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Robust boron nanoprotocol provokes potent tumoricidal activities via inhibiting heat shock protein

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A B S T R A C T

Near-infrared (NIR)-light-triggered photothermal therapy (PTT) is a promising treatment for breast cancer. However, its therapeutic efficiency is often compromised due to the heat-induced up-regulation of heat shock proteins, which confer photothermal resistance. To solve this urgent problem, PEGylated two-dimensional boron nanosheets (B-PEG)—which allow both multimodal imaging and photothermal conversion—were loaded with gambogenic acid (GA), which can inhibit heat shock protein 90 (Hsp90). Experimental findings indicated that this combination of B-PEG and GA could serve as an integrated drug delivery system for cancer diagnosis and treatment. It could be used to administer mild PTT as well as chemotherapy for breast cancer, provide improved anti-tumor effects, and reduce the toxicity of PTT, all while inhibiting breast cancer growth. This drug delivery system could offer a novel tool for administering chemotherapy combined with PTT while avoiding the adverse effects of traditional PTT.

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1. Introduction

Breast cancer is a serious illness with a high prevalence around the world. Most conventional cancer therapies cause unfavorable adverse effects [1,2]. Hence, new anti-cancer strategies with good therapeutic benefits and few adverse effects are required. Photothermal therapy (PTT) involves the use of near-infrared (NIR) light to activate photosensitizers that can ablate tumor tissue [3]. Recently, this strategy has gained serious attention recently owing to its high amenability and good curative efficacy [4]. Moreover, several studies have demonstrated the promising potential of PTT in cancer treatment [5,6].

The most important requirement for effective PTT is a suitable photosensitizer [7]. Boren nanosheets (B NSs), a novel two-dimensional (2D) material, offer several advantages as a photosensitizer. In our previous study, we showed that B NSs exhibit good biocompatibility, excellent photothermal performance, and a great drug-loading capability [8]. In addition, B NSs are also useful for achieving long-term tumor suppression via synergistic photothermal immune therapy [9]. B NSs are rapidly heated under 808-nm NIR irradiation, and heated B NSs can induce tumor cell necrosis and destroy tumor tissue. In conventional PTT, rapid heating and higher temperatures provide better anti-tumor effects in conventional PTT treatment [10,11]. However, these high temperatures can damage peritumor tissues and also induce secondary recurrence and tumor metastasis after treatment [12]. In order to address this issue, our study focused on the thermal tolerance of tumors, which is strongly linked to the expression of heat shock proteins (Hsps) [13,14]. When tumor cells are thermally stimulated, Hsps are significantly up-regulated, thereby mitigating heat-induced damage in tumor cells [15]. Among the different Hsps, Hsp90 is the primary contributor to tumor thermotolerance [16,17]. Therefore, the inhibition of Hsp90 may effectively reduce tumor thermotolerance and enable low-temperature thermotherapy. Gambogic acid (GA), an anti-tumor chemotherapeutic agent, is known to inhibit Hsps. Davenport et al. examined the mechanism underlying GA-induced Hsp90 inhibition. They showed that GA acts as a non-competitive inhibitor of Hsp90 and binds to the N-terminal region of this protein [18]. GA can cause a reduction in both the level and activity of Hsps after binding to them. Thus, it reduces the amount of Hsps secreted by tumor cells under stress, allowing the ablation of tumor cells at 43 °C and preventing damage to normal cells [19,20].

Fig. 1 - Schematic illustration showing the (A) the synthesis of B-PEG loaded with GA, (B) the multimodal imaging capability of B-PEG, and (C) their application in GA-mediated mild photothermal therapy.
In this study, PEGylated two-dimensional B NSs (B-PEG) with multimodal imaging capabilities were loaded with GA. This platform was used for combined PTT and chemotherapy to improve anti-tumor effects in models of breast cancer (Fig. 1). The loaded GA attenuated the thermotolerance of tumor cells, allowing low-temperature PTT while reducing adverse effects, thus preventing secondary tumor recurrence and metastasis.

