

Ho-doped Prussian Blue for MR imaging-guided enhanced NIR-II photothermal-chemodynamic therapy via HSP70 inhibition against triple-negative breast cancer

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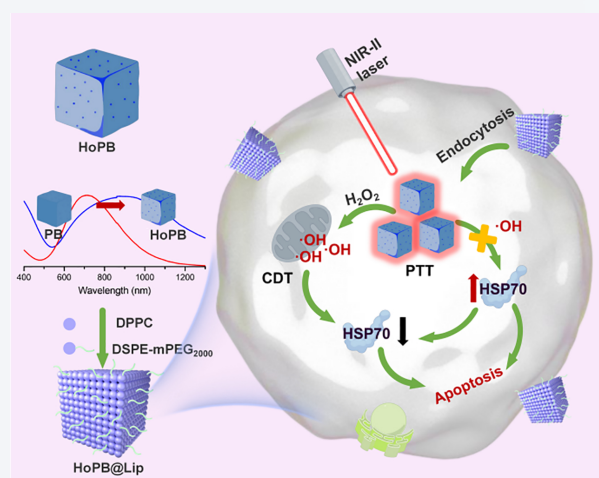
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ABSTRACT: Triple-negative breast cancer (TNBC) remains a challenging clinical challenge due to its chemoresistance, deep invasiveness, and thermal tolerance during photothermal therapy (PTT). Pristine Prussian Blue (PB) nanoparticles are limited by near-infrared I (NIR-I) absorption, weak nanozyme activity, and lack of imaging capability. Herein, we developed liposome-coated holmium-doped PB (HoPB@Lip) with an optimal Ho/Fe ratio of 0.4 as a multifunctional theranostic platform. Ho doping red-shifts the absorption to the NIR-II window with a high photothermal conversion efficiency of 68.3% and enhances peroxidase-like activity for chemodynamic therapy (CDT). It also boasts a T_2 relaxivity of $218.3 \text{ mM}^{-1} \cdot \text{s}^{-1}$, enabling high-contrast magnetic resonance (MR) imaging. *In vitro* and *in vivo* experiments on 4T1 TNBC models demonstrated that synergistic PTT-CDT inhibited tumor growth by $\sim 95\%$. Reactive oxygen species (ROS) generated by HoPB@Lip suppressed heat shock protein 70 (HSP70) expression via oxidative inactivation of heat shock factor 1, effectively overcoming tumor thermal tolerance. Notably, HoPB@Lip exhibited excellent systemic biocompatibility without organ toxicity. This work integrates NIR-II PTT therapy, CDT, and MR imaging into one platform, providing a safe and precise theranostic strategy for TNBC with great clinical translation potential.

KEYWORDS: Ho-doped Prussian Blue, triple-negative breast cancer, near-infrared II (NIR-II) theranostics, nanozyme synergy, photothermal therapy



1 Introduction

As the primary threat to global women's health, triple-negative breast cancer (TNBC), a subtype of breast cancer lacking estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 (HER2) expression, relies mainly on chemotherapy for clinical treatment [1–3]. However, its inherent high invasiveness, metastasis tendency, and multidrug resistance lead to significant systemic toxicity of chemotherapeutic drugs,

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limited efficacy, high recurrence rate, and poor prognosis in patients, making it a persistent challenge in breast cancer treatment [4]. Therefore, developing novel theranostic strategies with high selectivity, low toxicity, and real-time visualization capabilities to achieve precise treatment of invasive breast cancer lesions has become a critical unmet need in oncology. Photothermal therapy (PTT), a non-invasive therapeutic modality, has emerged as a promising option for precise breast cancer treatment [5–8]. It utilizes photothermal agents to convert light energy into thermal energy under specific laser irradiation, enabling local hyperthermia-induced tumor ablation, and exhibits prominent advantages such as high spatiotemporal controllability and minimal damage to normal tissues.

Among various photothermal agents, Prussian Blue (PB) is regarded as a promising candidate with great translational potential for breast cancer treatment, owing to its excellent photothermal stability, tunable size, and clinically proven biocompatibility, having been approved by the U.S. Food and Drug Administration (FDA) for clinical detoxification of cyanide poisoning [9–11]. However, intrinsic PB nanoparticles have multiple limitations that hinder their application in breast cancer treatment: First, their characteristic absorption peak is confined to the near-infrared I (NIR-I) window (650–700 nm). Intense scattering and absorption of NIR-I light by biological tissues (skin, blood, fat, etc.) result in a penetration depth of less than 1 cm, failing to reach deep chest wall invasive lesions commonly seen in breast cancer [12]. Second, the unfavorable surface charge and hydrophilic-hydrophobic property of PB nanoparticles lead to rapid clearance by the reticuloendothelial system (RES), poor circulation stability, and insufficient biocompatibility, which impairs efficient accumulation at tumor sites [13]. Third, the lack of imaging functionality prevents real-time monitoring of drug distribution and treatment progress, failing to meet the clinical demand for theranostic integration, thus severely limiting its application in advanced breast cancer treatment [14].

In contrast, the near-infrared II (NIR-II) window (1000–1700 nm) is known as the "optical transparency window" of biological tissues [15–17]. Light in this wavelength range possesses lower photon scattering coefficient and tissue absorption rate, enabling centimeter-level deep tissue penetration, along with a higher maximum permissible exposure (MPE), which can improve the therapeutic depth of breast cancer lesions while reducing the risk of photodamage [18]. Thus, developing novel PB-based derivatives with strong NIR-II absorption, integrated biocompatibility and imaging capabilities, is the key to breaking the bottleneck of breast cancer treatment. Existing studies have shown that doping metal ions into the PB lattice to regulate its energy band structure is an effective strategy for absorption peak redshift [19]. However, most current studies focus on doping common ions such as Fe and Co, which only achieve fine-tuning of the absorption peak to the end of the NIR-I region rather than stable redshift to the NIR-II region, and often lead to decreased photothermal stability or impaired biocompatibility after doping [20]. Meanwhile, most modified PB materials focus on a single photothermal function, lacking synergistic optimization of nanozyme activity, imaging performance, and biocompatibility, and fail to form an integrated system that can simultaneously address deep penetration, thermal tolerance, and real-time monitoring in breast cancer treatment [21]. Rare earth ions have emerged as promising dopants for addressing these limitations. The unique 4f electronic configuration of rare

earth ions can effectively regulate the energy band structure of PB to achieve significant absorption peak redshift, and their inherent paramagnetism can endow the nanoplatform with magnetic resonance imaging (MRI) capability, making them ideal dopants for the multifunctionalization of PB [22].

Although rare earth ion doping has also been explored for PB modification, most studies focus on single functional optimization rather than comprehensive performance enhancement. For instance, gadolinium (Gd) and thulium (Tm)³⁺ doping primarily improves T₁ MR imaging performance but only induces a limited absorption peak redshift to ~ 808 nm, failing to enter the NIR-II responsive region [23]. Ytterbium (Yb) doping mainly introduces upconversion luminescence properties, with their absorption peaks typically remaining below 816 nm, which cannot meet the requirements of deep-tissue photothermal therapy [24]. In contrast, holmium (Ho) with a [Xe]4f⁹ electronic configuration and moderate ionic radius (0.901 Å) can form strong orbital hybridization with the Fe-3d orbitals in the PB framework, significantly narrowing its band gap to achieve a substantial absorption peak redshift. Meanwhile, Ho doping induces appropriate lattice distortion to enhance nanozyme activity and preserves the cubic crystal structure of PB to maintain excellent photothermal stability. Additionally, the strong paramagnetism of Ho³⁺ endows the nanoplatform with high-performance T₂-weighted MRI capability, enabling the integration of multiple theranostic functions in a single system.

Beyond the limitations of physical penetration depth and single functionality, PTT also faces a critical biological challenge of "thermal tolerance" in cancer treatment [25–27]. The cancer cells are inherently highly invasive and have active stress responses; when subjected to sublethal hyperthermia, they rapidly activate stress pathways and overexpress heat shock proteins (especially heat shock protein 70 (HSP70)) [28]. As molecular chaperones, HSP70 can efficiently repair heat-damaged proteins and block apoptotic pathways, significantly enhancing the thermal resistance of cancer cells, leading to incomplete thermal ablation and rapid tumor recurrence and metastasis [29–31]. Studies have confirmed that the explosive increase in intracellular ROS levels can effectively interfere with the transcription and function of HSP70, breaking the tumor thermal tolerance mechanism [32]. Mechanistically, ROS specifically oxidize the conserved cysteine residues in the DNA-binding domain of heat shock factor 1 (HSF1), the master regulator of HSP70 expression, inhibiting its trimerization and nuclear translocation, thereby fundamentally blocking HSP70 gene transcription [33]. Meanwhile, ROS can directly oxidize the redox-sensitive cysteine residues in the ATPase and substrate-binding domains of HSP70 protein, causing conformational changes that abolish its molecular chaperone function [34]. This multi-pronged inhibition of the HSF1-HSP70 axis completely eliminates the tumor's thermal defense capability, enabling efficient photothermal ablation even at mild hyperthermia temperatures. PB itself exhibits weak peroxidase (POD)-like and catalase (CAT)-like activities, which can catalyze endogenous H₂O₂ in the tumor microenvironment to generate ROS [35, 36]. However, the nanozyme activity of intrinsic PB is too weak to produce sufficient ROS to inhibit the high expression of HSP70 in breast cancer cells when acting alone, and it does not form a synergy with NIR-II photothermal effect, failing to simultaneously solve the multiple challenges in breast cancer treatment.

