Electronic Supplementary Material (ESI) for Journal of Materials Chemistry B.
This journal is © The Royal Society of Chemistry 2022

Supporting Information

Targeted intelligent mesoporous polydopamine nanosystems for multimodal synergistic tumor treatment

Jiaxin Yang [a,1], Weinan Zeng [b,1], Xiaoxue Fu [a], Lu Chen [a], Xiaojuan Yu [a], Ping Xu [a], Wenyan Huang [a], Feng Leng [a], Chao Yu [a,], Zhangyou Yang [a,*]*

[a] Chongqing Key Laboratory for Pharmaceutical Metabolism Research, Chongqing pharmacodynamic evaluation engineering technology research center, College of Pharmacy, Chongqing Medical University, Chongqing 400016, P.R. China

[b] Orthopedic Research institution, Department of Orthopedics, West China Hospital, Sichuan University, Chengdu, 610041, P.R. China

* Corresponding authors. E-mail addresses:

yangzhangyou@cqmu.edu.cn (Z. Yang).

yuchao@cqmu.edu.cn (C. Yu).

1. Materials and Methods

Materials

Lauric acid (97%), stearic acid (95%), ethyl alcohol absolute, methyl alcohol, 3,5-trimethylbenzene (TMB) and aqueous ammonia were purchased from Aladdin; N,N-dimethylethylenediamine (DMEA), Pluronic F127, and dopamine (PDA) were purchased from Sigma; gambogic acid (GA) was purchased from MedChemExpress; SH-PEG (5000), FA-PEG-SH (5000) was purchased from Yare; NF-κB subunit p65 (siRNA-p65) (5'-GCCCUAUCCCUUUACGUCA TT-3' (sense)); siRNA-p65-cy3 (cy3-5'-GCCCUAUCCCUUUACGUCA TT-3' (sense)) was purchased from Sangon Biotech; and trypsin-EDTA, RPMI 1640 medium, penicillin and streptomycin and fetal bovine serum (FBS) were obtained from Gibco-BRL. RIPA Lysate and PageRuler Prestained Protein Ladder (10-180 kDa) were purchased from ThermoFisher. Lysotracker Red was purchased from Invitrogen. Agarose and Goldview were purchased from Yeasen. Trypan Blue was purchased from TianGen (Beijing, China). Anti-beita actin, anti-MMP9, anti-MMP2, and anti-NF-Kb p65 were purchased from Abcam.

Preparation of MPDA NPs

MPDA nanomaterials were synthesized by a previously reported method. Then, 0.1 g of F_{127}, 0.15 g of dopamine and 0.14 mL of TMB were added to a mixed solution of 5 mL of water and 5 mL of absolute ethanol, and the emulsion was formed by ultrasonication for 2 min. Then, 0.375 mL of ammonia water was added to the mixed solution with stirring. The product was collected by centrifugation and washed with

water and ethanol several times to obtain MPDA nanoparticles, Then the product was redispersed in 10 mL of water.

Synthesis of MPDA-PEG and surface modification

Briefly, 1 mL of FA-PEG-SH (5000) aqueous solution (5 mg/mL) was added to 1 mL of MPDA (1 mg/mL) solution, followed by adjustment of the pH to approximately 9. After stirring for 2 h, the solution was centrifuged and the supernatant was discarded, and washed with water several times. The product is dispersed in water and named MPDA-PEG. Subsequently, 1 mL of aqueous DMEA solution (5 mg/mL) was stirred and mixed with 1 mL of MPDA-PEG solution, followed by adjustment of the pH to approximately 9. After two hours of magnetic stirring, the material was collected, washed, purified and named PPMD.

Loading of PCM, GA and siRNA

Briefly, lauric acid and stearic acid (4:1 by weight) were first dissolved in methanol at a concentration of 4 mg/mL. GA was dissolved in methanol solution at a concentration of 1 mmol/L. Subsequently, 0.4 mL of PCM methanol solution was mixed with 40 μL of GA methanol solution, and then the above solution was added to 2 mL of PPMD (2 mg/mL) methanol solution and sonicated for 2 min. Then, the solution was transferred to a rotary evaporator to remove excess solvent, followed by methanol washing to remove PCM adsorbed on the outer surface of PPMD. The as-obtained nanoparticles were denoted PPMD@GA and were redispersed in 1 mL of DEPC water for further use. Finally, the siRNA and PPMD@GA were mixed in different W/Ws in 1 mL DEPC water (siRNA: PPMD@GA = 1:1, 1:4, 1:5 and 1:10),

and then vortexed for 3 min to obtain PPMD@GA/si. Gel retardation assay for testing siRNA-loading capacity. The different W/W PPMD@GA/si were loaded into 4% agarose gels and in 1 x TBE buffer at 80 V for 40 min. The gels were stained with GoldView, and the siRNA bands were detected using chemiluminescent imaging systems (C300, Azure).