2. Materials and methods

2.1. Materials

GA (purity: 99%) was obtained from Feiyu Biological Co., Ltd (Jiangsu, China). Boron powder, purchased from Maclim (Shanghai, China), was stored in an argon glove box. Isopropanol (IPA) and ethanol (anhydrous grade) were obtained from Sinopharm Chemical Reagents Co., Ltd. (Shanghai, China). PEG-NH₂ (MW = 2000) was provided by Xi’an Regal Biotech Co., Ltd. Ultrapure water (18.25 MΩcm, 25 °C) was used to prepare all solutions. Phosphate-buffered saline (PBS, pH 7.4), fetal bovine serum (FBS), Dulbecco’s modified eagle medium (DMEM), and trypsin-ethylenediaminetetraacetic acid (Trypsin-EDTA) were obtained from Thermo Fisher Scientific Inc. (USA). Cell counting kit-8 (CCK8) was purchased from DOJindo Laboratories (Japan). Cy5.5-PEG-NH₂ and FITC-PEG-NH₂ were obtained from Ruixi Biological Co., Ltd. (Shanxi, China). Propidium iodide (PI) and calcein acetoxymethyl ester (calcein-AM) were obtained from Solarbio Science & Technology Co., Ltd. (China). Methyl-β-cyclodextrin (MβCD), chlorpromazine (CPZ), hypertonic sucrose, and 5-(N-ethyl-N-isopropyl)-amiloride (EIPA) were purchased from Sigma Aldrich (St. Louis, MO, USA). 4′,6-diamidino-2-phenylindole (DAPI), Lyso Tracker, ER Tracker, and Mito Tracker were obtained from Molecular Probes Inc. (OR, USA).

2.2. Synthesis of B NSs, B-PEG, and B-PEG/GA

B NSs were fabricated using liquid-phase exfoliation in IPA, which has a low melting point and low toxicity. Briefly, a mixture of high-purity boron powder (50 mg) and IPA (15 ml) was added to a brown bottle, which was sealed with parafilm. Subsequently, the boron dispersion in the brown bottle was subjected to probe sonication (360 W) for 8 h and bath sonication (650 W) for 12 h. An ice bath and a built-in water-cooling system were used to prevent the oxidation of B NSs during probe and bath sonication, respectively. Next, two consecutive rounds of centrifugation (3000 rpm for 20 min, followed by 12 000 rpm, 20 min) were used to obtain B NSs of an appropriate size. Finally, the obtained B NSs were stored in a vacuum dry box under dark conditions.

B NSs were modified with PEG-NH₂ to improve their biocompatibility and dispersion stability. First, a B NS aqueous dispersion (1 mg/ml, 2 ml) was mixed with 20 mg of PEG-NH₂. The mixture was subjected to bath sonication for 2 min to increase its dispersibility and then stirred overnight in the dark. Subsequently, centrifugation (18 000 rpm, 5 min) was performed to remove excess PEG-NH₂. Finally, B-PEG was obtained and resuspended in ultrapure water. For visualization experiments, NH₂-PEG-FITC or NH₂-PEG-Cy5.5 was used instead of PEG-NH₂, with the subsequent procedure remaining identical to that used for B-PEG preparation.

The B-PEG aqueous dispersion (1 mg/ml, 1 ml) was added to GA (0.25 mg/ml, 1 ml). The mixture was subjected to bath sonication for 2 min to increase its dispersibility and then stirred overnight in the dark. Subsequently, centrifugation (10 000 rpm, 5 min) was performed to remove excess GA. Finally, B-PEG/GA nanoparticles were obtained and resuspended in ultrapure water.

2.3. Characterization

The morphology and size of B NSs were determined using transmission electron microscopy (TEM) (HITACH HT7700, 80 kV), and their thickness was determined using atomic force microscopy (AFM) (BRUKER Dimension ICON, 512 pixels per line). The AFM samples were prepared by dispersing B NSs into IPA and adding the dispersion onto a mica substrate. The size and zeta potential of B NSs, B-PEG, and B-PEG/GA were tested using the Malvern Mastersizer 2000 system (Zetasizer Nano ZS90, Malvern Instruments Ltd., UK). The concentrations of B NSs were characterized using Vis-NIR spectrophotometry (HITACHI, UH4150, Japan). Energy-dispersive X-ray spectroscopy (EDS) (JEOL F200, 200 kV) was performed to evaluate the distribution of elements in B-PEG/GA. Further, X-ray photoelectron spectroscopy (XPS) was performed using ESCALAB 250Xi (Thermo Fisher, USA).

To measure photothermal performance, B NSs were irradiated with a collimated 808-nm laser (power density, 2 W/cm²), and the temperature was measured using a thermometer at a sampling interval of 1 s [2122]. The B NS aqueous dispersion was added to a two-pass light quartz cuvette (1 ml) and directly irradiated with a laser. During a 10-min irradiation period, the photothermal effect of different B NS aqueous dispersions (25, 50, and 100 μg/ml) was tested. Furthermore, their photostability was also examined during six on-off laser irradiation cycles. The laser switching cycle was 20 min, and the duty cycle was 50%.