Based on the aforementioned core gaps in breast cancer

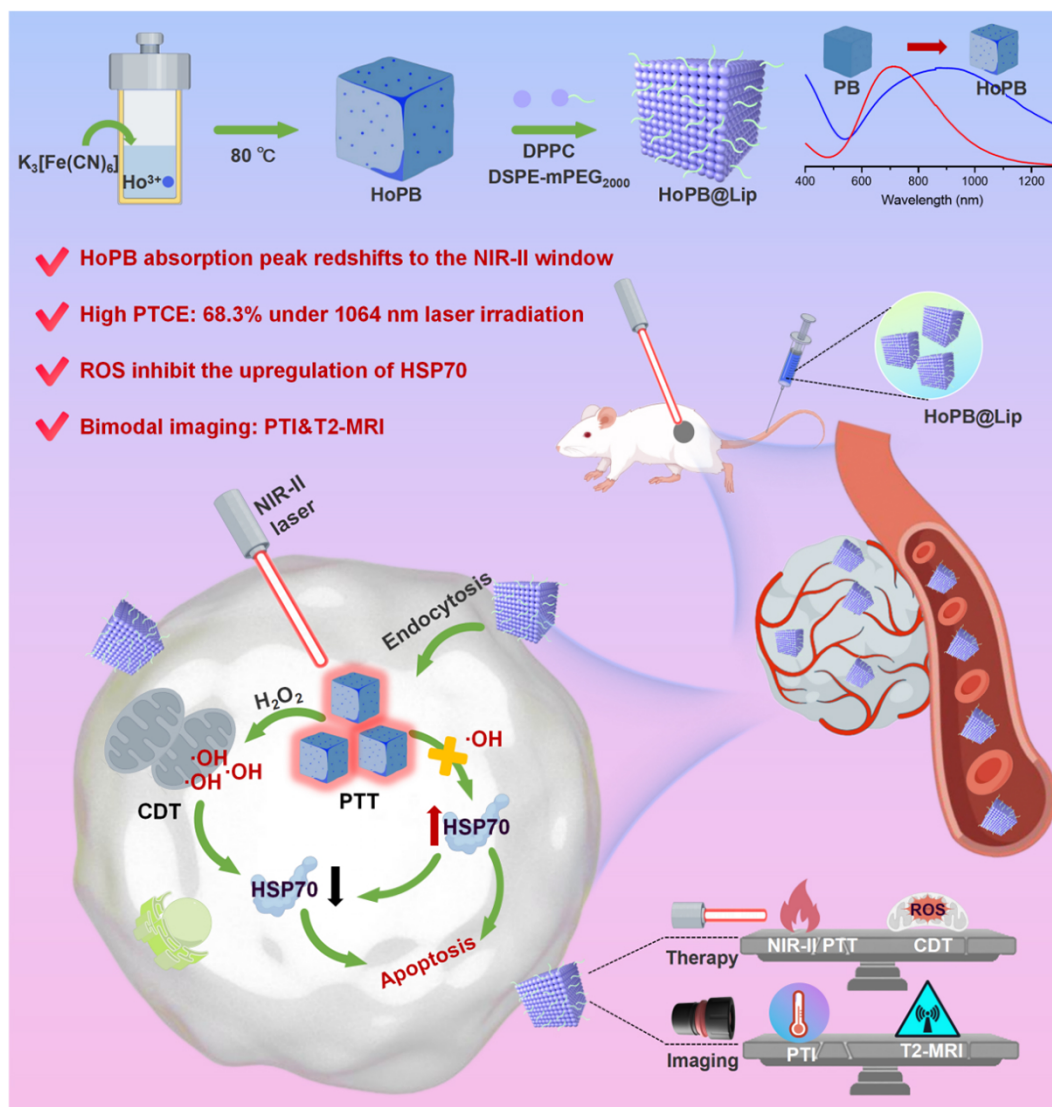
treatment, this study constructed a NIR-II photothermal-nanozyme synergistic theranostic system based on Ho-doped PB (HoPB), achieving integrated functions of anti-thermal tolerance and visual monitoring through multi-dimensional optimization, with core performances verified by a series of experiments (Scheme 1): Firstly, we adjusted different Ho ion doping ratios and combined spectroscopic and thermal performance characterization to finally screen out the optimal doping ratio of (Ho:Fe = 0.4:1). At this ratio, the 4f electronic configuration of Ho ions can precisely regulate the electronic energy band structure of PB, successfully and stably redshifting its characteristic absorption peak to the 1000 nm NIR-II window. Combined with NIR-II laser irradiation, efficient photothermal ablation of breast cancer tissue was achieved, with a photothermal conversion efficiency of 68.3% and a stability of over 95% after 5 cycles of irradiation. Meanwhile, Ho ions significantly enhance the POD activities of PB by inducing lattice distortion. Assisted by local hyperthermia induced by NIR-II photothermal effect, it catalyzes endogenous H_2O_2 in the breast cancer microenvironment to explosively generate ROS, forming an ROS storm. This ROS storm not only directly damages the breast cancer cells and induces apoptosis but also inhibits HSP70 expression. This ROS storm not only directly damages the breast cancer cells and induces apoptosis but also inhibits HSP70 expression,

completely breaking the thermal tolerance mechanism. To optimize *in vivo* performance, we modified the surface of HoPB with liposomes, which effectively reduces immunogenicity, prolongs *in vivo* circulation time, enhances passive targeted accumulation at 4T1 tumor sites, and improves biocompatibility. In addition, the paramagnetism of Ho^{3+} can significantly improve the T_2 relaxation rate of PB, endowing the platform with MR imaging capability, which provides visual support for real-time monitoring of 4T1 tumor sites and dynamic evaluation of treatment progress. Focusing on TNBC with high clinical difficulty and poor prognosis, this study will further verify the synergistic theranostic effects of the HoPB nano-platform through systematic material characterization, *in vitro* experiments on 4T1 TNBC cells, and *in vivo* nude mouse models of subcutaneous 4T1 breast cancer, providing a new material basis and strategy for the precise treatment of TNBC.

2 Experimental

2.1 Materials

Ethanol, phosphate-buffered saline (PBS), 2,2'-azobis(2-



Scheme 1 Schematic illustration of HoPB@Lip NPs to integrate NIR-II PTT therapy, CDT, and MR imaging, providing a safe and precise theranostic strategy for TNBC.

methylpropionamidine) dihydrochloride (ABDA), 3,3',5,5'-tetramethylbenzidine (TMB), cell counting kit-8 (CCK-8), 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), Calcein-AM/propidium iodide (PI) double staining kit, and hydrogen peroxide (H_2O_2) were purchased from Sinopharm Chemical Reagent Co., Ltd. Penicillin-Streptomycin, fetal bovine serum (FBS), Dulbecco's modified Eagle medium (DMEM), Roswell Park Memorial Institute (RPMI)-1640 medium, and trypsin-ethylenediaminetetraacetic acid (EDTA) were obtained from Gibco Life Technologies. 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-methoxy(polyethylene glycol)-2000, potassium ferricyanide ($\text{K}_3[\text{Fe}(\text{CN})_6]$), polyvinylpyrrolidone (PVP), molecular weight (M_w) $\sim 58,000$, holmium nitrate hexahydrate ($\text{Ho}(\text{NO}_3)_3 \cdot 6\text{H}_2\text{O}$), and chloroform were purchased from Aladdin Biochemical Technology Co., Ltd. DID (1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate salt) and JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide) were acquired from Thermo Fisher Scientific. Primary antibodies against HSP70 and β -actin, horseradish peroxidase (HRP)-conjugated secondary antibody, and enhanced chemiluminescence (ECL) detection kit were purchased from Cell Signaling Technology. Deionized water (18.2 $\text{M}\Omega\cdot\text{cm}$) was prepared using a Milli-Q Academic A10 ultra-pure water system.

2.2 Synthesis of Ho-doped Prussian Blue nanoparticles (HoPB NPs)

HoPB NPs were synthesized via a hydrothermal method. Briefly, 264 mg of $\text{K}_3[\text{Fe}(\text{CN})_6]$ and 3 g of PVP were dissolved in 40 mL of deionized water, followed by magnetic stirring at room temperature for 5 min to form a homogeneous solution. To prepare samples with different doping ratios, $\text{Ho}(\text{NO}_3)_3 \cdot 6\text{H}_2\text{O}$ was added at molar ratios (Ho:Fe) of 0, 0.1, 0.2, 0.4, and 0.6, with the final reaction volume adjusted to 40 mL. The mixed solution was reacted at 80 °C for 12 h. After the reaction, the product was collected by centrifugation (10,000g, 15 min), alternately washed with deionized water and ethanol three times to remove unreacted ions, and finally dried under vacuum for subsequent use. Pristine PB nanoparticles were synthesized using the same procedure without the addition of $\text{Ho}(\text{NO}_3)_3 \cdot 6\text{H}_2\text{O}$.

2.3 Preparation of HoPB@Lip nanocomposites

DPPC is a naturally occurring phospholipid with excellent biocompatibility and biodegradability, which minimizes the immunogenicity and systemic toxicity of the nanoplatform. DSPE-PEG₂₀₀₀ forms a hydrophilic "stealth" layer on the nanoparticle surface, which significantly reduces non-specific adsorption of serum proteins and opsonization. Liposome-coated HoPB (HoPB@Lip) was fabricated via the thin-film hydration method. DPPC and DSPE-PEG₂₀₀₀ were dissolved in chloroform at a molar ratio of 9:1. The organic solvent was removed by rotary evaporation (60 °C, 30 min) to form a uniform lipid film on the inner wall of the round-bottom flask. Subsequently, an aqueous solution containing HoPB NPs (1 mg/mL) was added, and the mixture was hydrated under ultrasonic treatment (100 W, 15 min) to facilitate self-assembly of the lipid bilayer around the nanoparticles. Free lipids were removed by centrifugation (8000g, 10 min) followed by dialysis (molecular weight cut-off (MWCO) = 10,000 Da) against PBS for 24 h, yielding HoPB@Lip nanocomposites.

2.4 Material characterization

The morphology and size of the nanoparticles were observed using a transmission electron microscope (TEM), Thermo Fisher Talos F200x, operated at 200 kV. X-ray diffraction (XRD) patterns were recorded on an X-ray diffractometer (Bruker D8 Advance) with Cu $K\alpha$ radiation ($\lambda = 1.5406 \text{ \AA}$) to analyze the crystal structure. Elemental distribution was characterized by energy-dispersive X-ray spectroscopy (EDS) mapping attached to the TEM. X-ray photoelectron spectroscopy (XPS), Thermo Fisher ESCALAB 250Xi, was used to determine the chemical valence states of elements. Ultraviolet-visible-NIR (UV-Vis-NIR) absorption spectra were measured using a spectrophotometer (TP-720, Beijing Purkinje General Instrument Co., Ltd.). Hydrodynamic diameter and zeta potential were determined by dynamic light scattering (DLS) using a Malvern Zetasizer Nano ZS at 25 °C.

2.5 Photothermal performance measurement

To evaluate the doping ratio-dependent photothermal effect, aqueous solutions of PB, 0.1HoPB, 0.2HoPB, and 0.4HoPB (100 $\mu\text{g/mL}$) were placed in centrifuge tubes and irradiated with a 1064 nm laser (1 W/cm^2) for 5 min. For power density-dependent studies, 0.4HoPB aqueous solution (100 $\mu\text{g/mL}$) was irradiated with 1064 nm laser at different power densities (0.5, 0.75, and 1 W/cm^2) for 5 min. Concentration-dependent photothermal effect was investigated by exposing 0.4HoPB solutions with varying concentrations (0, 50, 100, 150, and 200 $\mu\text{g/mL}$) to 1064 nm laser (1 W/cm^2) for 5 min. Temperature changes were monitored in real-time using a thermal imaging camera (FOTRIC 600C). Photothermal stability was assessed by laser on/off cycles: 0.4HoPB aqueous solution (75 $\mu\text{g/mL}$) was irradiated with 1064 nm laser (1 W/cm^2) for 5 min, followed by 5 min of laser off, and this cycle was repeated five times. The photothermal conversion efficiency (PTCE) was calculated using the formula reported previously [37].

2.6 Detection of $\cdot\text{OH}$ generation

TMB colorimetric assay was used to quantify $\cdot\text{OH}$ generation in HoPB suspensions in the presence of H_2O_2 (0.9 M). Specifically, TMB solution (2 mM in methanol) was mixed with PB, 0.1HoPB, 0.2HoPB, or 0.4HoPB (10 $\mu\text{g/mL}$) with different pH, and the time-dependent absorbance of TMB at 652 nm was monitored using a UV-Vis spectrophotometer.

2.7 Cell culture and animals

Mouse mammary epithelial cells (HC11) and triple-negative breast cancer cells (4T1) were cultured in RPMI-1640 medium supplemented with 10% FBS, 100 units/mL Penicillin, and 100 $\mu\text{g/mL}$ Streptomycin. Cells were maintained in a humidified incubator at 37 °C with 5% CO_2 . Six-week-old female Balb/c mice were purchased from Zhuhai Best Animal Technology Co., Ltd. All experimental protocols were approved by the Animal Care and Use Committee of Guangdong Second Provincial General Hospital (Approval No.: 2023-DW-KZ-130-02) and conducted in accordance with the Guide for the Care and Use of Laboratory Animals. Tumor models were established by subcutaneous injection of 1×10^6 4T1 cells into the right flank of mice.

