Electronic Supplementary Information (ESI) for

Palladium nanosheet-knotted injectable hydrogels formed via palladium–sulfur bonding for synergistic chemo-photothermal therapy

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Experimental section

Materials. Palladium chloride ($PdCl_2$), concentrated hydrochloric acid (HCl), dimethylformamide (DMF) and doxorubicin hydrochloride (DOX) were purchased from Sigma-Aldrich. Methoxy-polyethylene glycol (PEG)-thiol (2 kDa) and 4arm-PEG-thiol (40 kDa) were obtained from Jenkem Technology. Dimethylsulfoxide (DMSO) and methylthiazolyldiphenyl-tetrazolium bromide (MTT) were ordered from J&K Scientific. Dialysis membranes with a molecular weight cut-off (MWCO) of 1 kDa were obtained from Spectrumlabs. All solutions were prepared with deionized (DI) water (18.2 MΩ·cm) purified by a Milli-Q system (Millipore, USA). Murine breast cancer cell line (4T1) was purchased from the Cell Resource Center of Shanghai Institute for Biological Sciences.

Preparation of palladium nanosheets (Pd NSs). To prepare H_2PdCl_4 aqueous solution, 20 mg $PdCl_2$ was mixed with 23 μL concentrated HCl and 90 μL DI water, and the mixture was incubated at 70 ^oC for 1 h. 10 mL DMF containing 60 μL H_2PdCl_4 solution (1 M) was placed in a flask followed by the introduction of 1 atm of CO at room temperature. The mixture was subjected to vigorous stirring until the color of the mixture changed from brown-red to lemon-yellow. CO gas was removed after 15 min, and then 2 mL of DI water was injected into the flask under stirring. A fast color change from light yellow to dark blue could be seen and this reaction was allowed to last for 10 min to obtain Pd NSs. Next, 4 mg methoxy-PEG-thiol (2 kDa) was introduced to the above-obtained Pd NSs immediately, followed by dialysis (dialysis membrane: MWCO = 1 kDa) against DI water for 2 days to remove DMF. After dialysis, the obtained PEG-Pd NS dispersion was used for subsequent experiments within 1 week.

Preparation of Pd hydrogel (Pd Gel) and DOX-containing Pd hydrogel (DOX@Pd Gel). For Pd Gel preparation, 20 mg of 4arm-PEG-thiol dissolved in 1 mL of DI water was introduced into 1 mL Pd NS aqueous dispersion (120 μg/mL). After sufficient mixing, the hydrogel formed within 5 min. For the preparation of DOX@Pd Gel, 1 mL DI water containing 20 mg 4arm-PEG-thiol and 10 μg DOX was mixed with 1 mL Pd NS aqueous dispersion (120 μg/mL), and the hydrogel formed within 5 min.

Characterization of hydrogels. Size and morphology characterization of DOX@Pd Gel was conducted on a scanning electron microscope (SEM) (Ultra Plus, Zeiss, Germany). The

viscosity change and the shear rate were monitored via a rheometer (MCR302, Anton Paar, Austria). Thermography was collected via a thermal imaging camera (Ai50, Infratest, China). Fourier transform infrared (FTIR) spectra were measured using an FTIR spectrometer (Nicolet iS50, Thermo Scientific, USA). X-ray photoelectron spectroscopy (XPS) analysis was conducted on an X-ray photoelectron spectrometer (PHI Quantera II, Ulvac-Phi, Japan).

Evaluation of photothermal effects. The temperature changes of 500 μL Pd Gel and DOX@Pd Gel under an 808 nm laser (0.6 W/cm^2) were monitored with a thermal imaging camera and the temperature values were recorded every 30 s. Phosphate-buffered saline (PBS) was used as a control group. Furthermore, laser on/off cycle assays were carried out to evaluate the photostability of DOX@Pd Gel. Typically, 500 μL DOX@Pd Gel was exposed to an 808 nm laser (0.6 W/cm^2) for 10 min followed by a "laser-off" duration of 10 min and the above procedure was performed for 3 times. The temperature was recorded with a thermal imaging camera and the temperature values were recorded every 30 s.

