Electronic Supplementary Information

Noninvasive Magnetic Resonance/Photoacoustic Imaging for Photothermal Therapy Response Monitoring

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Reagents

Tungsten disulfide (WS_2, 99%), n-butyllithium (C_4H_9Li, 1.6 M in hexanes), hexane, Poly(ethylene glycol) methyl ether amine (mPEG-NH_2, molecular weight 5000 Da), lipoic acid (LA), dichloromethane (CH_2Cl_2), N,N-dicyclohexylcarbo-diimide (DCC), triethylamine (TEA), and sodium bicarbonate (NaH-CO_3, 0.1 M) were purchased from Sigma-Aldrich (USA). All reagents unless specified were used without further purification.

Synthesis of WS_2 nanosheets

For intercalation, 100 mg WS_2 powder was added into 30 mL n-butyllithium solution under nitrogen atmosphere. After 48 hour stirring, the resulting mixture was filtered and washed using hexane. The obtained sediments were re-suspended in distilled

deionized water (DI-H_2O), subject to 1 h bath sonication. The WS_2 nanosheets were synthesized during sonication and separated from the suspension *via* centrifugation. To remove residual ions, the as-synthesized WS_2 nanosheets were further treated with repeated washing and 24 h dialysis in DI-H_2O.

Preparation of lipoic acid-modified PEG (LA-PEG)

The method for LA-PEG preparation was reported in ref. [1]. In brief, 500 mg mPEG-NH_2 polymer, 45 mg LA, 10 mg DCC, and 6 μL TEA were added into 2 mL dichloromethane with the molar ratio of mPEG-NH_2 and LA kept at 1 : 2. LA-PEG precipitate was yielded as dichloromethane evaporation induced by blowing nitrogen. Such a precipitate was dissolved into 10 mL DI-H_2O, followed by a filtrating treatment. The pH value of collected solution was adjusted to 8.0 by adding sodium bicarbonate (0.1 M). Such a solution was subject to dichloromethane extraction for three times. Final products were dissolved in DI-H_2O and lyophilized after the organic solvent was removed.

PEGylation of WS_2 nanosheets

To achieve this step, 20 mg WS_2 nanosheets and 50 mg LA-PEG were added into 5 mL DI-H_2O, subject to 30 min sonication. After stirring overnight, the PEG decorated WS_2 nanosheets (WS_2-PEG) were prepared and washed clean *via* centrifugation.

Characterization for WS_2-PEG

UV-vis-NIR absorption spectra of the WS_2-PEG were acquired using a microplate reader (Multiskan GO, USA). To study photo-thermal effects of the WS_2-PEG, 1.5 mL PBS solutions containing 0.05, 0.1, and 0.2 mg/mL WS_2-PEG and pure PBS were photo-irradiated for 5 min using 808 nm NIR laser (1 W/cm^2). The thermal images were monitored using a FLIR A5 camera (FLIR Systems Inc., Wilsonville, OR). A BM-IR software was used to calculate the temperature change against irradiation time. To investigate photo-acoustic tomography imaging (PATI) performances of the WS_2-PEG, 200 μL PBS solutions containing 0.05, 0.1, and 0.2 mg/mL WS_2-PEG and 200 μL PBS were added into eppendorf tubes. The PATI signals were captured using Endra Nexus128 (Ann Arbor, MI, USA).