2.8 Cellular uptake study

To investigate the cellular uptake kinetics of HoPB@Lip NPs, 4T1 cells were seeded in 24-well plates at a density of 8000 cells per well and allowed to adhere overnight at 37 °C. The medium was then

replaced with fresh RPMI-1640 containing DID-labeled HoPB@Lip NPs (100 $\mu\text{g/mL}$). At predetermined time points (1, 2, 4, 6, and 8 h), cells were fixed with 4% paraformaldehyde for 15 min, and nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI, 1 $\mu\text{g/mL}$) for 5 min. Fluorescence images were acquired using a confocal laser scanning microscope (CLSM), Zeiss LSM 880, to observe the intracellular accumulation of nanoparticles.

2.9 Cell viability assay

The biocompatibility of HoPB@Lip NPs was evaluated using the CCK-8 assay in 4T1 cells and normal HC11 cells. Cells were seeded in 96-well plates at a density of 5×10^3 cells per well and incubated overnight for adhesion. Subsequently, cells were exposed to HoPB@Lip NPs at different concentrations (0, 12.5, 25, 50, 100, and 200 $\mu\text{g/mL}$) for 24 h. After treatment, 10 μL of CCK-8 reagent was added to each well, and the plates were incubated for an additional 2 h. The absorbance at 450 nm was measured using a microplate reader (Thermo Fisher Scientific) to determine cell viability.

2.10 Intracellular ROS detection

For quantitative detection of intracellular ROS, 4T1 cells were seeded in 24-well plates and allowed to adhere for 24 h. Cells were then treated with PBS (control), H_2O_2 (50 μM), PB@Lip + H_2O_2 (100 $\mu\text{g/mL}$), or HoPB@Lip + H_2O_2 (100 $\mu\text{g/mL}$) and incubated overnight. After treatment, cells were washed three times with PBS and loaded with DCFH-DA (10 μM) for 30 min at 37 $^\circ\text{C}$. After removing the staining solution and washing twice with PBS, intracellular ROS levels were observed using a fluorescence microscope (excitation: 480 nm, emission: 525 nm) and quantified using ImageJ software.

2.11 Mitochondrial membrane potential ($\Delta\Psi_m$) analysis

JC-1 dye was used to assess $\Delta\Psi_m$. 4T1 cells were seeded in 96-well plates at a density of 5×10^3 cells per well and adhered overnight under standard culture conditions. Cells were then treated with the following groups: PBS (control), H_2O_2 alone (50 μM), PB@Lip + H_2O_2 + laser irradiation (L), HoPB@Lip + H_2O_2 , HoPB@Lip + L, and HoPB@Lip + H_2O_2 + L. After treatment, cells were incubated with 100 μL of JC-1 working solution (10 $\mu\text{g/mL}$ in Hanks' Balanced Salt Solution (HBSS)) at 37 $^\circ\text{C}$ in the dark for 20 min. After loading the dye, cells were washed twice with HBSS to remove excess probe, and fluorescence images were immediately acquired using a CLSM with 488 nm excitation and dual emission filters (530 nm for green fluorescence (JC-1 monomers) and 590 nm for red fluorescence (JC-1 aggregates)). The red-green fluorescence intensity ratio was quantified using image analysis software to determine changes in $\Delta\Psi_m$, with a higher ratio indicating hyperpolarized mitochondria and a lower ratio reflecting depolarization.

2.12 In vitro therapy

To evaluate the anticancer efficacy, 4T1 cells were seeded in 96-well plates at the same density and treated with different experimental groups: PBS (control), H_2O_2 alone (50 μM), PB@Lip, HoPB@Lip, PB@Lip + H_2O_2 , PB@Lip + H_2O_2 + L, HoPB@Lip + H_2O_2 , HoPB@Lip + L, and HoPB@Lip + H_2O_2 + L. After 6 h of incubation, cells designated for laser treatment were irradiated with a 1064 nm laser (1 W/cm^2) for 5 min at room temperature. Finally, cell viability was determined using the CCK-8 assay.

2.13 Live/dead staining

For live/dead cell viability assessment, 4T1 cells were seeded in 24-well plates at a density of 1×10^5 cells per well. Cells were then subjected to different treatment groups: PBS (control), H_2O_2 alone (50 μM), PB@Lip + H_2O_2 + L, HoPB@Lip + H_2O_2 , HoPB@Lip + L, and HoPB@Lip + H_2O_2 + L. After 6 h of incubation, laser-treated groups were irradiated with a 1064 nm laser (1 W/cm^2 , 5 min). Subsequently, all groups were stained with the Calcein-AM/PI viability kit to distinguish live cells (green fluorescence) and dead cells (red fluorescence), and live/dead images were acquired using a fluorescence microscope.

2.14 Western blotting

To investigate the activation of HSP70, 4T1 cells were seeded in 12-well plates and treated with PBS (control), H_2O_2 alone (50 μM), HoPB@Lip, HoPB@Lip + L, and HoPB@Lip + H_2O_2 + L for 24 h. After treatment, cells were harvested, washed with ice-cold PBS, and lysed with radioimmunoprecipitation assay (RIPA) buffer supplemented with protease and phosphatase inhibitors. Cell lysates were centrifuged at 12,000g for 15 min at 4 $^\circ\text{C}$, and the protein concentration of the supernatant was determined using the bicinchoninic acid (BCA) assay. Equal amounts of protein (30–50 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were blocked with 5% non-fat milk in Tris-buffered saline with Tween 20 (TBST) at room temperature for 1 h, then probed with primary antibodies against HSP70 and β -actin (loading control) at 4 $^\circ\text{C}$ overnight. After washing, membranes were incubated with HRP-conjugated secondary antibody at room temperature for 1 h. Protein bands were visualized using ECL reagent.

2.15 In vivo imaging and pharmacokinetic study

When the volume of 4T1 tumors reached approximately 200 mm^3 , mice were administered DID-labeled HoPB@Lip (HoPB@Lip-DID) via tail vein injection at a dose of 10 mg/kg . Before imaging, mice were anesthetized with isoflurane, and the hair over the tumor area was removed using commercial depilatory cream to minimize autofluorescence interference. Fluorescence images were acquired at 0.1, 3, 12, 24, 36, and 48 h post-injection using an *in vivo* imaging system (IVIS) Spectrum (PerkinElmer). At predetermined time points (12, 24, and 36 h), mice were euthanized, and major organs (heart, liver, spleen, lungs, kidneys, and tumor) were excised for *ex vivo* fluorescence imaging. Fluorescence intensity was quantified using ImageJ software, with background subtracted and normalized to tissue area.

2.16 In vitro and in vivo MR imaging of HoPB

Prepared PB and HoPB solutions were carefully diluted in plastic tubes to concentrations of 0, 10, 20, 50, 100, and 200 $\mu\text{g/mL}$. Serial T_2 -weighted MRI scans were then performed using a multi-slice spin-echo sequence (repetition time (TR) = 2000 ms, echo time (TE) = 20–100 ms, slice thickness = 2 mm). After acquiring T_2 -weighted MRI images, the signal intensity of each sample within the region of interest was measured. Relaxivity r_2 ($1/T_2$) was calculated from T_2 values at different concentrations. To evaluate the accumulation of HoPB in tumors, mice bearing 4T1 tumors (approximately 200 mm^3) were treated with PBS (Control), PB@Lip, or HoPB@Lip. T_2 -weighted MRI images were acquired 12 h after intravenous injection (10 mg/kg).



2.17 *In vivo* photothermal effect of HoPB

Mice bearing 4T1 tumors were injected with HoPB@Lip via tail vein, and the tumor site was exposed to laser irradiation (1064 nm, 1 W/cm²) for 5 min. Mice injected with PB@Lip served as the negative control group. Temperature changes and images of the tumor site were captured simultaneously using an infrared thermal imager.

2.18 *In vivo* antitumor activity of HoPB

Four groups ($n = 5$) of 6–8-week-old female Balb/c mice were randomly assigned: (1) PBS group; (2) PB@Lip + L group; (3) HoPB@Lip group; and (4) HoPB@Lip + L group. At the start of the experiment, each animal received a subcutaneous injection of 1×10^6 4T1 cells, and tumors were allowed to grow to a size of 80 mm³. Each group was intravenously injected with PB@Lip + L, HoPB@Lip, HoPB@Lip + L, or an equal volume of saline. Twenty-four hours after injection, mice in the PB@Lip + L and HoPB@Lip + L groups were exposed to a 1064 nm laser (1 W/cm², 5 min). Body weight and tumor volume were measured every two days. The formula for calculating tumor volume was: $V \text{ (mm}^3\text{)} = L \times W^2/2$, where L represents the longest dimension and W represents the shortest dimension. At the end of treatment, tumors were excised and stained with hematoxylin and eosin (H&E), Ki67, and HSP70. Blood samples were collected for standard blood tests and biochemical analysis, including red blood cells (RBC), white blood cells (WBC), platelets (PLT), hemoglobin (HGB), aspartate transaminase (AST), alanine transaminase (ALT), and creatinine (CREA). After mouse dissection, major organs were preserved in 4% formaldehyde. After a 24 h fixation period, tissues were sectioned, stained with H&E for histological evaluation, including vital organs such as the heart, liver, spleen, lungs, and kidneys.

2.19 Statistical analysis

Data are reported as mean $\pm$ standard deviation (SD). Differences between groups were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$.

3 Results and discussion

3.1 Fabrication and characterization of HoPB NPs

To investigate the structural and compositional evolution of Ho-doped PB nanoparticles, the materials with varying Ho/Fe molar ratios (0, 0.1, 0.2, 0.4, and 0.6) were systematically characterized. The TEM images in Fig. 1(a) and Fig. S1 in the Electronic Supplementary Material (ESM) reveal that PB nanoparticles maintain a well-defined cubic structure when the Ho/Fe ratio is ≤ 0.4 . At a Ho/Fe ratio of 0.6, however, the particles transform into a spherical morphology. The average particle size (calculated from TEM) increases gradually with Ho doping: ~ 120 nm (PB), ~ 160 nm (0.1HoPB), ~ 180 nm (0.2HoPB), ~ 200 nm (0.4HoPB), and ~ 180 nm (0.6HoPB). This morphological transition at high Ho ratios may arise from the disruption of PB's cubic lattice during excessive rare-earth ion incorporation, consistent with previous reports that high doping concentrations of large-radius rare-earth ions can induce shape evolution in PB-based nanomaterials [38]. At a Ho/Fe ratio of 0.6, excess Ho³⁺ ions preferentially adsorb onto the (100) planes of growing PB nanocrystals, passivating these planes and equalizing the growth rates of all crystallographic directions to

break the inherent anisotropic growth pattern [39]. Meanwhile, the severe lattice distortion induced by high-concentration Ho³⁺ doping lowers the nucleation energy barrier and accelerates nucleation rate, while competing for precursor ions to suppress crystal growth, resulting in smaller particle size.