Characterization of PPMD@GA/si

MPDA micromorphology was examined by SEM (SU8020, Hitachi) and TEM (Talos F200X, FEI). Zeta potentials and DLS were analysed by particle size meter (Malvern Instruments Ltd, ZEN3600). The particle size distribution was determined by means of a physisorption instrument (TriStar II 3020, Micromeritics) with an analysis temperature of -195.80°C. Melting point of PPMD@GA/si and PCM determined by a differential scanner (DSC214. NETZSCH), equilibrate 0.00°C, ramp 10.00°C/min to 100.00°C. Fourier transform infrared (FT-IR) spectral detection was performed by FT-IR spectroscopy (Nicolet iS5, Thermo Fisher Scientific) and ultraviolet–visible (UV–vis) absorption spectroscopy on a UV–vis spectrophotometer (Ultra6600A, Rigol).

Photothermal effect evaluation of PPMD@GA/si

The PBS, ICG (100 μmol/L) and MPDA (250 μg/mL) solutions were irradiated with NIR laser for 3 cycles of 10 min each, and the temperature was measured and recorded every minute during irradiation. In addition, the PBS, MPDA and PPMD@GA/si solutions were irradiated by a laser for 10 min and images were taken with an infrared thermal imager (220 s, FOTRIC) during irradiation.

Photothermal conversion performance of PPMD@GA/si: The PPMD@GA/si

(1mg/mL) is scattered in the water, and then the 808 nm laser was used to irradiate the solution for 5 min, pure water is used as a negative control. According to the equation, the photothermal conversion efficiency (η) is calculated.

$$\eta = \frac{hS\left(\Delta T_{max,\,mix} - \Delta T_{max,\,water}\right)}{I\left(1 - 10^{-A_{808}}\right)}$$

NIR laser-triggered GA release from PPMD@GA/si

PPMD@GA/si was irradiated under an 808nm NIR laser (0.6 W/cm^2) for 10 min and then after a 10 min interval, four cycles were repeated. The PPMD@GA/si was centrifuged before each measurement and the supernatant was analysed using high performance liquid chromatography (1260, Agilent).

Cellular uptake and intracellular localization

For cell uptake assessment, 4T1 cells were inoculated into 12-well culture plates at a density of 5 × 10^4 cells per well. siRNA-CY3 or MMPD@GA/si-CY3 (100 μg/mL) were added separately and incubated at different time points. Then pictures were taken by inverted fluorescence microscopy (Eclipse Ti-S, Nikon) and further analysed for fluorescence intensity within the cells at different time points using a flow cytometer (CytoFLEX S, Beckman Coulter). Next, for the folic acid inhibition assay, 4T1 cells were first incubated in advance with medium with or without folic acid (FA:5 mg/mL). After 24 hours of incubation, flow cytometry was used to analyse the fluorescence intensity changes in cells.

To observe the intracellular distribution of MMPD@GA/si nanoparticles, specifically, 4T1 cells were treated with PPMD@GA/si (100 μg/mL) for 2h, followed by NIR laser irradiation (808 nm, 0.6W/cm^2) for 10 min, and then washed 3 times with

PBS after 2 h. The cells were incubated with DAPI (C1006, Beyotime) and LysoTracker (75 nM) for 20 min in turn. Finally, the cells were observed using confocal laser scanning microscope (TCS SP8 CARS, Leica).

Cytotoxicity assay

The CCK-8 assay was used to assess the cytotoxicity of 4T1 cells. Cells were seeded at a density of 5 x 10^3 cells per well on 96-well plates and incubated at 37°C for 24 h prior to performing the cytotoxicity assay. The normal, light groups and the high temperature group (43°) were set up separately, where fresh cell medium containing different concentrations of GA, MPDA, PPMD@GA/si were substituted for the culture medium. The high temperature group was incubated at 43°C for 24 h, the normal and light groups were incubated at 37°C for 24 h, and then the light group cells were exposed to NIR laser for 10 min (808 nm, 0.6W/cm^2). Subsequently, the culture medium was removed and washed 3 times with PBS. Subsequently, CCK-8 (10 μL) and fresh medium (100 μL) were added and the cells were further incubated for 1.5 h. Finally, the absorbance of each well at 450 nm was measured using a microplate reader (Varioskan LUX, Thermo Fisher Scientific). The viability of the 4T1 cells was further evaluated using Calcein/PI cell viability Assay kit (C2015L, Beyotime).

