample collected from the lateral tail vein of KM mice. Firstly, the whole blood sample was centrifuged at 3000 rpm for 5 min, and the obtained precipitate was rinsed for four times with 1×PBS to harvest erythrocytes. Then, 0.25 mL of erythrocytes suspension (4% v/v) was mixed with 0.25 mL of HMPB@PEI/ICG NPs dispersed in 1×PBS at various concentrations (50, 100, 200 and 300 μg·mL⁻¹), followed by incubation at 37 °C for 0.5 h or 6 h. Erythrocyte suspensions in DI water and 1×PBS served as positive and negative control groups, respectively. The samples were centrifuged at 10000 rpm for 5 min, and optical absorbance intensity of the supernatants was recorded at the wavelength of 570 nm.

**In vitro cytotoxicity**

To evaluate the therapeutic efficacy of combined chemo/photothermal/photodynamic treatment *in vitro*, standard MTT assay was conducted on five groups treated with (1) free DOX, (2) ICG, (3) hollow PB NPs, (4) HPID NPs without NIR irradiation, and (5)
HPID NPs with NIR irradiation (equivalent DOX concentration: 10 μg·mL⁻¹), respectively. Briefly, 4T1 cells were firstly seeded in a 96-well culture plate at the density of 10⁴ per well, followed by incubation at 37°C for 12 h. Afterwards, the cells were incubated with corresponding aforementioned agents for 4 h, and cells of group (2), (3) and (5) were subsequently exposed to NIR laser irradiation (808 nm, 2 W·cm⁻²) for 5 min. After further incubation for 12 h, cell viability of each group was determined by MTT cell viability assay.