The B-PEG aqueous dispersion (1 mg/ml, 1 ml) was mixed with different GA concentrations (0.25, 0.5, 1, 2, 3, 4, and 5 mg/ml; 1 ml). This mixture was subjected to bath sonication for 2 min to increase its dispersibility and then stirred overnight in the dark. Subsequently, centrifugation (10 000 rpm, 5 min) was performed to remove excess GA. The concentration of excess GA in the supernatant was evaluated using a UV spectrophotometer (Nicomp 380 Z3000, Particle Sizing Systems). The drug loading capacity (DL%) was evaluated as follows:

\[ DL\% = \frac{W_{GA}}{W_{B}} \times 100 \]

where \( W_{GA} \) and \( W_{B} \) signify the weights of GA loaded onto B NSs and the gross weight of B NSs, respectively.

2.4. Cell culture and in vitro cytotoxicity

Murine mammary carcinoma (4T1) and human hepatocellular carcinoma (HepG2) cells (Science and Technology Innovation
Center of Guangzhou University of Chinese Medicine) were plated in DMEM containing 10% FBS, 50 units/ml penicillin, and 50 units/ml streptomycin. Cells were incubated at 37 °C in humidified air containing 5% CO₂. Before all evaluations, the 4T1 and HepG2 cells were separately seeded in 96-well plates (5000 cells/well) and incubated for 24 h.

To evaluate the toxicity of B-PEG, the original medium was replaced with DMEM containing different concentrations of B-PEG. The cells were incubated with B-PEG for 24 h. Cell survival was measured using the CCK8 kit (Multiskan FC, Thermo Fisher).

To evaluate photothermal efficiency, DMEM containing different concentrations of B-PEG was added to the cells. After 120 min, the cells were treated with 808-nm NIR at 1 W/cm² for 10 min. After an additional 22 h of incubation, the CCK8 reagent was added to assess the viability of 4T1 and HepG2 cells.

To evaluate the cytotoxicity of GA, cells were treated with different concentrations of GA. After 24 h of incubation, the CCK8 test was performed to assess the viability of 4T1 and HepG2 cells.

To evaluate the photothermal efficiency of GA, cells were treated with different concentrations of GA and B-PEG/GA (0.125 μM B-PEG/GA with 0.15 mg/ml GA and 3 mg/ml B-PEG; 0.25 μM B-PEG/GA with 0.3 mg/ml GA and 6 mg/ml B-PEG; 0.5 μM B-PEG/GA with 0.6 mg/ml GA and 12 mg/ml B-PEG; and 1 μM B-PEG/GA with 1.2 mg/ml GA and 24 mg/ml B-PEG). Cells were incubated with the treatment medium for 18 h at different temperatures. Finally, the CCK8 reagent was added and cell viability was assessed.

To evaluate the overall treatment effects, cells were divided into different treatment groups (0.0625 μM B-PEG/GA with 0.075 mg/ml GA and 1.5 mg/ml B-PEG; 0.125 μM B-PEG/GA with 0.15 mg/ml GA and 3 mg/ml B-PEG; and 0.25 μM B-PEG/GA with 0.3 mg/ml GA and 6 mg/ml B-PEG; NIR, cells exposed to 808-nm NIR at 1 W/cm², 10 min). After 24 h of incubation, CCK8 assays were performed to assess the viability of 4T1 and HepG2 cells.

2.5. In vitro HSP90 expression

Western blots were utilized to evaluate Hsp90 expression in different treatment groups. Proteins extracted from 4T1 cells using RIPA lysis buffer were quantified using the BCA protein assay kit (Thermo Fisher Scientific). The obtained proteins were separated using SDS-PAGE and transferred onto PVDF membranes. After blocking with 5% non-fat-dry milk for 2 h, the PVDF membranes were incubated with an anti-Hsp90 antibody overnight. The membranes were washed and incubated with an HRP-coupled secondary antibody. Protein bands were visualized using the ECLplus system (Chemidoc XRS+, Bio-Rad). GAPDH was used as the reference and control.

2.6. Cellular uptake and internalization

The cellular uptake and internalization of B-PEG were visualized via confocal laser scanning microscopy (CLSM, Leica). 4T1 cells were seeded in 12-well plates on a sterile coverslip (8000 cells/well) and incubated for 24 h.