Apoptosis/necrosis assay: In brief, 4T1 cells from different treatments were collected, washed three times with PBS, treated with Annexin V-FITC/PI Apoptosis Assay Kit (C1062L, Beyotime) and finally analysed using flow cytometry.

Migration and invasion assay

4T1 cells were inoculated at the appropriate density in six-well plates subjected to

different treatments (PBS, MPDA, MPDA+laser, PPMD@GA/si, PPMD@GA/si+laser) and the cell layer formed in the well plates was scratched with a pipette tip. Cell migration was observed under a microscope at different time points and migration rates were calculated using ImageJ software. A Transwell (Corning) migration assay was performed to determine the effect of PPMD@GA/si on 4T1 migration capacity. Pretreated 4T1 cells were dispersed in serum-free medium and inoculated in the upper chamber of the Transwell, and medium containing 10% serum was added to the lower chamber. After 24 hours of incubation, the cells were removed, fixed in paraformaldehyde and stained with crystal violet. Subsequently, ethanol was added to dissolve the cells with bound dye and the absorbance of the samples at 590 nm was measured by a microplate reader.

Western Blot analysis

After different treatments, 4T1 cells were lysed in RIPA protein extraction buffer with 1% protease inhibitor (PMSF) on ice. Next, the total protein concentration of each sample was measured using the Protein Analysis Kit (,Biosharp). After cell lysis, equal amounts of protein from each sample were loaded onto sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. After electrophoresis was completed, the resulting gel was transferred to a polyvinylidene difluoride (PVDF) membrane for 1 hour. The blotted PVDF membranes were then blocked in a TBST solution containing 5% defatted milk for 2 hours. The PVDF membranes were then incubated with the corresponding horseradish peroxidase-conjugated secondary antibodies for 2 hours at room temperature using primary antibodies (HSP90, NF-κb,

MMP9) overnight at 4°C, shaken and washed 3 times with TBST. After incubation with enhanced chemiluminescence reagents, target proteins were visualized using a chemiluminescence imaging system (Tanon 5200, China).

Hemolysis test

Clean red blood cells (RBCs) were obtained by removing the serum from the blood by centrifugation (1000 r/min). Then 900 μL of RBC solution was mixed with 100 μL of PPMD@GA/si to obtain final concentrations of 50, 100, 200, 400 and 500 μg/mL. Red cells incubated with PBS solution and 1% Triton X-100 solution were set up as negative (A_n) and positive controls (A_p), respectively. The mixture was then incubated in 37° water for 1 h and then centrifuged at 1000 rpm for 5 minutes. Finally, the absorbance (A_t) of these supernatants at 450 nm was measured using a microplate reader. The percentage of hemolysis was calculated by the formula: Hemolysis rate (%) = $(A_t-A_n) / (A_p-A_n)$.

***In vivo* fluorescence imaging**

Female BALB / c mice (20-25 g) were provided by the Experimental Animal Centre of Chongqing Medical University. All animal experiments were approved by the Animal Ethics Committee of Chongqing Medical University (Chongqing, China). A 4T1 subcutaneous tumor model was established by inoculating 1×10^7 cells in 25 μL PBS. When the tumor grew to approximately 80 cm^3, 100 μL each of ICG, PPMD@GA/si-1 and PPMD@GA/si-2 solutions were injected into the tail vein, and then the distribution of nanoparticles *in vivo* was observed by VISQUE Smart system (Vieworks). 24 h later, the mice were sacrificed to take organs and tumor tissues for

fluorescence imaging to observe the distribution of nanoparticles.

***In vivo* antitumor effect and biocompatibility of PPMD@GA/si**

For *in vivo* cancer treatment, tumor-bearing mice were randomly divided into 7 groups (n=7): I: PBS, II: GA, III: MPDA+Laser, IV: PPMD@si, V: PPMD@si+Laser, VI: PPMD@GA/si, VII: PPMD@GA/si+Laser. The drugs were injected into the tail vein continuously for the first three days, and the laser group was laser irradiation (808 nm,0.6W/cm^2) 24h after drug injection for 10 min each time. The tumor volume and body weight of the mice were monitored every other day for 14 days. The mice were finally sacrificed, and the tumor tissue was photographed and weighed to record the tumor size. The collected tumors were then fixed and sectioned for hematoxylin-eosin (H&E) and TUNEL staining analysis. Then, major organs were collected from the PBS and PPMD@GA/si groups by H&E staining for histological analysis, and blood samples were collected for routine blood and biochemical parameter examination.