In addition, the UV-Vis-NIR absorption spectra (Fig. 1(b)) show that the characteristic absorption peak of PB (centered at ~ 715 nm) undergoes a gradual red-shift with increasing Ho doping (to ~ 910 nm for 0.4HoPB), which is attributed to the altered electron delocalization in the PB framework upon Ho incorporation. Notably, the absorption peak of 0.6HoPB blue-shifts back to ~ 800 nm, likely correlated with the morphological transition (from cubic to spherical) observed in Fig. 1(a); morphology-dependent light scattering/absorption effects are known to modulate optical properties of nanomaterials [40]. Given the optimal red-shifted absorption (favorable for photothermal applications) and retained cubic structure, the 0.4HoPB sample was selected for subsequent PTT studies. Moreover, PXRD patterns (Fig. 1(c)) confirm that all Ho-doped samples retain the face-centered cubic (FCC) crystal structure of pristine PB (Joint Committee on Powder Diffraction Standards (JCPDS) No. 73-0687), with no additional impurity peaks. This indicates that Ho³⁺ ions are successfully incorporated into the PB lattice (instead of forming separate phases) at the tested doping ratios. Elemental mapping (Fig. 1(d)) of 0.4HoPB shows uniform spatial distribution of C, N, Fe, and Ho across the nanoparticles, directly verifying the homogeneous doping of Ho. The EDS spectrum (Fig. 1(e)) further confirms the presence of Fe, Ho, C, and N in the sample, with no extraneous elements detected. Next, XPS was used to analyze the chemical states (Fig. 1(f)). As shown in Fig. 1(g), the distinct Ho 4d peaks (at ~ 160 and ~ 163 eV) in 0.4HoPB (absent in pristine PB) confirm Ho incorporation, with binding energies consistent with Ho³⁺ [41]. The Fe 2p peaks (Fe²⁺/Fe³⁺) of 0.4HoPB are identical to those of pristine PB, indicating that Ho doping does not alter the oxidation state of Fe (Fig. 1(h)). The N 1s peak of 0.4HoPB shifts from ~ 399.5 eV (pristine PB) to ~ 398.0 eV, which arises from the coordination of N (in [Fe(CN)₆]⁴⁻) with Ho³⁺ (replacing partial Fe ions in the PB framework). Deconvolution of the high-resolution N 1s spectrum further confirms this coordination: the spectrum can be fitted into two distinct peaks at 399.3 eV (Fe-C $\equiv$ N-Fe) and 397.8 eV (Fe-C $\equiv$ N-Ho) (Fig. 1(i)). This peak shift and deconvolution result provide direct evidence for the integration of Ho into the PB structure via N-Ho coordination.

To enhance biocompatibility, 0.4HoPB was coated with a lipid bilayer (DPPC/DSPE-PEG). In the TEM image (Fig. 1(j)), a low-contrast layer (~ 5 nm thick) surrounding the cubic 0.4HoPB nanoparticles confirms successful lipid coating. DLS results show that the hydrated diameter increases from ~ 213 nm (uncoated 0.4HoPB) to ~ 221 nm (lipid-coated 0.4HoPB), consistent with the presence of the lipid shell (Fig. 1(k)). Furthermore, the zeta potential of 0.4HoPB shifts from ~ -9.29 mV (uncoated) to ~ -14.8 mV (lipid-coated), which is typical for PEGylated lipid bilayers and indicates improved colloidal stability in biological media (Fig. 1(l)). Furthermore, we evaluated the long-term colloidal stability of HoPB and HoPB@Lip in RPMI-1640 medium containing 10% FBS at 37 °C for 7 days. As shown in Fig. S2 in the ESM, uncoated HoPB exhibited severe aggregation with the average diameter increasing from ~ 210 to ~ 630 nm over 7 days. In contrast, HoPB@Lip maintained a stable hydrodynamic diameter of ~ 220 – 240 nm throughout the incubation period, demonstrating excellent colloidal stability in physiological media. This superior stability arises from the steric hindrance provided by the PEGylated

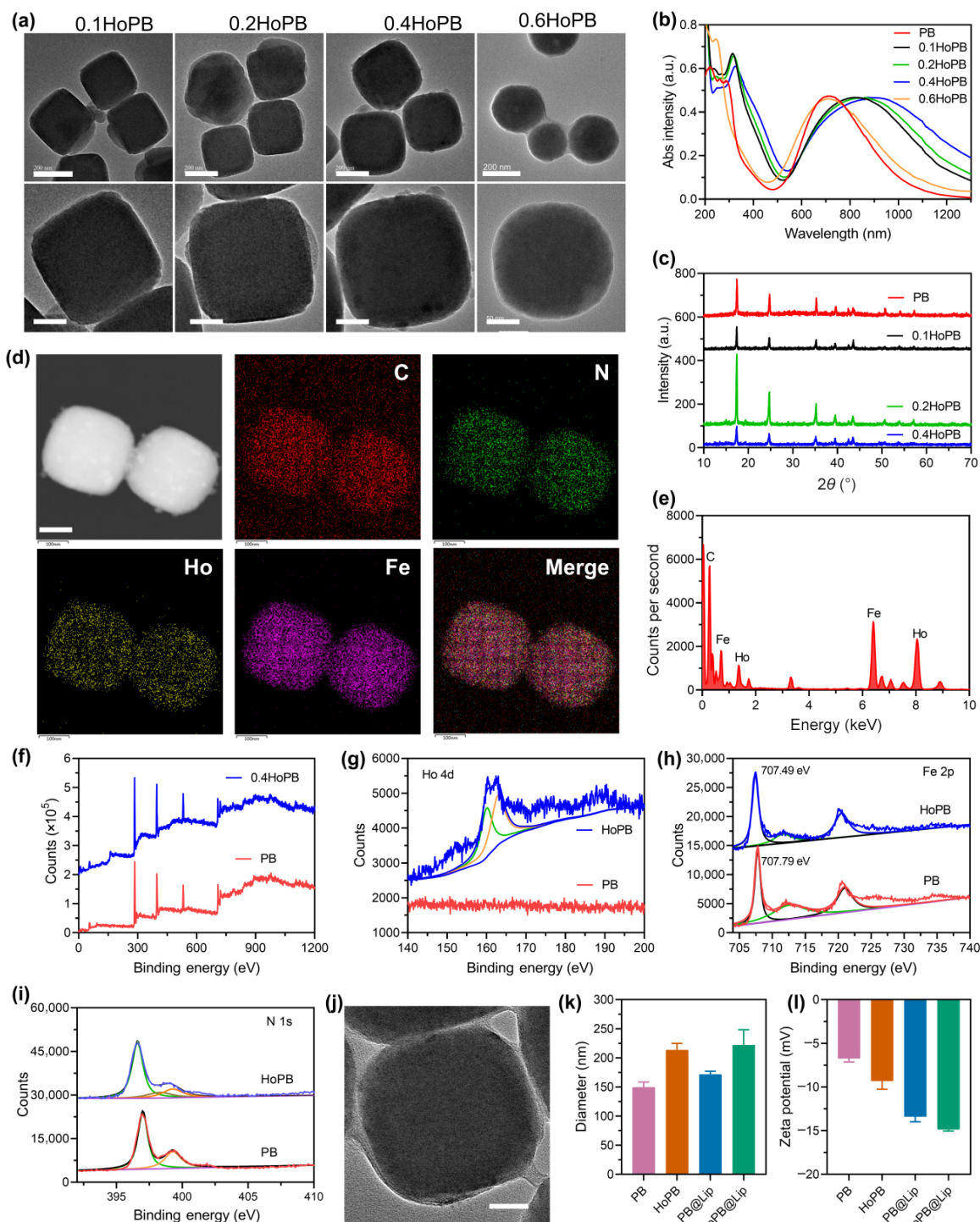


Figure 1 (a) TEM images of HoPB (Ho/Fe = 0.1, 0.2, 0.4, and 0.6); scale bars: 200 nm (top row) and 100 nm (bottom row). (b) UV-Vis-NIR absorption spectra of PB and HoPB samples. (c) Powder X-ray diffraction (PXRD) patterns of PB and HoPB samples. (d) Scanning transmission electron microscopy (STEM) image and corresponding elemental mapping of C, N, Ho, Fe, and merged distribution for 0.4HoPB, scale bar: 100 nm. (e) EDS spectrum of 0.4HoPB. XPS spectra: (f) C 1s, (g) Ho 4d, (h) Fe 2p, and (i) N 1s of PB and 0.4HoPB. (j) TEM image of lipid-coated (DPPC/DSPE-PEG) 0.4HoPB, scale bar: 50 nm. (k) Hydrodynamic diameter (measured via DLS) of PB, HoPB, and lipid-coated HoPB samples. (l) Zeta potential of PB, HoPB, and lipid-coated HoPB samples.

lipid bilayer, which inhibits non-specific protein adsorption and nanoparticle aggregation. Overall, these characterizations demonstrate that Ho^{3+} is homogeneously doped into the PB lattice (≤ 0.4 Ho/Fe ratio) while preserving the cubic structure, and lipid coating successfully enhances the biocompatibility of 0.4HoPB for potential biomedical applications.

3.2 Properties of HoPB@Lip NPs for photothermal/chemodynamic effect

Following the structural and compositional validation of Ho-doped Prussian blue (HoPB) nanoparticles, we next interrogated their functional properties, specifically, the NIR-II photothermal effect

(under 1064 nm laser, a window enabling deep-tissue light penetration) and peroxidase-like nanozyme activity, both of which are core to realizing synergistic PTT-chemodynamic therapy (CDT) for cancer. The photothermal capacity of HoPB was systematically characterized across key variables (doping ratio, laser power, material concentration) to optimize therapeutic utility. First, doping ratio-dependent photothermal heating (Fig. 2(a)) was evaluated at a fixed condition (100 $\mu\text{g/mL}$, 1 W/cm^2 1064 nm laser): among samples with Ho/Fe ratios of 0 (pristine PB), 0.1, 0.2, and 0.4, 0.4HoPB exhibited the most robust temperature elevation, reaching $\sim 54^\circ\text{C}$ ($\Delta T \sim 25^\circ\text{C}$) within 5 min. This outperformed pristine PB ($\Delta T \sim 11^\circ\text{C}$) and lower-doped variants (0.1HoPB: $\Delta T \sim 13^\circ\text{C}$; 0.2HoPB: $\Delta T \sim 18^\circ\text{C}$). This enhancement directly traces to the red-shifted NIR-II absorption of 0.4HoPB (Fig. 1(b)): its absorption peak aligns more closely with the 1064 nm laser wavelength, maximizing photon energy capture and subsequent conversion to thermal energy consistent with the structure-optical property correlation established earlier.

Next, laser power density optimization (Fig. 2(b)) was conducted for 0.4HoPB (100 $\mu\text{g/mL}$) to balance efficacy and biocompatibility. At 0.5 and 0.75 W/cm^2 , the maximum temperature only reached ~ 40 and $\sim 48^\circ\text{C}$, respectively, insufficient for PTT (which typically requires $> 50^\circ\text{C}$ to induce tumor cell necrosis). In contrast, 1 W/cm^2 (a clinically validated skin-safe power density for NIR-II laser exposure [42]) drove 0.4HoPB to $\sim 52^\circ\text{C}$: this temperature is effective for PTT (tumor cells are more thermosensitive than normal cells) while avoiding excessive damage to adjacent healthy tissue. Thus, 1 W/cm^2 was selected for all subsequent experiments. We then confirmed concentration-dependent photothermal response (Figs. 2(c) and 2(d)) for 0.4HoPB (1 W/cm^2 laser): temperature elevation scaled linearly with material concentration (50–200 $\mu\text{g/mL}$). For example, 200 $\mu\text{g/mL}$ 0.4HoPB reached $\sim 61^\circ\text{C}$ after 4 min (sufficient for rapid thermal ablation), while 50 $\mu\text{g/mL}$ only reached $\sim 34^\circ\text{C}$ (suited for mild hyperthermia). Infrared thermal images (Fig. 2(d)) visually validated this trend, showing progressively brighter (hotter) signals with increasing concentration, enabling tunable photothermal effects for diverse therapeutic scenarios (e.g., mild hyperthermia for sensitization vs. thermal ablation for direct killing).