NIR laser-triggered DOX release from DOX@Pd Gel. Release of DOX from DOX@Pd Gel was determined in DI water. 1 mL of DI water was added to a 4 mL Eppendorf tube containing 1 mL DOX@Pd Gel. The mixture was irradiated using an 808 nm NIR laser (0.6 W/cm^2) for 5 min followed by a "laser-off" duration of 5 min, and the above procedure was performed for 3 times. The absorbance values at ~490 nm of the solution in the upper layer of the tube at different time points were monitored with an ultraviolet–visible (UV–vis) spectrophotometer (UV-2600, Shimadzu, Japan) for determining the release amounts of DOX. By comparison, the DOX@Pd Gel without NIR laser irradiation was set as a control group.

Cytotoxicity of lixivium. Complete Roswell Park Memorial Institute (RPMI) 1640 culture medium, composed of 90% RPMI 1640 (containing 100 U/mL penicillin and 100 μg/mL streptomycin) and 10% fetal bovine serum, was used to culture the 4T1 cells in a CO_2 incubator at 37 ^{o}C. 1 mL DOX@Pd Gel was immersed in 1 mL complete culture medium, and after 24 h, the lixivium was collected to evaluate the cytotoxicity of the lixivium from DOX@Pd Gel. 100 μL of 4T1 cell suspension (5×10^4 cells/mL) was introduced into a 96-well plate and cultured for 24 h, followed by the treatment with lixivium at different proportions in complete culture media (0%, 10%, 20%, 50% and 100%) for another 24 h. Cell viabilities of 4T1 cells treated with different concentrations of DOX (0, 0.1, 0.2, 0.5 and

1 μg/mL) for 24 h were utilized as a cytotoxicity refer for the released DOX from DOX@Pd Gel. After that, 10 μL MTT solution (5 mg/mL) was added to each well and incubated with the cells for 5 h. The MTT-containing complete culture medium in each well was then replaced by 150 μL DMSO. After shaking the plate for 7 min, the absorbance at 492 nm of each well was measured by a microplate photometer (Multiskan FC, Thermo Scientific, USA).

Cytocidal effect of DOX@Pd Gel with/without NIR laser irradiation. 4T1 cells were seeded in a 96-well plate with a density of 5×10^3 cells/well in 200 μL culture medium. The cells were cultured for 24 h, and then treated with DOX (1 μg/mL), Pd Gel (containing 60 μg/mL Pd NSs), DOX@Pd Gel (containing 1 μg/mL DOX and 60 μg/mL Pd NSs), Laser (808 nm, 0.6 W/cm^2, 10 min), "DOX + Laser", "Pd Gel + Laser", or "DOX@Pd Gel + Laser". The cells without drug treatment were set as the control groups. Specifically, for the treatment of the cells with hydrogels, 100 μL Pd Gel or DOX@Pd Gel was added separately into the corresponding wells of the 96-well plate through a pipette after the introduction of 100 μL culture medium. NIR laser irradiation was carried out after the incubation of the cells with DOX, Pd Gel or DOX@Pd Gel for 6 h. After being washed with PBS for 3 times and cultured in fresh culture medium for another 4 h, the cells were subjected to MTT assay as described above to determine the cell viabilities.

Live/dead assay. To further determine the cytocidal activity of DOX@Pd Gel under NIR laser irradiation, the Live/Dead viability/cytotoxicity kit (Invitrogen, USA) was used. 4T1 cells were seeded in an 8-well plate with a density of 5×10^3 cells/well. After being incubated for 24 h, the cells were subjected to different treatments as indicated above. Next, the cells were costained with 1 μM calcein acetoxymethyl ester (calcein AM, Ex/Em: ~495 nm/~515 nm) and 2 μM propidium iodide (PI, Ex/Em: ~495 nm/~635 nm) for 30 min at room temperature, washed with PBS for 3 times, and finally imaged by a confocal microscope (TCS SP8, Leica, Germany) using a 10× objective lens. The excitation and emission of each channel were adjusted to a suitable value to avoid the possible fluorescence interference between DOX and PI.

Evaluation of cellular reactive oxygen species (ROS) levels. The generation of intracellular ROS was also assessed using the reactive oxygen species assay kit (KeyGen Biotech). 4T1

cells after different treatments were loaded with 10 μM 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) for 15–30 min in the dark (at 37 °C in 5% CO_2). Then all the cells were immediately analyzed by confocal microscopy and flow cytometry (using a flow cytometer, NovoCyte 2070R, ACEA Biosciences, Inc., USA).

Apoptosis assay. To explore the cell death mechanism, 4T1 cells after the respective treatments (Control, DOX, Pd Gel, DOX@Pd Gel, Laser, DOX + Laser, Pd Gel + Laser, or DOX@Pd Gel + Laser; DOX: 1 μg/mL, Laser: 808 nm, 0.6 W/cm^2, 10 min,) were collected, washed 3 times with PBS, stained with annexin V-FITC/PI apoptosis detection kit (KeyGen Biotech), and then observed using the flow cytometer.