For uptake assays, media containing different concentrations of B-PEG/FITC (FITC concentration 2.5, 5 and 10 μg/ml) were added to the cells. Following different incubation periods (10, 30 and 60 min), the medium was removed, and the cells were carefully washed thrice in ice-cold PBS. Further, DAPI was used to stain the nuclei, and the cells were fixed with 4% paraformaldehyde (PFA). The coverslips were washed thrice with PBS, mounted, and observed using CLSM.

For internalization assays, 1 ml medium containing different inhibitors was added to block endocytosis pathways. After 30 min of incubation, another 1 ml of medium containing different concentration of B-PEG/FITC (FITC concentration 2.5, 5 and 10 μg/ml) was added. Following an additional 30 min of incubation, the medium was removed, and cells were carefully washed thrice in ice-cold PBS. Further, DAPI was used for nuclear staining, and the cells were fixed with 4% PFA [23]. The coverslips were washed thrice with PBS, mounted, and observed using CLSM.

2.7. Tumor model establishment

BALB/c mice (4 weeks, female) were obtained from Viton Lever. The study protocols were approved by the Institutional Animal Care and Use Committee of the Guangzhou University of Chinese Medicine Animal Experimentation Center. All experimental procedures complied with the Regulations on the Administration of Laboratory Animals by the State Council of the People’s Republic of China. To establish a 4T1 in situ transplantation model, 100 μl saline loaded with 4T1 cells (1 × 10⁷ per ml) was injected subcutaneously into the right axilla of female BALB/c mice (18–22 g, 4 weeks of age). When the tumor volume reached ~100 mm³, the mice were randomized into various groups. Tumor volume was calculated as follows:

\[ \text{Tumor volume} = (\text{axial length}) \times (\text{axial width})^2 \times 0.5 \]

2.8. B-PEG in vivo imaging and biodistribution

All treatments and assessments were performed in live animals after their tumor volumes reached 100 mm³. For tumor activity assessments, tumors were injected with B-PEG or B-PEG/GA and irradiated with an 808-nm laser for 10 min (1.0 W/cm²). Infrared thermal images were recorded using a FLIR Ax5 camera (FLIR C2) every 2 min.

To evaluate the photoacoustic imaging capabilities of B-PEG, the tumors were injected with B-PEG (2 mg/ml, 100 μl). Signals were recorded using a photoacoustic imager at 1, 2 and 3 h post-injection (n = 3).

B-PEG/Cy5.5, prepared using the method used to prepare B-PEG/GA, revealed the biodistribution of B-PEG. B-PEG/Cy5.5 (2 mg/ml, 100 μl) was injected into mouse tumors, and fluorescence signals were recorded using a small animal in vivo imager (LB983, Berthold) at 1, 2 and 4 h post-injection (n = 3). Some mice were sacrificed to observe fluorescence signals in their tumors and major organs (heart, liver, spleen, lung, and kidney) (excitation, 650 nm; emission, 700 nm) [14].
2.9. Assessment of anti-tumor effect in vivo

When the tumor volume reached 100 mm³, 4T1 tumor-bearing mice were randomized into five groups: (1) PBS, (2) GA (GA = 0.5 mg/kg), (3) B-PEG/GA (B = 10 mg/kg, GA = 0.5 mg/kg), (4) B-PEG+NIR (B = 10 mg/kg), and (5) B-PEG/GA+NIR. Thirty minutes after injection, the NIR groups were irradiated with an 808-nm laser (1.0 W/cm²) for 10 min. Tumor temperatures and infrared thermographic maps were recorded using an infrared thermal camera. Temperatures were maintained at 42–45 °C during irradiation.

The tumor volume and body weight of the mice in the six groups were recorded every 2 d. At 2 d post-treatment, some mice were sacrificed (n = 4). Their tumors were taken for immunofluorescence and pathological analysis; major organs were used for pathological analysis, and blood was used for blood biochemistry and hematology tests. On Day 30, tumors from one mouse per group were imaged to record tumor size. Then, all mice were sacrificed (n = 6). Tumors and lungs were imaged to examine tumor size and lung metastases. Lung tissue was used for pathological analysis. Blood was used for blood biochemistry and hematology tests.

2.10. Statistical analysis

One- and two-way ANOVAs were used to compare data among groups. Data are represented as the means ± SD. The significance threshold was P < 0.05.