Finally, photothermal stability and conversion efficiency (PTCE) (Figs. 2(e)–2(g)) were quantified to assess long-term utility. Photothermal cycling stability (critical for repeated PTT sessions) was confirmed: 0.4HoPB maintained a consistent ΔT ($\sim 29^\circ\text{C}$) over 5 on/off laser cycles (Fig. 2(e)), indicating no structural degradation under repeated thermal stress. To quantify performance, PTCE was calculated using the cooling curve method (Fig. 2(f)): by fitting $\ln(\theta)$ ($\theta = (T - T_0)/(T_{\text{max}} - T_0)$, where T_0 is ambient temperature) against time (Fig. 2(g)), the PTCE of 0.4HoPB was determined to be 68.3%. This value is substantially higher than most NIR-II photothermal agents reported to date (e.g., pristine PB-based nanoparticles: PTCE $< 50\%$ [43]; black phosphorus quantum dots: $\sim 40\%$ [44]), highlighting 0.4HoPB's superiority as a PTT agent.

To objectively evaluate the performance advantages of HoPB@Lip among rare earth-doped PB-based photothermal agents, we systematically compared it with representative recently reported platforms in terms of PTCE, laser wavelength, imaging modality, and core innovation (Table S1 in the ESM). Most reported rare earth-doped PB agents operate at 808 nm (NIR-I window) with PTCE values ranging from 39.66% to 55.0% [22–24]. In contrast, HoPB@Lip achieves a record-high PTCE of 68.3% at

1064 nm (NIR-II window), which enables deeper tissue penetration and higher maximum permissible exposure compared to NIR-I lasers. This high PTCE allows efficient tumor ablation at lower laser power densities, minimizing damage to adjacent normal tissues.

We next assessed 0.4HoPB's peroxidase-like activity (a key CDT mechanism, which catalyzes H_2O_2 to generate cytotoxic $\cdot\text{OH}$ using TMB, a chromogenic substrate that oxidizes to a blue product ($\lambda_{\text{max}} = 652\text{ nm}$) in the presence of $\cdot\text{OH}$. As shown in Fig. 2(h), negligible TMB oxidation was observed in the TMB-only or TMB+ H_2O_2 groups (H_2O_2 decomposes slowly spontaneously, producing insufficient $\cdot\text{OH}$ to drive TMB oxidation). Pristine PB induced weak 652 nm absorbance, but 0.4HoPB dramatically amplified this signal—indicating accelerated H_2O_2 decomposition. Quantitative analysis (Figs. 2(i) and 2(j), and Fig. S3 in the ESM) confirmed that 0.4HoPB exhibited ~ 4 -fold higher peroxidase-like activity than pristine PB ($***P < 0.001$). Furthermore, we evaluated the pH-dependent POD activity of HoPB to verify its tumor microenvironment (TME)-responsive characteristics. As shown in Fig. S4 in the ESM, the catalytic activity of HoPB at pH 6.0 was 1.42-fold higher than that at pH 7.4. This enhancement arises from Ho^{3+} : as a Lewis acid, Ho^{3+} acts as an additional catalytic site to promote H_2O_2 cleavage into $\cdot\text{OH}$, supplementing the Fe-mediated catalysis of PB. This doping-induced catalytic activity enhancement strategy modulates the local electronic structure of active sites and can significantly improve catalytic performance [45–47].

Collectively, 0.4HoPB combines high-performance NIR-II photothermal properties and enhanced peroxidase-like activity, two functionalities that enable synergistic PTT-CDT. Critically, the $\cdot\text{OH}$ generated by 0.4HoPB's nanozyme activity may also inhibit HSP expression (a well-documented PTT resistance mechanism [48]), which we later validated in cellular experiments, further amplifying therapeutic efficacy.

3.3 Antitumor evaluation of HoPB@Lip NPs *in vitro*

To bridge the gap between the physicochemical properties of HoPB@Lip and their therapeutic utility, we systematically evaluated their cellular behavior and antitumor efficacy in 4T1 breast cancer cells, focusing on uptake, biocompatibility, ROS-mediated damage, mitochondrial dysfunction, synergistic cell killing, and HSP modulation. First, cellular uptake and biocompatibility were assessed. For uptake, 4T1 cells were incubated with DiO (3,3'-diiododecylcarbocyanine perchlorate)-labeled HoPB@Lip (DiO: red fluorescent membrane probe; DAPI: blue nuclear stain), and time-dependent imaging (Fig. 3(a)) revealed a gradual intensification of red fluorescence over 8 h: at 1 h, faint red signal localized to the cell periphery, while by 8 h, robust red fluorescence colocalized with nuclear DAPI, confirming efficient endocytic internalization of HoPB@Lip into 4T1 cells. This high uptake efficiency ensures sufficient delivery of the material's photothermal and nanozyme functionalities to target tumor cells. Parallel biocompatibility assays (Fig. 3(b)) tested HoPB@Lip cytotoxicity in both normal HC11 mammary epithelial cells and 4T1 cancer cells across concentrations (0.9–200 $\mu\text{g/mL}$, 24 h treatment): cell viability remained $> 90\%$ in both cell lines even at the highest concentration, demonstrating excellent biocompatibility. To further evaluate the long-term biocompatibility, we also assessed HC11 cell viability after 72 h of exposure to HoPB@Lip. Consistent with the 24 h results, cell viability remained above 90% at all tested concentrations (Fig. S5 in the ESM), indicating no time-dependent cytotoxicity of HoPB@Lip on normal cells. This safety profile is attributed to the PEGylated lipid coating, which minimizes nonspecific interactions with healthy cellular membranes.

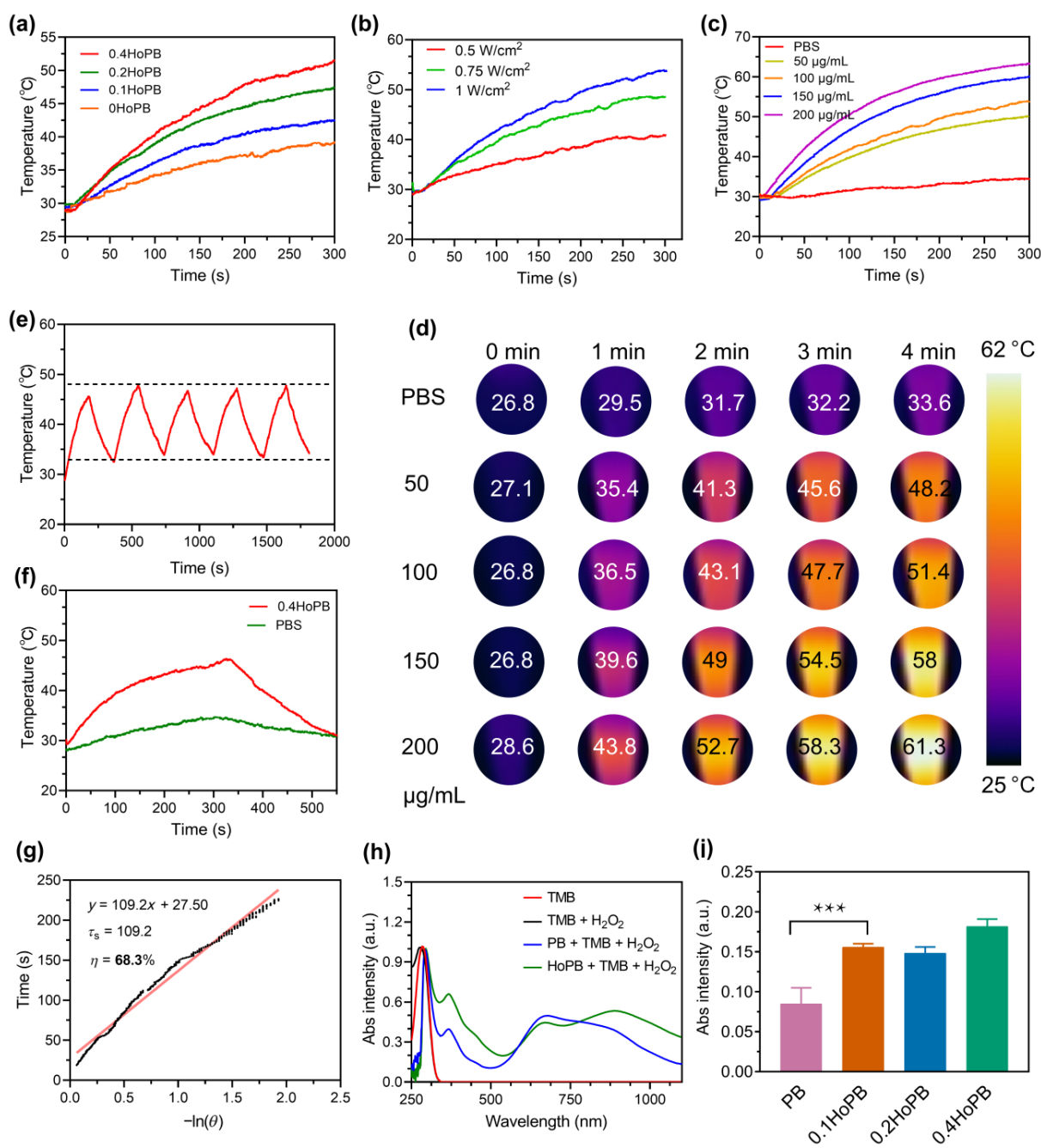


Figure 2 (a) Temperature elevation curves of PB and HoPB (Ho/Fe = 0.1, 0.2, and 0.4) (100 µg/mL) upon 1064 nm laser irradiation (1 W/cm²). (b) Power density-dependent (0.5, 0.75, 1 W/cm²) temperature profiles of 0.4HoPB (100 µg/mL) under 1064 nm laser. (c) Concentration-dependent (50–200 µg/mL) temperature elevation of 0.4HoPB under 1064 nm laser (1 W/cm²). (d) Infrared thermal images of 0.4HoPB solutions (various concentrations) at 0–4 min post 1064 nm laser irradiation (1 W/cm²). (e) Photothermal cycling stability of 0.4HoPB (100 µg/mL) over 5 on/off cycles of 1064 nm laser (1 W/cm²). (f) Temperature evolution and cooling curve of 0.4HoPB (100 µg/mL) after laser cessation. (g) Linear fitting plot ($\ln(\theta)$ vs. time) for calculating the PTCE of 0.4HoPB. (h) UV-Vis-NIR absorption spectra of TMB oxidation products in systems: TMB only, TMB+H₂O₂, PB+H₂O₂+TMB, and 0.4HoPB+H₂O₂+TMB. (i) Quantitative absorbance (652 nm) of TMB oxidation for PB and HoPB samples, showing peroxidase-like activity (***) $P < 0.001$, one-way ANOVA).