***In vivo* inhibition of tumor migration**

The 4T1 cells were first pretreated and divided into 7 groups (n=5) (I: PBS, II: GA, III: MPDA+Laser, IV: PPMD@si, V: PPMD@si+Laser, VI: PPMD@GA/si, VII: PPMD@GA/si+Laser), and the laser group was given NIR laser irradiation (808 nm, 0.6W/cm^2). After 24 hours of treatment, the cells were collected and resuspended in PBS. Each mouse was injected with 100 μL of 4T1 suspension in the tail vein to construct a lung metastasis model. After 14 days all mice were sacrificed, and their lungs were taken for assessment of metastasis. Finally, the lungs of each group of mice were fixed and sectioned for H&E staining and immunohistochemical (MMP2, MMP9)

analysis.

Statistical analysis

Each experiment was repeated at least three times. All results are reported as the mean ± standard deviation. All data were analysed by one-way analysis of variance (ANOVA) followed by Tukey's test in the Statistical Package for the Social Sciences (SPSS), version 20 (SPSS Inc., Chicago, IL). *P < 0.05, **P < 0.01, and ***P < 0.001 were considered statistically significant between the different groups (NS, not significant).

2. Supplementary figures

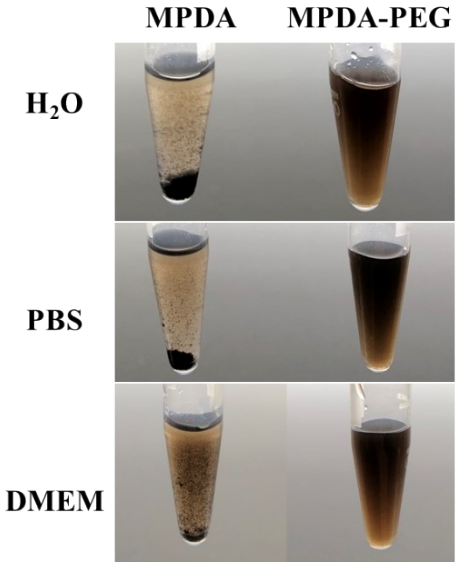

Figure S1. Dispersion image with MPDA and MPDA-PEG in different solutions.

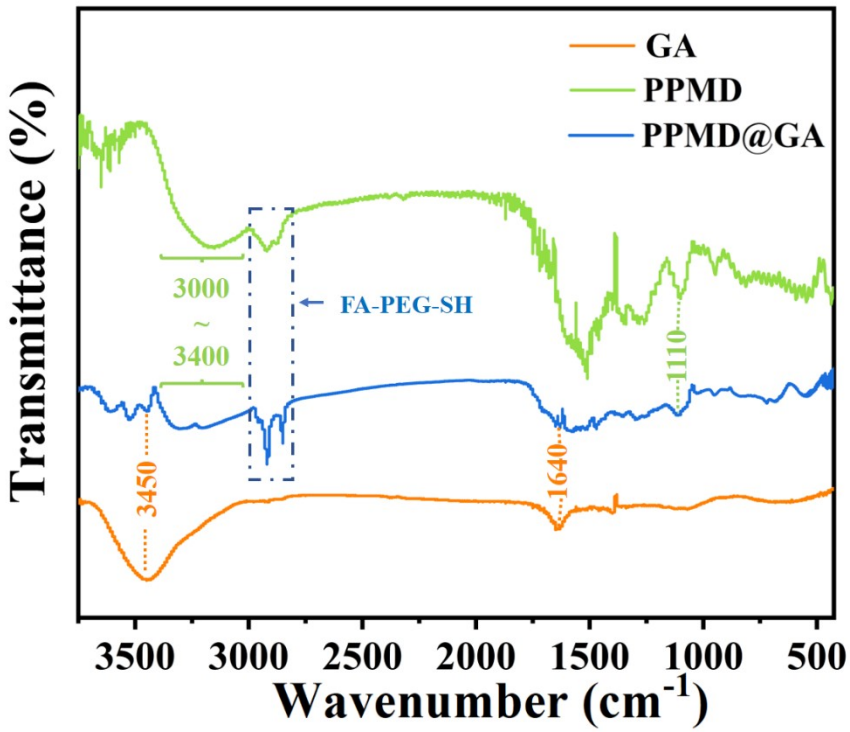

Figure S2. FTIR spectrum of the GA, PPMD and PPMD/GA.