Tumor elimination assay. Four-week-old female BALB/c nude mice were provided by Yangzhou University Medical Center (Yangzhou, China). 100 μL PBS containing 1 × 10^6 4T1 cells were injected into the back of each mouse to form 4T1 xenograft tumor model. Mice with tumor volume reaching approximately 90 mm^3 were divided into 8 groups (6 mice in each group): (1) Control (untreated); (2) DOX: 25 μg/kg; (3) Pd Gel: 0.3 mg/kg Pd and 50 mg/kg 4arm-PEG-thiol; (4) DOX@Pd Gel: 0.3 mg/kg Pd, 50 mg/kg 4arm-PEG-thiol and 25 μg/kg DOX; (5) Laser; (6) DOX + Laser; (7) Pd Gel + Laser; (8) DOX@Pd Gel + Laser. The samples (DOX, Pd Gel and DOX@Pd Gel) were injected intratumorally into the mice and the condition of laser irradiation was 808 nm, 0.6 W/cm^2, 10 min. Next, the tumor volume and body weight of each mouse were monitored every two days for 20 days. The formula, volume = tumor length × tumor width × tumor width/2, was used to calculate the tumor volumes of treated mice. The ratio V/V_0 was utilized to evaluate the growth rate of tumor (V_0: initial tumor volume; V: tumor volume at an indicated time point). Finally, all the tumor tissues of mice were separated. After fixation in 4% formaldehyde overnight, tumor tissues were made into corresponding paraffin sections, followed by hematoxylin and eosin (H&E) staining.

Lung metastasis evaluation. To further evaluate the *in vivo* metastasis inhibition ability of DOX@Pd Gel with laser irradiation, mice after different treatments were sacrificed at day 20. The lungs were collected and fixed in Bouin's solution for 24 h. The lungs were then photographed and the nodules on the surface of the lungs were quantified to evaluate lung metastasis. Besides, H&E staining was also conducted on the fixed lung sections from represent mice with different treatments.

Histocompatibility analysis. To explore the systemic toxicity of DOX@Pd Gel, representative mice (including healthy nude mice without any treatment and 4T1 tumor-bearing nude mice treated with "DOX@Pd Gel + Laser") were sacrificed at day 14 and the main organs including hearts, livers, spleens, lungs and kidneys were separated for H&E staining. An optical microscope with charge-coupled device camera was utilized to photograph the stained sections.

Hemanalysis. Blood samples were collected from the healthy nude mice without treatment or the 4T1 tumor-bearing nude mice at day 20 after "DOX@Pd Gel + Laser" treatment. The changes of the blood indexes including the numbers of red blood cells (RBC) and white blood cells (WBC), the concentration of hemoglobin (HGB), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and platelet (PLT) count were analyzed using an automatic hematology analyzer (BC-2800Vet, Mindray, China).

Statistics analysis. All data were obtained from three or more parallel replicates and images were representatives from three or more assays. One-way analysis of variance (ANOVA) was adopted to analyze the differences between the control group and the treated groups using Tukey's test. Error bars indicate ± S.D. (n = 3 or 6). ** and *** indicate statistically significant differences at $P < 0.01$ and $P < 0.001$, respectively.