Next, we validated intracellular ROS generation and associated mitochondrial damage. Using DCFH-DA (a non-fluorescent probe oxidized to fluorescent DCF by $\cdot\text{OH}$), we compared ROS levels across groups (Fig. 3(c)): The PBS and H₂O₂ only groups showed negligible green fluorescence (H₂O₂ alone is poorly decomposed into $\cdot\text{OH}$ in cells without catalytic agents), while the PB@Lip+H₂O₂ group exhibited moderate DCF signal. In contrast, the HoPB@Lip+H₂O₂ group displayed intense, widespread green

fluorescence, quantified in Fig. 3(d) (***) $P < 0.001$ vs. PB@Lip+H₂O₂, confirming that Ho doping enhances $\cdot\text{OH}$ production via accelerated H₂O₂ decomposition. To link ROS generation to cellular damage, we assessed mitochondrial membrane potential ($\Delta\Psi_m$) via JC-1 staining (red aggregates: healthy mitochondria, green monomers: depolarized/damaged mitochondria, Fig. 3(e)): the PBS and H₂O₂ only groups showed dominant red fluorescence (intact mitochondria), while the

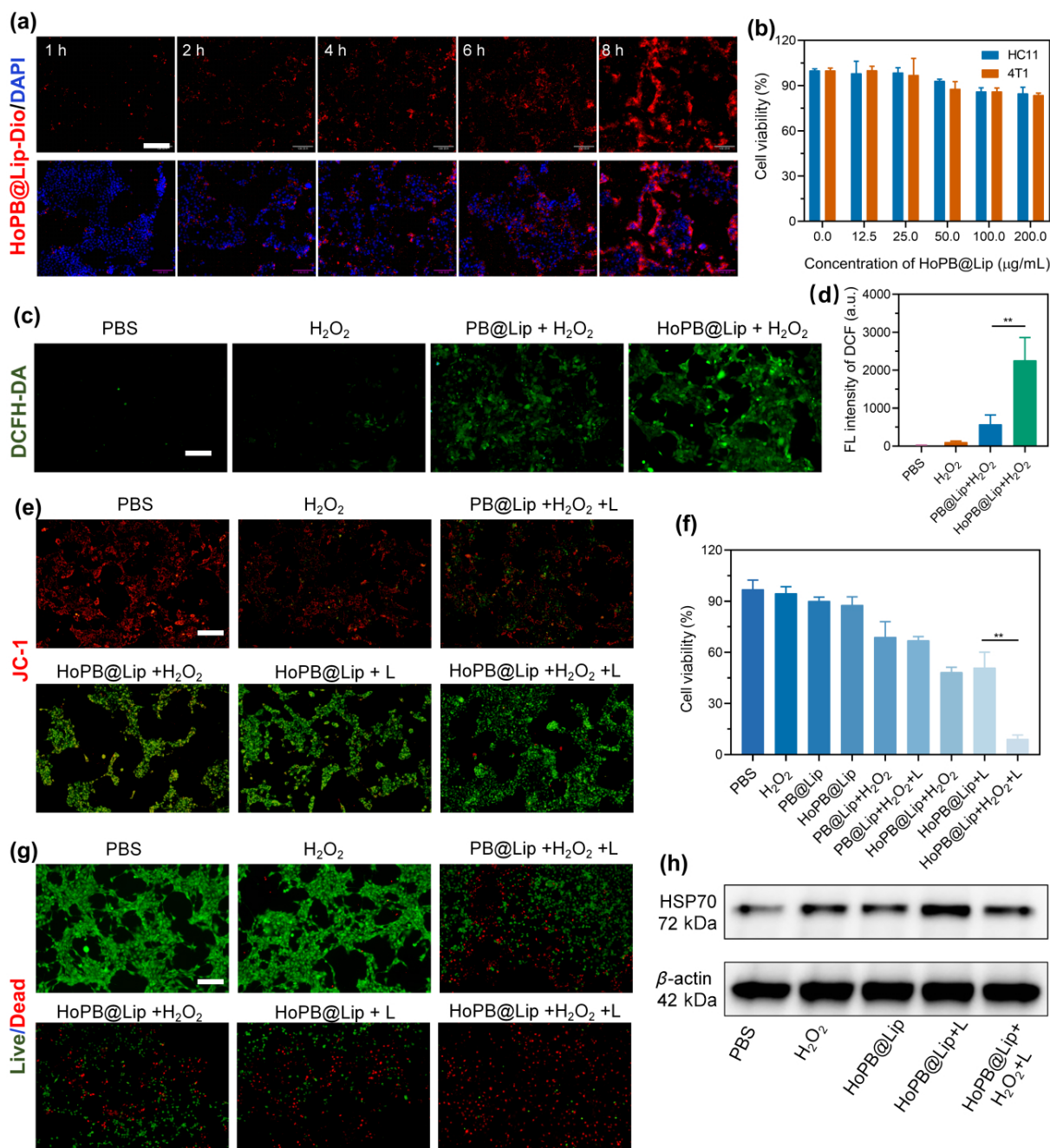


Figure 3 (a) Time-dependent cellular uptake of DiO-labeled HoPB@Lip in 4T1 cells (red: DiO, blue: DAPI, scale bars: 50 μ m). (b) Cytotoxicity of HoPB@Lip in normal HC11 mammary epithelial cells and 4T1 cancer cells at various concentrations (24 h treatment), assessed by CCK-8 assay. (c) Intracellular ROS detection via DCFH-DA fluorescence imaging (green: dichlorofluorescein (DCF), scale bar: 50 μ m) in groups: PBS, H_2O_2 , PB@Lip + H_2O_2 , HoPB@Lip + H_2O_2 . (d) Quantitative DCF fluorescence intensity (ROS) corresponding to panel (c) (** P < 0.001, one-way ANOVA). (e) Mitochondrial membrane potential ($\Delta\Psi_m$) assessment via JC-1 staining (red: aggregated JC-1 (healthy mitochondria), green: monomeric JC-1 (damaged mitochondria), scale bar: 50 μ m) in groups: PBS, H_2O_2 , PB@Lip + H_2O_2 + Laser, HoPB@Lip + H_2O_2 , HoPB@Lip + Laser, and HoPB@Lip + H_2O_2 + Laser. (f) 4T1 cell viability (CCK-8 assay) post different treatments (** P < 0.001, one-way ANOVA). (g) Live/Dead staining (green: live cells, red: dead cells, scale bar: 50 μ m) of 4T1 cells after various treatments. (h) Western blot (WB) analysis of HSP70 expression in 4T1 cells (β -actin as loading control) post different treatments.

HoPB@Lip + H_2O_2 group and HoPB@Lip + Laser group exhibited partial green signal. Critically, the HoPB@Lip + H_2O_2 + Laser group displayed overwhelming green fluorescence, indicating severe mitochondrial depolarization, driven by synergistic ROS and thermal stress that disrupts mitochondrial homeostasis.

We then quantified synergistic antitumor efficacy using CCK-8 viability assays and Live/Dead staining. CCK-8 results (Fig. 3(f) and

Fig. S6 in the ESM) revealed that single-modal treatments induced moderate 4T1 cell death: CDT alone (HoPB@Lip + H_2O_2) achieved a cell killing rate of ~42.5%, and PTT alone (HoPB@Lip + Laser) achieved a cell killing rate of ~45.2%. The PB@Lip + H_2O_2 + Laser group showed limited efficacy with a cell killing rate of ~40%. In stark contrast, the HoPB@Lip + H_2O_2 + Laser group (synergistic PTT-CDT) drove cell viability down to ~9.0%, corresponding to a

cell killing rate of ~ 91.0%. Quantitative analysis using the Chou-Talalay combination index (CI) method yielded a CI value of 0.286, confirming a strong synergistic effect between PTT and CDT. This synergistic effect arises from a positive feedback loop: PTT accelerates the POD-like catalytic activity of HoPB@Lip to generate more ROS, while ROS-mediated HSP70 inhibition eliminates tumor thermal tolerance to enhance PTT efficacy. The combination of thermal necrosis and ROS-induced apoptosis further maximizes tumor cell elimination. Live/Dead staining (Fig. 3(g), green: live cells, red: dead cells) visually corroborated these results: the HoPB@Lip+H₂O₂+Laser group showed extensive red fluorescence and minimal green signal, aligning with the quantitative CCK-8 data and confirming potent synergistic tumor cell killing.

Finally, we investigated ROS-mediated inhibition of HSP70 (a key PTT resistance factor). HSP70 is upregulated by thermal stress to protect cells from PTT-induced damage, but ROS can suppress its expression [4]. Western blot analysis (Fig. 3(h)) showed: HSP70 levels were elevated in the HoPB@Lip+Laser group, but significantly reduced in ROS-generating groups (e.g., HoPB@Lip+H₂O₂, HoPB@Lip+H₂O₂+Laser). This result confirms that HoPB@Lip-derived ROS not only drives CDT but also eliminates PTT resistance by inhibiting HSP70, further amplifying the synergistic therapeutic effect.

Collectively, these cellular experiments demonstrate that HoPB@Lip combines efficient uptake, excellent biocompatibility, ROS-mediated CDT, photothermal PTT, and HSP70 inhibition to achieve potent synergistic antitumor efficacy in 4T1 cells, laying a strong foundation for subsequent *in vivo* therapeutic evaluations.

3.4 *In vitro* and *in vivo* imaging performance of HoPB@Lip

For clinical translatability, nanotherapeutics benefit greatly from integrated theranostic capabilities, enabling noninvasive tracking of material distribution, tumor visualization, and real-time monitoring of therapeutic responses. Here, we characterized HoPB@Lip's performance as a fluorescence tracer, T₂-weighted MR contrast agent, and photothermal imaging probe, complementing its PTT-CDT therapeutic functions. To determine the optimal time window for subsequent imaging and therapy, we tracked the biodistribution of DID-labeled HoPB@Lip (DID: NIR fluorescent dye) in 4T1 tumor-bearing mice via *in vivo* fluorescence imaging (Fig. 4(a)). At early time points (0.1–3 h), fluorescence signal was predominantly localized to the liver, but by 12 h, signal began to accumulate at the tumor site (marked by the yellow circle). The tumor signal peaked at 24 h, exhibiting strong, localized fluorescence that persisted through 48 h.

This 24 h peak accumulation time is rationally explained by the enhanced permeability and retention (EPR) effect and the blood circulation characteristics of PEGylated liposomes [49, 50]: The hydrodynamic diameter of HoPB@Lip is approximately 221 nm, which falls within the effective particle size range for the EPR effect (100–250 nm) and is close to the optimal range (100–200 nm); the blood circulation half-life of PEGylated liposomes is approximately 6–12 h, so the peak tumor accumulation is achieved at 24 h post-injection. Quantitative analysis of average radiant efficiency (Fig. 4(b)) confirmed this trend: tumor signal increased steadily from 0.1 to 24 h (reaching maximum enrichment) before gradually declining. *Ex vivo* fluorescence imaging of major organs (heart, liver, spleen, lung, and kidney) and tumors at 12, 24, and 36 h further confirmed preferential tumor localization at 24 h (Fig.

S7 in the ESM). This 24 h peak accumulation time point was selected for all subsequent *in vivo* experiments, as it ensures sufficient HoPB@Lip delivery to the tumor for both diagnostic visualization and therapeutic efficacy.

Given the paramagnetic properties of Ho³⁺ and Fe ions in HoPB@Lip, we evaluated its performance as a T₂-weighted MR contrast agent. First, *in vitro* T₂ MR characterization (Figs. 4(c)–4(e)) revealed concentration-dependent signal darkening for both PB and HoPB solutions, but HoPB induced far more dramatic attenuation: at 100 µg/mL, HoPB solutions appeared nearly black (maximal T₂ relaxation enhancement), while PB solutions retained moderate brightness. Quantitative T₂ signal intensity (Fig. 4(d)) showed HoPB's signal decreased ~ 10-fold from 0 to 200 µg/mL, compared to only ~ 2-fold for PB. To quantify this effect, we calculated T₂ relaxivity (r_2 , a key metric for contrast agents, defined as the slope of 1/T₂ vs. concentration, Fig. 4(e)): HoPB@Lip exhibited an r_2 of 218.3 mM⁻¹·s⁻¹, 7.7-fold higher than PB@Lip (28.9 mM⁻¹·s⁻¹). This enhancement stems from Ho³⁺: its strong local magnetic field inhomogeneities accelerate water proton transverse relaxation far more efficiently than Fe ions alone, validating HoPB@Lip as a high-performance T₂ contrast agent. Next, *in vivo* T₂ MR imaging (Figs. 4(f) and 4(g)) was performed 24 h post injection: the PBS-treated control group showed bright tumor signal, PB@Lip induced mild signal darkening, but HoPB@Lip caused profound tumor signal attenuation. Quantitative analysis (Fig. 4(g)) confirmed HoPB@Lip reduced tumor T₂ signal by ~ 70% (vs. ~ 20% for PB@Lip), demonstrating its ability to enable high-contrast, noninvasive tumor visualization *in vivo*.