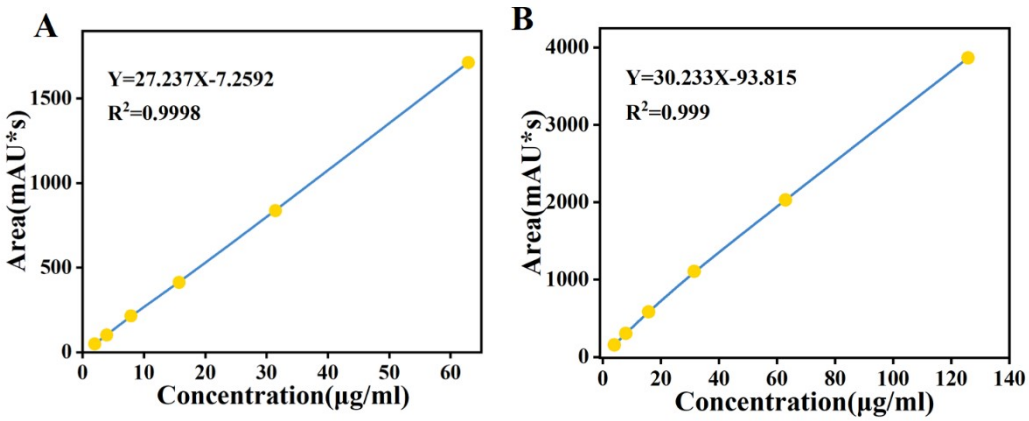

Figure S3. Standard curve for GA solution of 50% methanol phase (A) and pure methanol phase (B) by high performance liquid chromatography.

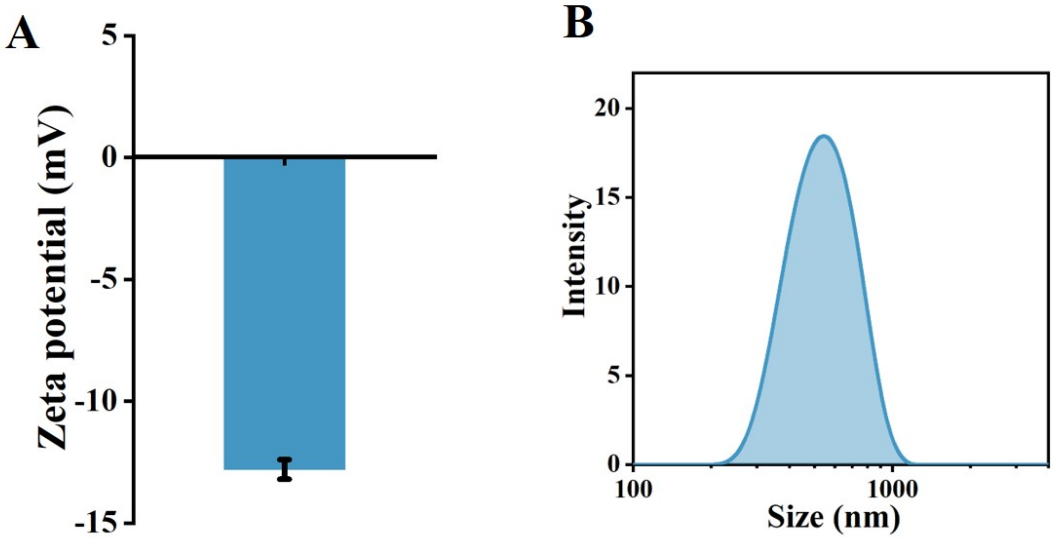

Figure S4. A) Zeta potential and B) Size distribution of PPMD@GA/si.