3. Results and discussions

3.1. Preparation and characterization

In this study, we used the simple liquid-phase exfoliation technique reported previously to prepare B NSs [24]. Compared with other solvents, IPA is easier to remove from the surface of B NSs and offers good biocompatibility due to its low melting point [25]. After exfoliation, various methods were used to characterize the B NSs. First, the morphology and structure of B NSs were examined using TEM and AFM. According to TEM findings, the size width of the B NSs was 106–200 nm (Fig. 2A). AFM measurements indicated that the thickness of the B NSs was less than 5 nm (Fig. 2B and 2C). Such an ultra-thin nature is considered conducive for effective drug loading and optical responses [26]. Moreover, when the B NSs were examined using high-resolution XPS (Fig. 2E), the binding energy of the B 1s peak was detected at 189 eV. This proved that no obvious oxidation occurred during the exfoliation process.

Subsequently, PEG-NH₂ was used to modify B NSs. PEGylated B NSs (B-PEG) showed improved biocompatibility and dispersion stability. As shown in Fig. 2F, the introduction of PEG-NH₂ altered the zeta potential of B NSs, demonstrating successful modification [27]. When B-PEG were treated with GA (250–5000 μg/ml, 24 h, room temperature), the loading capacity appeared to increase almost linearly with an increase in the feeding ratio (Fig. S7&S8). Moreover, at 400 mg/ml, the zeta potential declined to ~30 mV owing to the negative charge of GA under neutral conditions [28]. The serial changes in the zeta potential and hydrodynamic diameter (Fig. S1) demonstrated the successful generation of B-PEG/GA. This was further validated by the co-localization of different elements (O, N from the surface coating PEG-NH₂, and B from B NSs) detected on TEM-EPS (Fig. 2D). To examine the effect of PEGylation on B NS stability, the dispersibility of B NSs and B-PEG was examined under physiological conditions. As shown in Fig. S3, compared with bare B NSs, B-PEG showed negligible agglomeration in an aqueous solution. Moreover, the dispersibility of B-PEG in PBS and culture medium (FBS) was significantly better than that of bare B NSs. These results proved that PEG-NH₂ modification can improve B NS stability and dispersibility.

To achieve an excellent photothermal performance, strong absorption in the NIR region is necessary. The concentration dependent Vis-NIR spectrum of B NSs indicated the great potential of B NSs in photothermotherapy-related applications (Fig. 2G). In this study, a collimated 808-nm NIR laser was used for evaluating the photothermal performance and photostability of B NSs. This laser was selected owing to its good tissue permeability [29]. The heat generated was observed to increase as the sample concentration increased (Fig. 2H). Under irradiation (2 W/cm², 10 min), the temperature of the 100 mg/ml B NS aqueous solution rose to 43 °C, while the temperature of pure water rose only by 4.5 °C. In addition, B NSs were also found to have excellent light stability under 808-nm NIR laser irradiation. After six on-off cycles, the temperature showed a negligible change (Fig. 2I). The photothermal conversion efficiency (PTCE) of B NSs was 25.9%, as calculated by a previous reported method [30]. In addition, the photothermal stability (Fig. S4) and photothermal heating curves (Fig. S5) of B-PEG/GA confirmed that the loading of GA did not affect the photothermal activity of B NSs.

3.2. Anti-tumor effects of combined chemotherapy and mild PTT in vitro

The toxicity of B-PEG was assessed using the CCK8 assay. B-PEG were found to be nontoxic to 4T1 (Fig. 3A) and HepG2 (Fig. S9) cells when used at a concentration of 48–96 μg/ml. To determine the appropriate power density and duration of irradiation, different concentrations of B-PEG were irradiated using different combinations of power density and duration. Subsequently, temperature changes were observed (Fig. S6). Based on these findings, 4T1 and HepG2 cells were incubated with different concentrations of B-PEG and irradiated with 808-nm (1 W/cm²) NIR light for 10 min (Fig. 3B). The survival rate of 4T1 and HepG2 cells (Fig. S10) was correlated with the concentration of B-PEG (Fig. 3C). These findings demonstrated that B-PEG exerted significant photothermal treatment effects.

When tumor cells are thermally stimulated and stressed, they secrete large amounts of Hsps, such as Hsp90, to avoid apoptosis [31]. The inhibition of Hsp90 production in tumors can effectively increase their sensitivity to thermal stimulation, allowing the thermal ablation of tumors under low-temperature conditions (no more than 45 °C) [32]. As shown in Fig. 3D and 3E, GA could effectively reduce the intracellular levels of Hsp90. Under temperature conditions
of 43 °C or under 808-nm NIR irradiation, tumors produced higher amounts of Hsp90, indicating that thermal stimulation drives Hsp90 production in tumor cells. However, in tumor cells treated with B-PEG/GA, the amount of Hsp90 protein produced after irradiation was similar to that in non-irradiated cells (normal group). This indicated that GA can inhibit Hsp90 production in tumor cells despite continuous thermal stimulation.