Photothermal imaging enables real-time tracking of temperature distribution during PTT, critical for ensuring therapeutic temperatures (> 50 °C) while avoiding healthy tissue damage. We tested this by irradiating 4T1 tumor-bearing mice (post PB/HoPB@Lip injection) with a 1064 nm laser (1 W/cm², Figs. 4(h) and 4(i)). Mice injected with PB showed only mild tumor heating (~ 4 °C elevation over 5 min), but those injected with HoPB@Lip exhibited rapid, robust heating: the tumor temperature reached ~ 52 °C within 5 min. This capability allows clinicians to verify that tumors reach therapeutic temperatures in real time, improving PTT safety and efficacy. In summary, HoPB@Lip functions as a versatile theranostic nanomaterial: it enables noninvasive biodistribution tracking via fluorescence imaging, high-contrast tumor visualization via T₂-weighted MR imaging, and real-time PTT monitoring via photothermal imaging—all while retaining potent synergistic PTT-CDT therapeutic activity. This integrated platform addresses a key gap in cancer therapy: the ability to diagnose, treat, and monitor responses in a single, minimally invasive system.

3.5 *In vivo* antitumor efficacy of HoPB@Lip

After validating HoPB@Lip's theranostic imaging capabilities, we investigated its *in vivo* antitumor performance and systemic biocompatibility in 4T1 tumor-bearing mice—critical steps toward clinical translation. The experimental workflow (Fig. 5(a)) involved establishing 4T1 tumors (~ 100 mm³) in mice, followed by tail-vein injection of formulations (day 1); PTT groups received 1064 nm laser irradiation (1 W/cm², 5 min) 24 h post-injection (day 2), and tumor/body weight were monitored over 17 days.

The PBS control group exhibited unimpeded tumor expansion, reaching ~ 1100 mm³ by day 17. As shown in Fig. 5(b), the PB@Lip + L group showed mild inhibition (final volume ~ 700 mm³), limited by PB's lower photothermal conversion efficiency

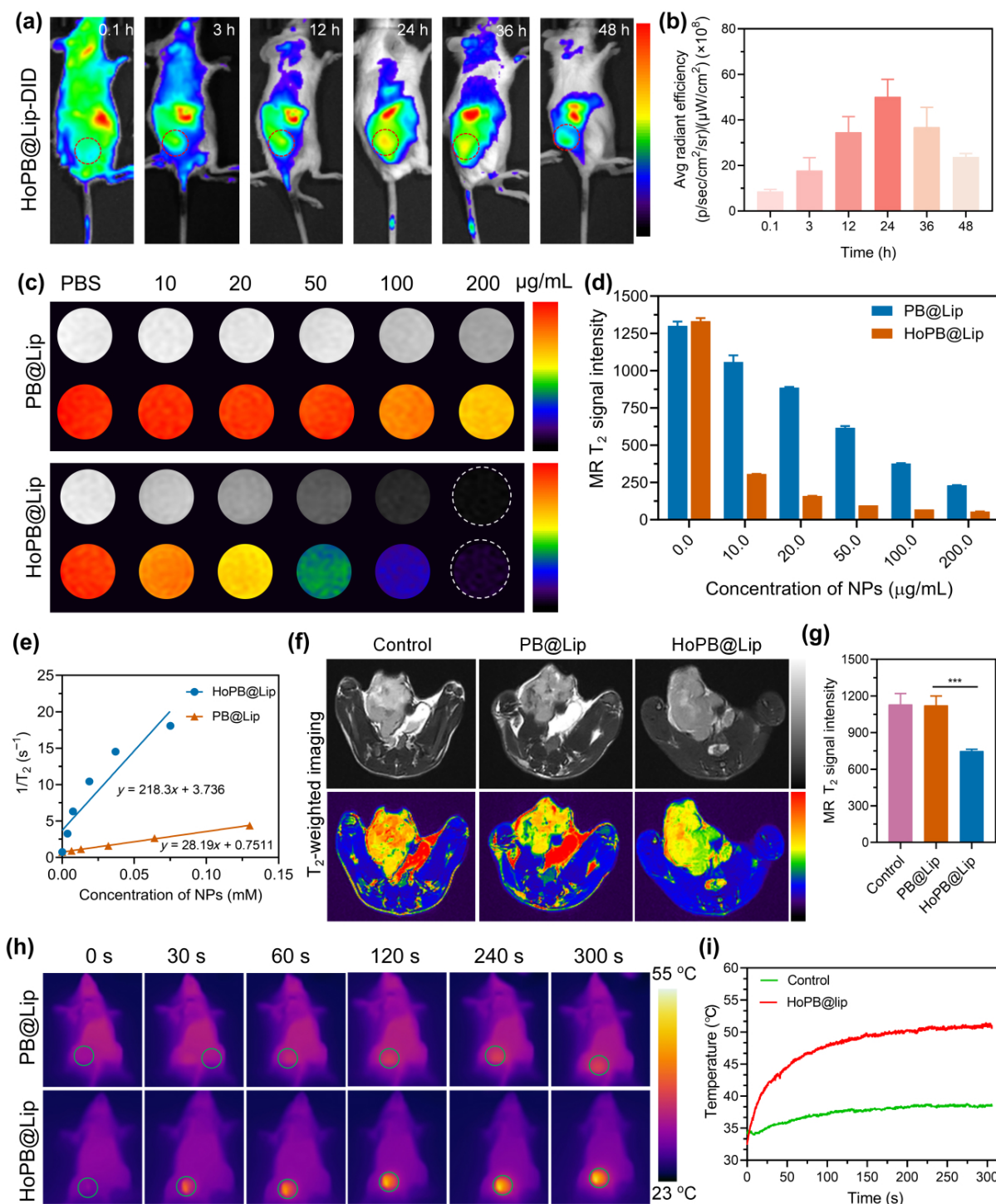


Figure 4 (a) *In vivo* fluorescence images of 4T1 tumor-bearing mice at 0.1–48 h post tail-vein injection of DID-labeled HoPB@Lip (yellow circle denotes tumor site). (b) Quantitative average radiant efficiency at the tumor site corresponding to panel (a). (c) *In vitro* T₂-weighted MR images of PB and HoPB solutions across concentrations (0–200 μg/mL). (d) Quantitative T₂ MR signal intensity of PB and HoPB solutions at varying concentrations. (e) Linear fitting of 1/T₂ (relaxivity) vs. concentration for PB@Lip and HoPB@Lip, used to calculate T₂ relaxivity (r₂). (f) *In vivo* T₂-weighted MR images (axial views: top = anatomical; bottom = T₂-weighted) of 4T1 tumor-bearing mice 24 h post injection of PBS (Control), PB@Lip, or HoPB@Lip (yellow circle denotes tumor site). (g) Quantitative T₂ MR signal intensity at the tumor site corresponding to panel (f) (****P < 0.0001, one-way ANOVA). (h) *In vivo* photothermal images of 4T1 tumor-bearing mice (yellow circle denotes tumor site) during 1064 nm laser irradiation (1 W/cm²) post injection of PB or HoPB@Lip. (i) Quantitative temperature elevation curves at the tumor site corresponding to panel (h).

(Fig. 2(g)). The HoPB@Lip group (CDT alone) induced moderate suppression (final volume ~ 300 mm³), driven by Ho-mediated ·OH generation. Most notably, the HoPB@Lip + L group (synergistic PTT-CDT) achieved ~ 95% tumor growth inhibition

relative to PBS: its final tumor volume was < 100 mm³. This enhancement stems from combined thermal ablation and ROS-mediated damage-induced HSP70 suppression (Fig. 3(h)), which eliminates PTT resistance.

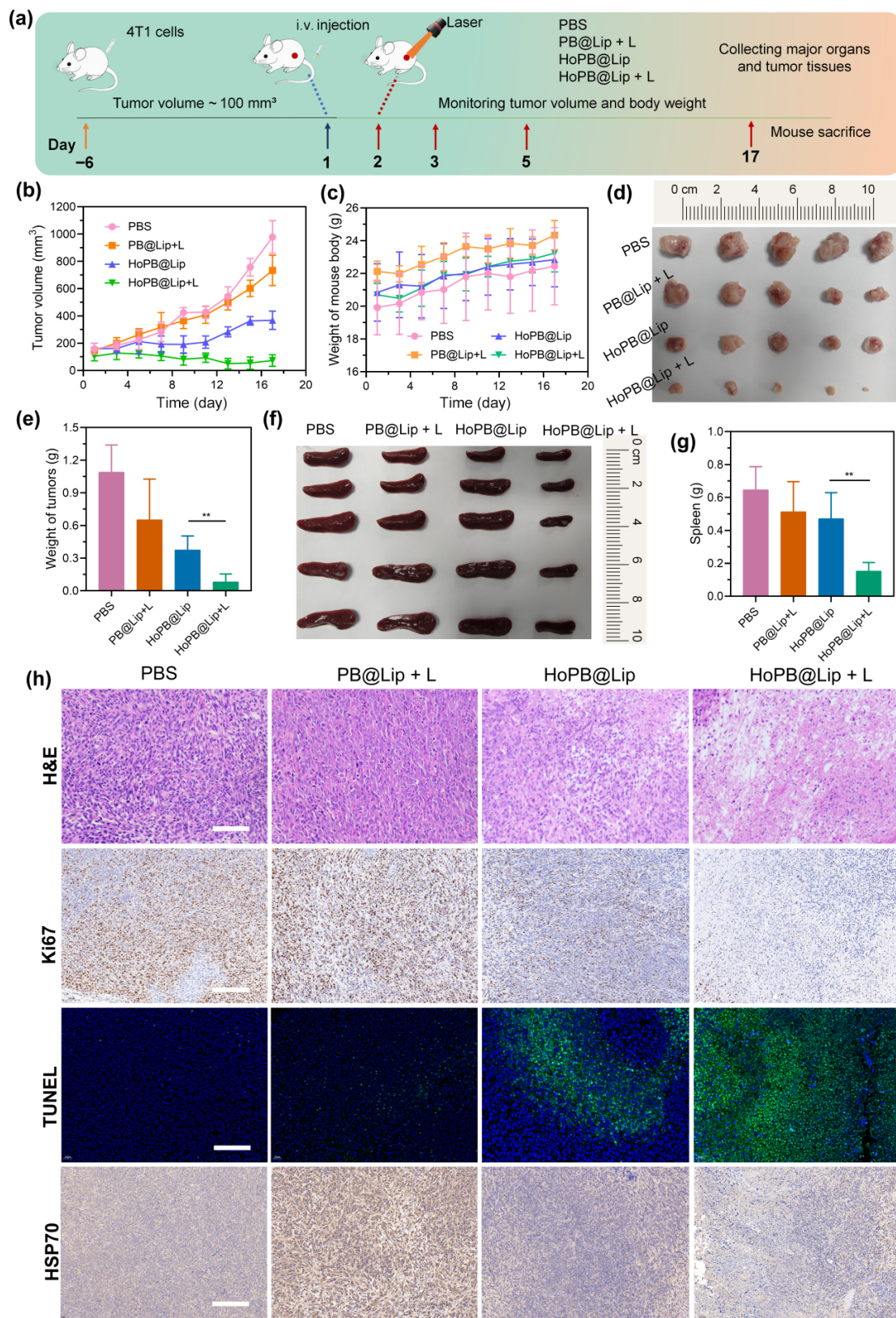


Figure 5 (a) Schematic of the *in vivo* therapeutic protocol: 4T1 tumor-bearing mice (tumor volume ~ 100 mm³) received tail-vein injection of test formulations (day 1). PTT groups underwent 1064 nm laser irradiation (1 W/cm², 5 min) 24 h post-injection (day 2); tumor volume and body weight were monitored every 2 days, and mice were sacrificed on day 17 for tissue collection. (b) Tumor volume growth curves over 17 days ($n = 5$ per group). (c) Body weight changes of mice in each group over 17 days ($n = 5$ per group). (d) Photographs of excised tumors from each group on day 17. (e) Quantitative weight of excised tumors (** $P < 0.01$, *** $P < 0.001$, one-way ANOVA; $n = 5$ per group). (f) Photographs and (g) quantitative analysis of excised spleens from each group. (h) Histological analysis of tumor tissues: H&E staining (scale bar: 100 μ m), Ki67 immunostaining (scale bar: 100 μ m), TUNEL staining (scale bar: 50 μ m), and HSP70 immunostaining (scale bar: 100 μ m).