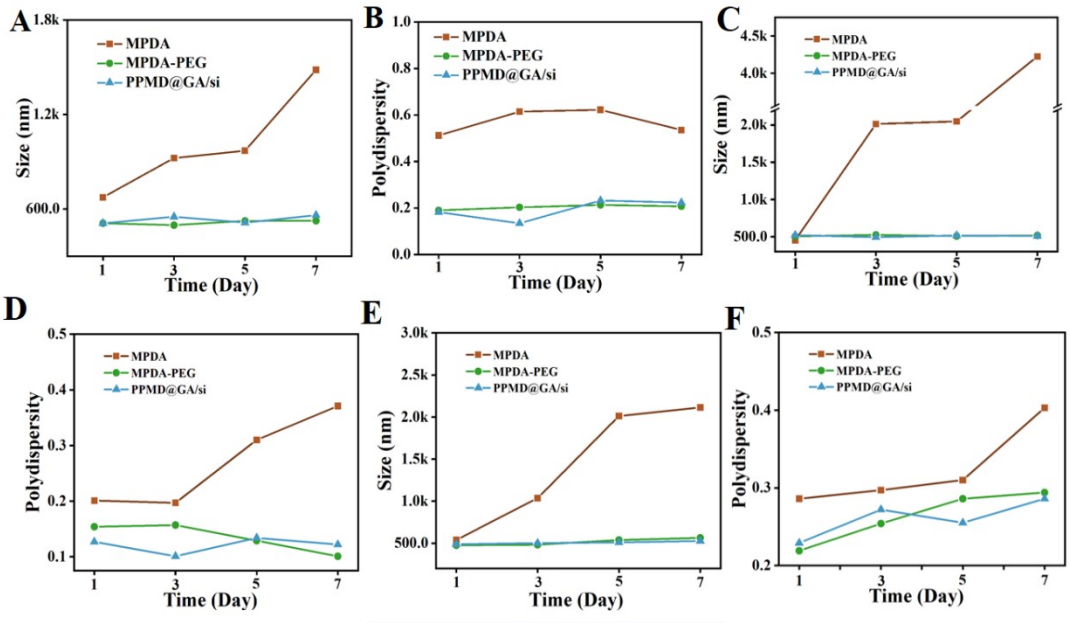

Figure S5. A) Hydrated particle size and B) polydispersity of the particles in PBS at different time points. C) Hydrated particle size and D) polydispersity of the particles in DMEM medium at different time points. E) Hydrated particle size and F) polydispersity of the particles in 10%FBS at different time points.

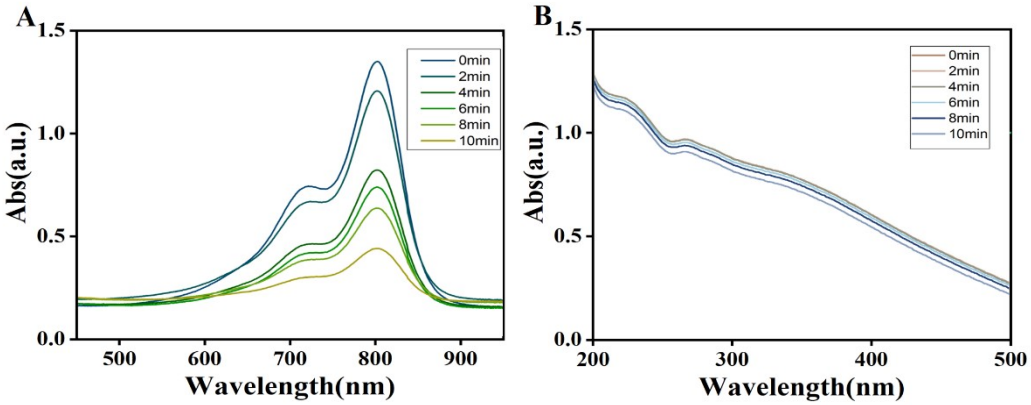

Figure S6. UV–vis absorption spectra of ICG and MPDA after the laser irradiations (808nm, 0.6W/cm^2) different time.

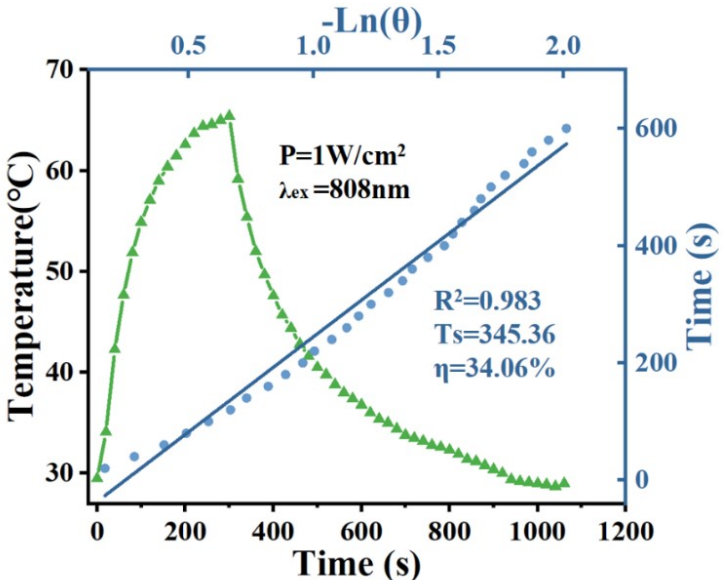

Figure S7. The photothermal performance of PPMD@GA/si and the corresponding linear relationship between -Ln (θ) and time (s).

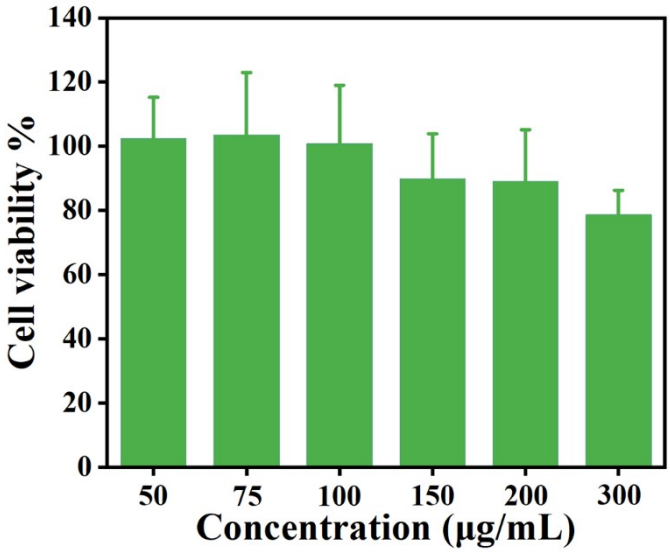

Figure S8. Relative viability of 4T1 cells treated with different concentrations PPMD@GA/si.