GA is a chemotherapeutic agent that can inhibit tumor proliferation [33]. The CCK8 assay demonstrated that GA could inhibit the proliferation of 4T1 (Fig. 3F) and HepG2 cells (Fig. S11). Moreover, its inhibitory effect was concentration dependent.

GA was incubated with 4T1 cells (Fig. 3G) and HepG2 cells (Fig. S12) at 43 °C (mild PTT temperature) and 37 °C. The total incubation period was 18 h, and the period of incubation at 43 °C was gradually extended. The survival rate of 4T1 cells incubated at 43 °C for 2.5 h without any GA intervention was greater than 80% (Fig. 3G). This indicated that mild photothermal conditions had no inhibitory effect on tumor cells. However, the survival rate of 4T1 cells decreased with an increasing GA concentration after incubation at 37 °C for 18 h, even in the absence of photothermal intervention. This indicated that GA had chemotherapeutic effects. When mild PTT was administered, the inhibitory effect of GA on 4T1 tumor cells gradually increased in a time-dependent manner. This suggested that GA can inhibit Hsp90 under low-temperature conditions and provide the anti-tumor effects induced by mild PTT [34]. Similar Hsp90 inhibition was observed in the B-PEG/GA group, which also showed lower tumor cell survival than the GA group at the same treatment concentration.

Fig. 2 – Characterization of B-PEG/GA. (A) TEM-based analysis of the B NSs (scale bar, 200 nm). (B) AFM images and (C) thickness of B NSs (scale bar, 100 nm). (D) TEM-EDS maps of B-PEG/GA (scale bar, 100 nm). (E) Magnified view of the B 1s region in the XPS spectrum. (F) Zeta potential measurements. (G) UV–vis absorbance spectra of different concentrations of B NSs. (H) Photothermal heating curves of different concentrations of B NSs and ultrapure water after 808-nm laser irradiation (2 W/cm²) for 10 min. (I) Temperature changes in the B NS suspension (0.1 mg/ml) after irradiation with an 808-nm laser at 2 W/cm² over six on-off cycles.
To evaluate the effect of combined chemotherapy and mild PTT, five groups were created: blank, GA, B-PEG/GA, B-PEG+NIR, and B-PEG/GA+NIR. Within each group, four GA concentrations were used. As shown in Fig. 3H and S13, the findings in the B-PEG/GA+NIR group and the other groups were similar until the GA concentration reached 0.125 μM. This indicates that a certain concentration of GA is required to effectively inhibit the production of the Hsp90 protein. When the GA concentration reached 0.125 μM and 0.25 μM, the anti-tumor effect of combined chemotherapy and mild PTT was better than that of chemotherapy or PTT alone. Hence, the combination of B-PEG/GA and NIR appeared to provide low-temperature thermo-therapy and improved anti-tumor effects.

### 3.3 Cellular uptake and intracellular distribution

The uptake of B-PEG in 4T1 cells was assessed using B-PEG loaded with the fluorescent marker FITC. In Fig. 4, green represents B-PEG/FITC and blue represents nuclei. First, 5 μg/ml of B-PEG/FITC was incubated with 4T1 for different durations. The intracellular fluorescence increased as the incubation period increased. Moreover, there was no co-localization between the green and blue fluorescence, indicating that B-PEG were mainly concentrated in the cytoplasm (Fig. 4A). With an increase in the B-PEG/FITC concentration, the intracellular fluorescence in the cytoplasm became stronger (Fig. 4B). Figs. S14 and S15 show the cellular fluorescence signals under a low magnification. Together, the results showed that B-PEG are taken up by 4T1 cells after approximately 10 min incubation. After entering the cells, B-PEG mainly remain concentrated in the cytoplasm. The uptake of B-PEG by 4T1 cells is time- and concentration-dependent.