Body weight (Fig. 5(c)) is a sensitive metric of systemic toxicity. All groups showed stable, gradual weight gain (final weight ~ 24 g, matching healthy mice), confirming that HoPB@Lip have excellent *in vivo* biocompatibility, consistent with earlier cellular safety data (Fig. 3(b)). Excised tumors (Fig. 5(d)) visually confirmed the therapeutic outcome: the HoPB@Lip + L group's tumors were drastically smaller compared to other groups. Quantitative tumor weight (Fig. 5(e)) reinforced this: the HoPB@Lip + L group's tumor weight (~ 0.1 g) was $\sim 90\%$ lower than the PBS group's (~ 1.0 g), providing robust quantitative evidence of synergistic efficacy. Next, the photographs of excised spleens (Fig. 5(f)) further support biocompatibility. Spleen size was normal across all groups (Fig. 5(g)). Spleen enlargement often correlates with high tumor burden or systemic inflammation; the preserved spleen morphology here indicates that the therapeutic regimen does not induce overt immunotoxicity or organ damage.

Histological analysis (Fig. 5(h)) elucidated the therapeutic mechanism. H&E staining showed that the HoPB@Lip + L group displayed extensive tumor necrosis, whereas the PBS group showed dense, viable tumor tissue. In addition, the Ki67 immunostaining displayed that the HoPB@Lip + L group had minimal Ki67-positive cells, indicating potent suppression of tumor cell proliferation. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) apoptosis staining further confirmed the therapeutic mechanism: the HoPB@Lip + L group exhibited intense and widespread green fluorescence, indicating massive tumor cell apoptosis, while only weak or moderate fluorescence was observed in the other groups. Moreover, HSP70 immunostaining showed that the HoPB@Lip + L group showed weak HSP70 signal, confirming ROS-mediated HSP70 inhibition—key to overcoming PTT resistance. Therefore, HoPB@Lip enables safe, potent synergistic PTT-CDT therapy *in vivo*: it achieves $\sim 95\%$ tumor growth inhibition, maintains excellent systemic biocompatibility, and acts via a multi-modal mechanism. These results establish HoPB@Lip as a promising theranostic nanoplatform for clinical cancer treatment.

3.6 Systemic biocompatibility evaluation of HoPB@Lip

To confirm the systemic safety of HoPB@Lip, we first evaluated hematological and serum biochemical indices (Figs. 6(a)–6(h)): key metrics (including WBC, RBC, platelet counts, hemoglobin, and liver/kidney function markers ALT, AST, CREA) remained within normal physiological ranges across all groups, with no significant deviations from the PBS control. These data demonstrate that HoPB@Lip (and its synergistic PTT-CDT regimen) does not disrupt hematopoietic homeostasis or induce organ dysfunction.

We further validated safety via H&E staining of major organs (heart, liver, spleen, lungs, kidneys, Fig. 6(i)): all organs from treated groups (including HoPB@Lip + L) retained intact, normal tissue morphology, with no signs of necrosis, inflammation, or structural damage. Together, these results confirm HoPB@Lip's excellent systemic biocompatibility, complementing its potent theranostic efficacy to position it as a safe and promising nanoplatform for cancer therapy.

4 Conclusions

In summary, this work rationally designs and constructs a Ho-doped Prussian blue liposome (HoPB@Lip) as a multifunctional theranostic nanoplatform integrating NIR-II PTT, CDT, and

multimodal imaging for synergistic cancer treatment. Structurally, Ho³⁺ is homogeneously incorporated into the PB lattice (at Ho/Fe ≤ 0.4) while preserving the cubic framework, and PEGylated lipid coating enhances biocompatibility and colloidal stability. Functionally, HoPB@Lip exhibits superior NIR-II photothermal performance (68.3% conversion efficiency) with tunable, stable heating; Ho doping also amplifies its POD-like nanozyme activity, catalyzing tumor H₂O₂ to generate cytotoxic $\cdot\text{OH}$ for CDT. As a theranostic agent, it enables fluorescence biodistribution tracking, high-contrast T₂-weighted MR imaging ($r_2 = 218.3 \text{ mM}^{-1}\cdot\text{s}^{-1}$, 7.7-fold higher than pristine PB), and real-time photothermal monitoring. In 4T1 tumor-bearing mice, HoPB@Lip mediates synergistic PTT-CDT to achieve $\sim 95\%$ tumor growth inhibition; critically, its ROS generation suppresses HSP70 expression, overcoming PTT resistance to amplify efficacy. Comprehensive hematological/biochemical assays and organ histology confirm its excellent systemic biocompatibility. Collectively, this work demonstrates that Ho doping endows PB with integrated enhancements in photothermal, nanozyme, and imaging properties, and the HoPB@Lip platform offers a safe, potent, theranostic strategy for synergistic cancer therapy, holding significant potential for clinical translation.

Electronic Supplementary Material: Supplementary material (TEM image of PB nanoparticles, UV–Vis–NIR absorption spectra of TMB, cytotoxicity of HoPB@Lip, and *ex vivo* fluorescence images of major tissues) is available in the online version of this article at <https://doi.org/10.26599/NR.2026.94908898>.

Data availability

Additional data related to this paper may be requested from the corresponding author upon request.

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Declaration of competing interest

All the contributing authors report no conflict of interests in this work.

Author contribution statement

H. L., W. Z. H., X. R. Z., and C. H. S.: Data curation, project administration, validation, writing manuscript, experimental design. P. Y. L. and C. L.: Data curation, project administration. S. F.: validation. J. X. L., M. Y. L., H. Y. L., and S. H.: Project administration, funding acquisition, writing manuscript. All the authors have approved the final manuscript.

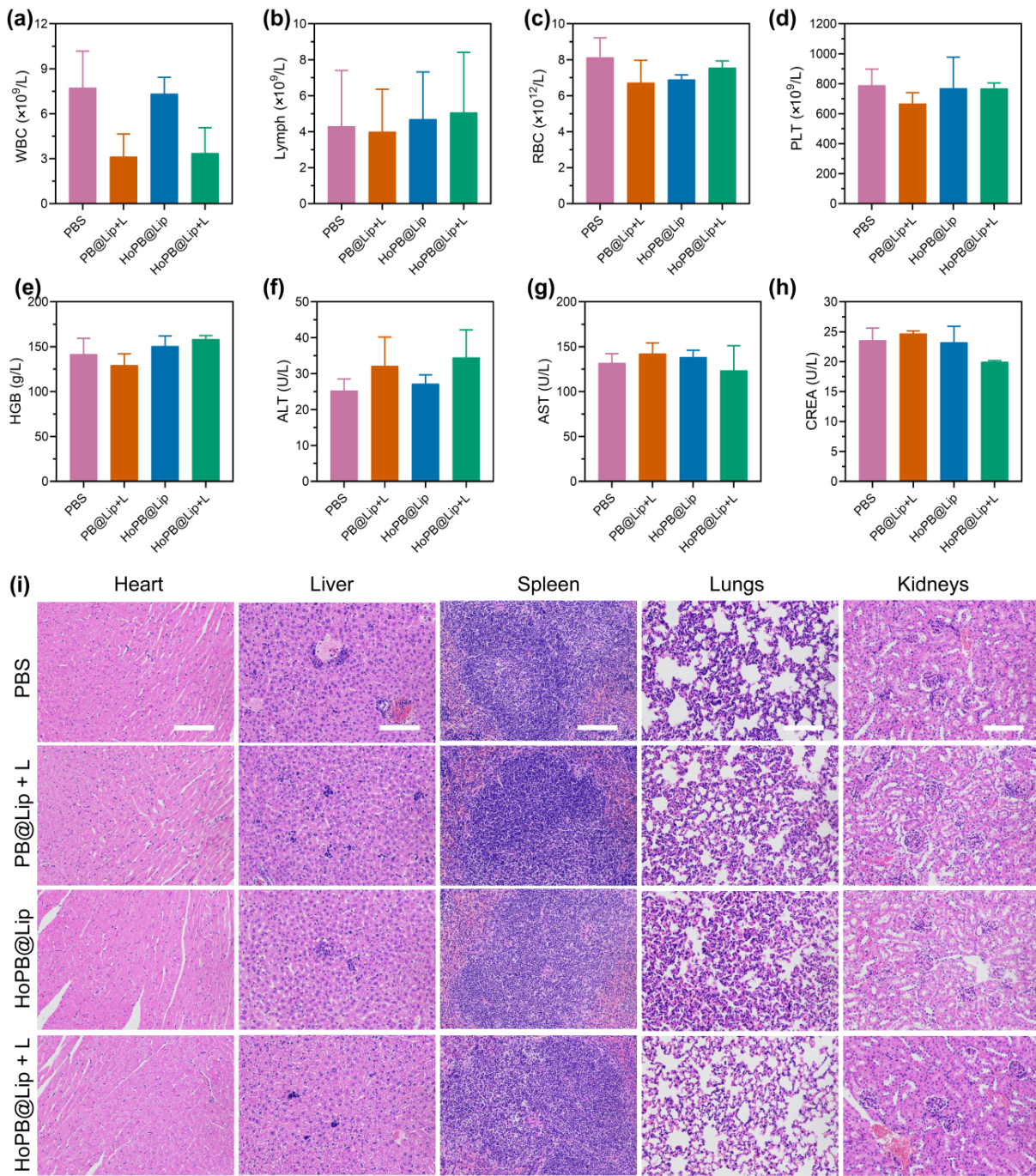


Figure 6 Hematological and serum biochemical indices of 4T1 tumor-bearing mice (sacrificed on day 17): (a) WBC, (b) Lymph, (c) RBC, (d) PLT, (e) HGB, (f) ALT, (g) AST, and (h) CREA. Data are presented as mean $\pm$ SD ($n = 5$ per group). (i) H&E stained sections of major organs (heart, liver, spleen, lungs, kidneys) from mice in each group (PBS, PB@Lip + L, HoPB@Lip, HoPB@Lip + L), scale bars: 100 μm .

Informed consent

Not applicable.

Ethics statement

The animal experiments were approved by the Animal Care and Use Committee of Guangdong Second Provincial General Hospital (Approval No.: 2023-DW-KZ-130-02) and conducted in accordance with the Guide for the Care and Use of Laboratory Animals.

Use of AI statement

None.

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