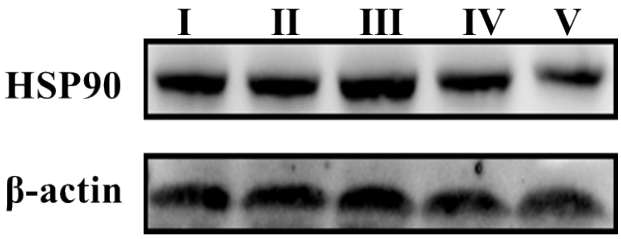

Figure S9. The HSP90 expression levels was performed by β-actin as an internal reference for 4T1 after different treatments as indicated.

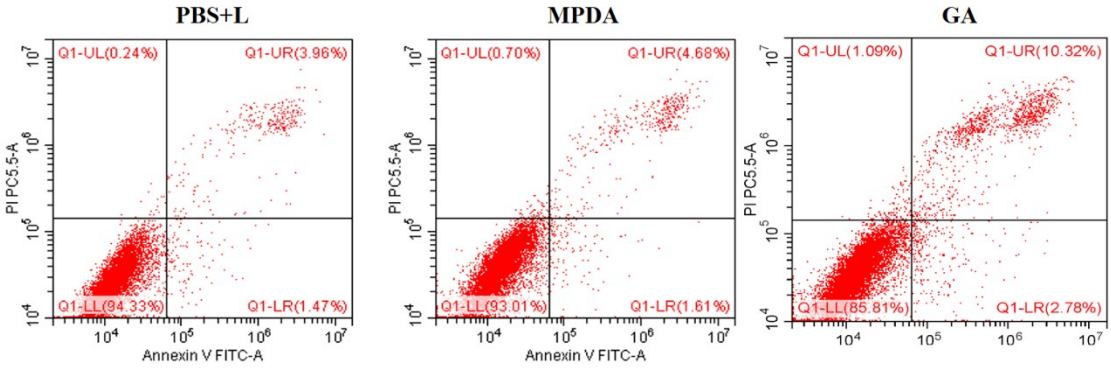

Figure S10. Apoptotic levels of 4T1 cells after different treatments as indicated measured by the annexin V-FITC/PI apoptosis detection kit.

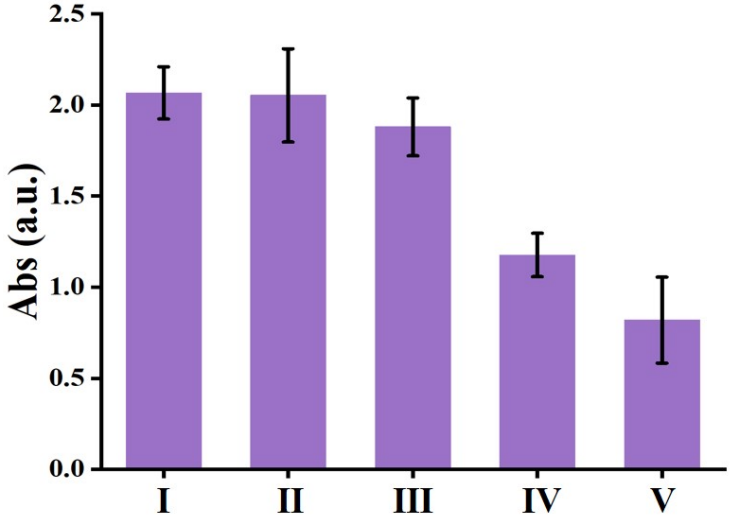

Figure S11. Absorbance of migrating cells after different treatments. (I: PBS, II: MPDA III: MPDA+L, IV: PPMD@GA/si, V: PPMD@GA/si+L).