Cells take up extracellular material primarily through caveolin-mediated endocytosis, the clathrin-mediated endocytosis pathway, and macropinocytosis. The intracellular distribution of drugs differs depending on the endocytic
pathway mediating their entry. Our findings showed that 4T1 cells can rapidly endocytose B-PEG. Therefore, we proceeded to explore the endocytic pathway via which B-PEG enters 4T1 cells. 4T1 cells were treated with different endocytosis inhibitors, and changes in intracellular fluorescence signals were observed to determine the mechanism of entry. Intracellular fluorescence was affected after treatment with all three endocytosis inhibitors (Fig. 4C), indicating that the uptake of B-PEG occurs via caveolin-mediated endocytosis, the clathrin-mediated endocytosis pathway, and micropinocytosis. However, the intracellular fluorescence signal was highly attenuated after treatment with caveolin and micropinocytosis inhibitors. This indicated that caveolin-mediated endocytosis and micropinocytosis were the primary pathways of B-PEG uptake, with the clathrin-mediated endocytosis pathway driving a smaller proportion of B-PEG entry. After entering the cells through caveolin-mediated endocytosis, drugs are processed via the Golgi-endoplasmic reticulum (ER) signaling pathway, which facilitates their intracellular utilization [35]. In contrast, entry via the clathrin-mediated endocytic pathway is followed by lysosomal phagocytosis [36]. Therefore, we hypothesized that most of the cytoplasmic B-PEG would be distributed in the ER, while a small portion would be distributed in lysosomes.

The distribution of B-PEG in the cytoplasm was determined based on co-localization with different subcellular organelle markers. The subcellular organelles selected in the experiment were the lysosomes (Lysosome), ER, and mitochondria (Mito). As shown in Fig. 4D, B-PEG were mainly distributed in the mitochondria. The heat generated after the irradiation of B-PEG with 808-nm NIR light would destroy the mitochondria, thus inhibiting the growth of tumor cells. Moreover, some of the B-PEG were distributed in the ER, consistent with the findings on the uptake mechanism of B-PEG.

3.4. Multimodal imaging in vivo

The intratumoral temperature during photothermal treatment in mice was recorded using an infrared imager. B-PEG became heated upon irradiation. As a result, the temperature of the tumor becomes higher than that of surrounding tissue, and these differences can be captured using an infrared camera (Fig. 5A). The infrared imager recorded the temperature change during 10 min of 808-nm NIR light irradiation, and the temperature was maintained below 45 ℃ throughout. Hence, the conditions for mild PTT were satisfied (Fig. S16).
Most two-dimensional materials, such as black phosphorus and 2D transition metal carbides and nitrides (MXenes), have photoacoustic imaging capabilities [37,38]. In clinical diagnosis, materials with photoacoustic imaging capabilities provide visual information as well as real-time data. In particular, materials with photoacoustic imaging capabilities provide accurate diagnostic information and also help in monitoring drug distribution in the body [39]. Moreover, some of these materials also exert therapeutic effects. Therefore, we analyzed the photoacoustic imaging capabilities of B-PEG. We found that B-PEG indeed have photoacoustic imaging capabilities (Fig. 5B). Hence, they could therefore be applied in photoacoustic imaging-guided chemotherapy and photothermal combination therapy.

The in vivo distribution of B-PEG loaded with Cy5.5 was assessed based on fluorescence signals in live animals. The fluorescence signal was recorded at various points after the intratumoral injection of these NSs. Although its intratumoral fluorescence signal weakened gradually, B-PEG/Cy5.5 was retained in the tumor for more than 4 h (Fig. 5C and 5E).

After injection, the mice were sacrificed at different time points, and major organs and tumors were obtained and analyzed using a small animal live imager. As observed in the in vivo experiments, the findings showed that B-PEG were retained in the tumor for more than 4 h (Fig. 5D and F). The concentration of B-PEG in the lung tissue increased slightly with time. The distribution of B-PEG was greatest in the liver, peaking at 2h. However, these nanoparticles were gradually metabolized after 2 h.

3.5. Anti-tumor effect of chemotherapy combined with mild PTT and suppression of pulmonary metastases

As shown in Fig. 6A, tumor volumes were measured at regular intervals. The following conclusions could be drawn from the trends in tumor volume: (1) The GA group and mild photothermal treatment B-PEG+NIR group showed tumor inhibition for 11 d. However, after 11 d, tumors recurred, and their growth rate increased. (2) In the combined chemotherapy with mild photothermal treatment group (B-PEG/GA+NIR), mild PTT could be achieved and secondary tumor recurrence could be inhibited. At the end of the experiment, one randomly selected mouse from each group was examined, and its tumors were photographed. (3) The GA group, B-PEG/GA group, and B-PEG+NIR group all showed tumor recurrence. However, the mice in the B-PEG/GA+NIR group had a clean tumor surrounding area and showed no recurrence (Fig. 6B). Subsequently, the tumors were extracted, and the findings were consistent with the previous experimental results (Fig. 6C).