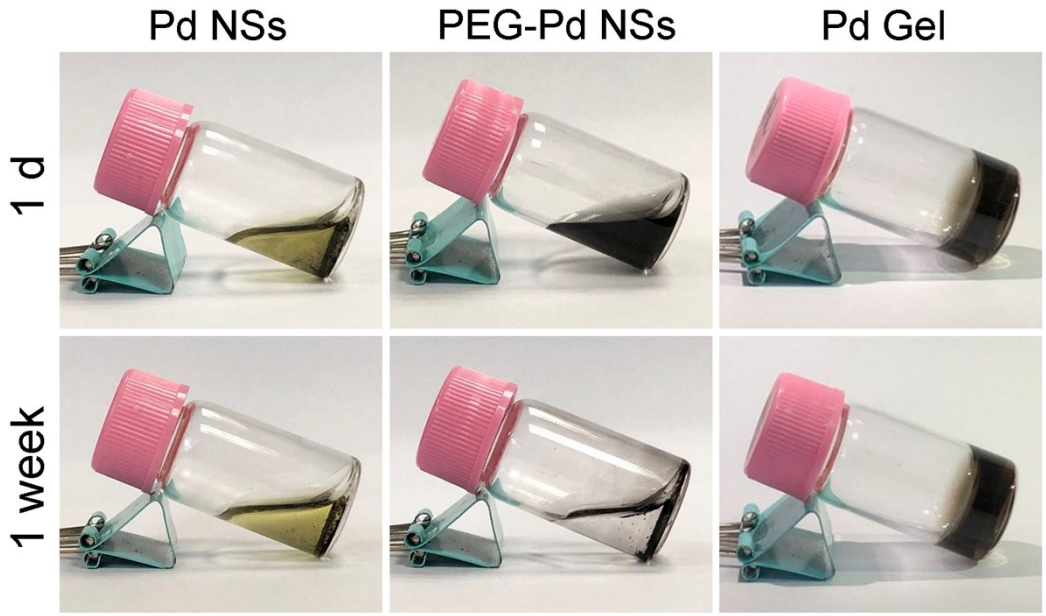

Fig. S1 Photographs of Pd NS aqueous dispersion, PEG-Pd NS aqueous dispersion and Pd Gel after standing for 1 day and 1 week, respectively.

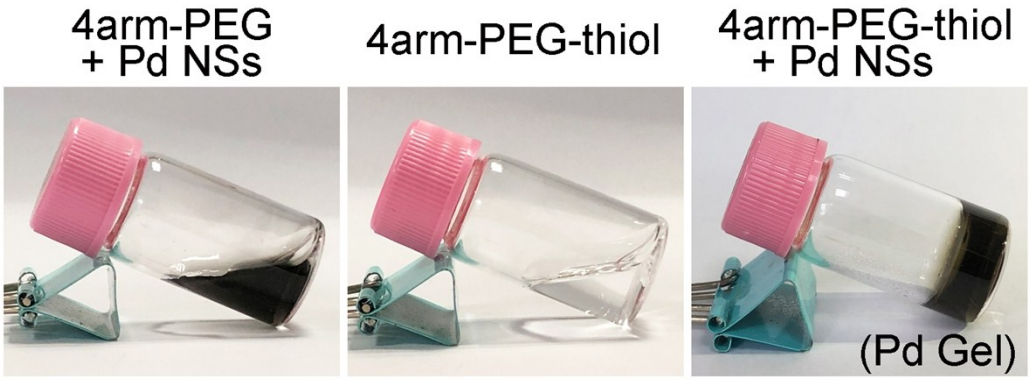

Fig. S2 Photographs of "4arm-PEG + Pd NSs" dispersion, 4arm-PEG-thiol solution and "4arm-PEG-thiol + Pd NSs" (Pd Gel) taken 1 week after preparation.

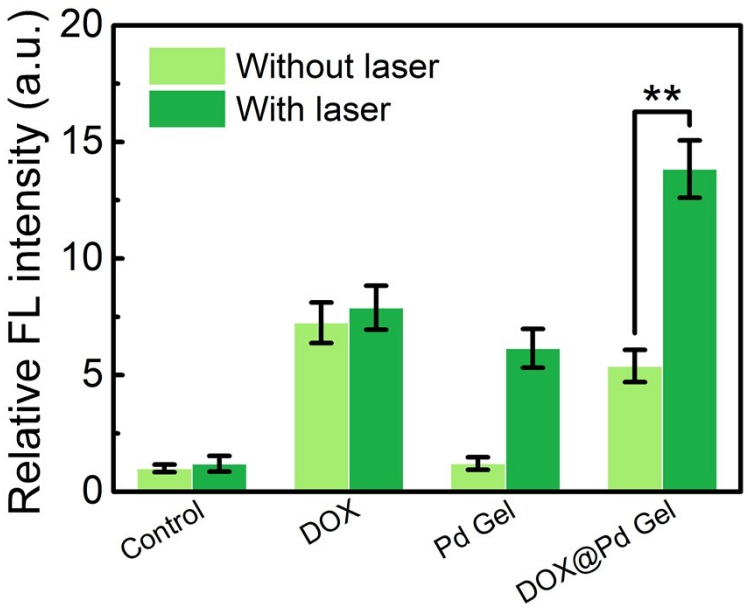

Fig. S3 Corresponding mean fluorescence intensities derived from the flow cytometric data in Figure 3c. Error bars indicate ± S.D. (n = 3). $^{**}P < 0.01$.