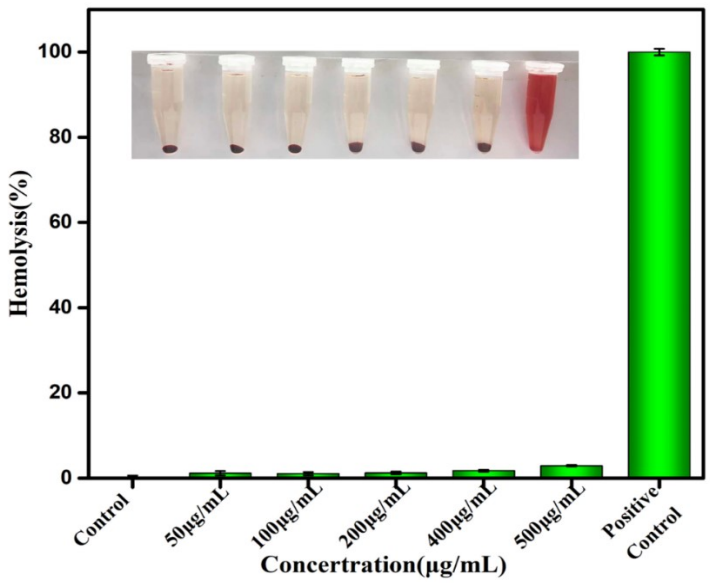

Figure S12. Results of hemolysis experiments with PPMD@GA/si at different concentrations.

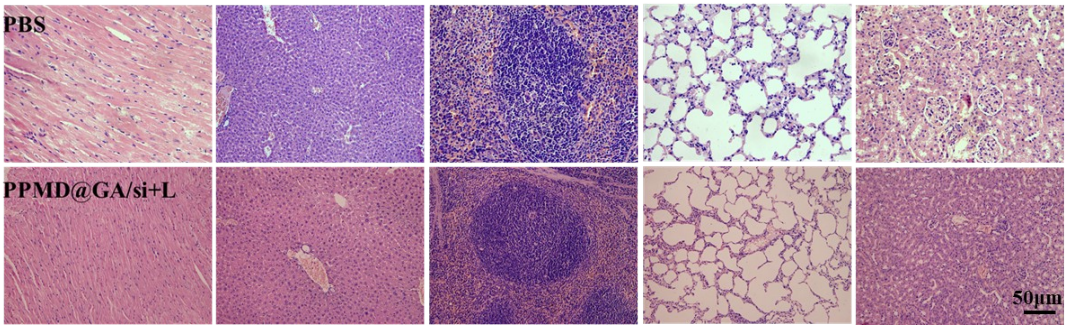

Figure S13. H&E-stained tissue slices of major organs from PBS and PPMD@GA/si+L groups.

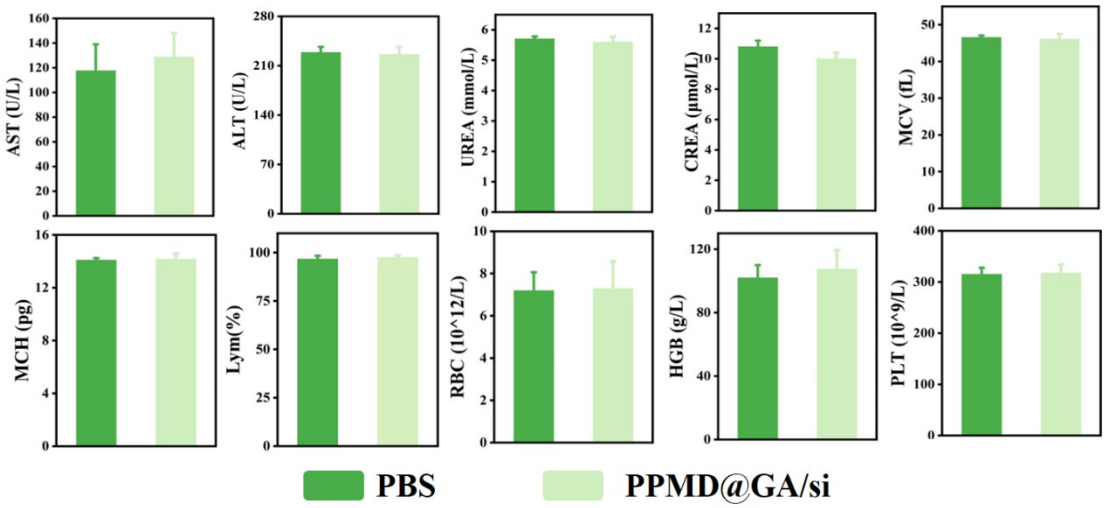

Figure S14. Results of routine blood analysis: mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), lymphocyte (Lym), red blood cells (RBC), hemoglobin (HGB), platelets (PLT), alanine transferase (ALT), aspartate transferase (AST), blood urea nitrogen (BUN), and creatinine (CRE).

3. Supplementary table

Table 1. PPMD@GA/si CI-Fraction affected (Fa) relationship table.

Total Dose(μg/ml)	Relative viability (Fa)	CI Value
50	0.96	1.61
75	0.80	0.74
100	0.55	0.12
150	0.38	0.08
200	0.19	0.04
300	0.14	0.04