On the second day post-treatment, tumor tissues were obtained and sectioned for H&E staining. Compared with the nuclei in the model group, the nuclei in the GA, B-PEG/GA, and B-PEG+NIR groups were sparse, indicating that tumor growth was inhibited and the individual cells had started undergoing apoptosis (Fig. 6E). However, the number of nuclei was still high, suggesting that mild photothermal or chemotherapeutic treatment alone was insufficient, and the tumor could still show secondary recurrence. Pathological sections from the B-PEG/GA+NIR group showed a very small
Fig. 6 – Anti-tumor effect in vivo. (A) Growth of 4T1 tumors in mice from different groups (n = 6). p < 0.001 between two groups. (B-D) Representative tumors (B), tumor morphology (C), and morphology of lung nodules (D) examined 30 d after treatment in different groups. (E) Representative images of H&E-stained sections of tumor tissue extracted after 2 d of different treatments (n = 4). (F) Representative images of Hsp90-stained sections of tumor tissue extracted after 2 d of different treatments. (G) Quantification of Hsp90 expression (n = 4). **P < 0.01 and ***P < 0.001 vs. the model group. (H) Representative images of H&E-stained sections of lung tissue extracted after 30 d of different treatments (n = 3).
number of nuclei, indicating that combined chemotherapy with mild PTT was effective in ablating tumor tissues more completely. Subsequently, the amount of Hsp90 in the tumor tissue was detected using immunofluorescence assays. The GA and the B-PEG/GA+NIR groups exhibited significant Hsp90 inhibition in vivo (Fig. 6F and 6G).

Thirty days after treatment, the tumors in the model, GA, B-PEG/GA, and B-PEG+NIR groups showed different degrees of proliferation, and there were several tumor nodules in the lung tissue. Using pathological staining (Fig. 6H), we found that the lung tissues from mice in these groups exhibited tumor occupancy and were compressed. In contrast, the lung tissues of mice from the B-PEG/GA+NIR treatment group were similar to those of mice from the normal group, with normal lung function and no tumor occupancy. This indicated that combined chemotherapy with mild PTT could inhibit the lung metastasis of breast cancer. This could be because some B-PEG/GA reaches lung tissues, as shown by biopsy findings (Fig. 6D). However, the mechanisms underlying this phenomenon need to be experimentally validated.

### 3.6. In vivo safety evaluation

To evaluate the biocompatibility, some mice were sacrificed on 2 d after treatment. Pathological sections from major organs were obtained for analysis. As shown in Fig. 7A, no significant inflammatory reaction was observed in any group. This indicated that none of the treatments caused any acute reaction in mice.

Then, 2 d and 30 d after treatment, blood was obtained for blood biochemistry and hematology tests, and the levels of inflammatory factors were tested. As shown in Fig. 7, all immune factors were within normal levels 2 d after treatment, indicating that the treatments did not cause any immune-related effects (Fig. 7B). At 30 d after treatment, all indicators in the mouse model group were abnormal, indicating that tumor growth significantly affected the health of mice in this group (Fig. 7D&7F). Moreover, blood urea nitrogen and creatinine levels showed slight elevations after treatment. This suggested a slight but insignificant short-term increase in the renal burden after treatment (Fig. 7C and 7D). Finally, the mean erythrocyte protein concentration decreased at 2 d
post-treatment, indicating the presence of transient anemia. However, after 30 d, these levels returned to normal in all mice except those in the model group (Fig. 7E and 7F).

4. Conclusions

In this study, GA was loaded onto B-PEG to provide combined mild PTT and chemotherapy. Given their good photothermal conversion abilities, the BSNs could rapidly heat up under 808-nm NIR irradiation and therefore thermally ablate and kill tumor cells. In addition, we demonstrated that B-PEG have multimodal imaging characteristics, including photothermal imaging, photoacoustic imaging, and fluorescence imaging capabilities. Thus, B-PEG could serve as a platform for the integration of diagnosis and treatment. Moreover, our findings showed that GA can inhibit Hsp90 and reduce the thermal tolerance of tumor cells. It can also lower the temperature required for PTT. Therefore, GA can be used to provide mild PTT. Across experimental models, the findings of this study indicated that the nano-delivery system consisting of GA and B-PEG has good multimodal imaging capabilities, good biocompatibility, and high bioavailability. Therefore, this system can reduce the negative effects associated with PTT, improve anti-tumor therapeutic effects, and inhibit the lung metastasis of breast cancer.

Conflicts of interest

The authors have declared that no competing interest exists.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ajps.2022.06.003.

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