Cytotoxicity and cellular uptake

The 4T1 and HUH-7 cells were cultured with DMEM/high glucose medium containing 10% FBS and 1% antibiotic solution at 37℃ in a 5% CO_2-95% air humidified atmosphere. Following dissociation with pancreatic enzymes, 4T1 and HUH-7 cells were separately seeded into 96-well culture plates at a density of 5000 cells per well. After incubation overnight for cells to adhere, the culture medium was replaced with fresh medium containing 0, 6.3, 12.5, 25, 50, 100, and 200 μg/mL WS_2-PEG. The wells containing 0 μg/mL WS_2-PEG were considered as the control group. After 24 h incubation, cytotoxicity was evaluated using a standard 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay. Optical density (OD) values at 490 nm wavelength were recorded using a microplate reader (Multiskan GO, USA). The relative cell viability can be described as: relative cell viability (%) = (OD value of the WS_2-PEG group / OD of the control group) × 100%. PATI was used to investigate the cellular uptake of WS_2-PEG. In experiments, 4T1 cells and HUH-7 cells went through 1, 2, 4, 8, 12 and 24 h incubation in the culture medium containing 200 μg/mL WS_2-PEG. Collected cells were embedded in 100 μL 1% agarose gel for PATI.

Establishment of tumor model

All animal experiments were conducted in strict accordance with protocols approved by the Animal Care and Use Committee (ACUC) of Xiamen University in China. Female Balb/c (20-22 g) mice and male nude mice (18-20 g) were obtained from Laboratory Animal Center of Xiamen University. 4T1 mouse breast tumor cells (ca. $3x10^6$) and HUH-7 human hepatocellular carcinoma cells (ca. $1x10^6$) were subcutaneously inoculated to the right hind leg of the female Balb/c and male nude mouse, respectively. Tumor volume was monitored using a vernier caliper according to the formula: tumor volume = $A \times B^2/2$, where A and B represent the measured length and width of tumors. In experiments, tumor volume was required to reach ca. 80-90 mm^3.

Dynamic contrast-enhanced MRI (DCE-MRI)

DCE-MRI was performed using 9.4 T small animal MR scanner (Bruker 94/20,

Germany) equipped with a respiratory monitoring system. Before MRI scanning, experimental animals were anesthetized with 2.5% isoflurane/air mixture and placed on a prone position of the MR bed. To allow rapid injection before DCE-MRI scanning, a remaining needle filled with a 100 μL 46.901 mg/mL gadopentetate dimeglumine (Gd-DTPA, Magnevist, a gadolinium-based contrast agent) was inserted into the mouse caudal vein. Coronal and axial two-dimensional fast spin-echo T_2-weighted imaging (TR/TE, 2500/33 ms; Field of view, 4 cm×4 cm; Matrix, 256×256; Slicethickeness, 1 mm; Averages, 2) was used to determine the location and size of the tumor. The DCE scanning was performed using a RARE T_1-weight spin-echo sequence (TR/TE, 960/10 ms; Field of view, 4 cm×4 cm; Matrix, 256×256; Slice thickness, 1 mm; Averages, 1). With a baseline image acquired, the prepared 100 μL Gd-DTPA contrast agents were injected to the mouse in 8-12 seconds *via* a manual manner. Sequential T_1-weighted imaging in the axial plane lasted for 15 min at 46 s intervals.

CD31 immunofluorescence staining

Following the DCE-MRI evaluation, three mice of each group were euthanized with a cervical dislocation for tumor excision. The excised tumors were embedded in optimal cutting temperature (O.C.T.) glue and preserved at -80℃ for subsequent frozen section. In CD31 staining experiments, the tumors were sliced into 4 μm thickness using a frozen microtome (Germany, Leica CM1860) and fixed for 20min in -20℃ acetone. Going through air-drying and PBS washing, the obtained slices were blocked for 30min in 1% bovine serum albumin (BSA) to avoid nonspecific staining, followed by 1 h incubation with PBS-diluted Anti-CD31 antibodies (abcam, ab28364, dilution 1 : 50) in a humidified atmosphere. Subject to PBS washing for three times, the pretreated slices were ready to be stained. The CD31 staining was performed with 1 h incubation of pretreated slices in PBS-diluted FITC-labeled goat anti rabbit secondary antibody (Beyotime, A0562, dilution 1:200), and nuclei counterstaining was carried out with 30-min incubation in 6-diamidino-2-

phenylindole (DAPI, Sigma-Aldrich, USA). All steps for immunofluorescence staining were performed at room temperature. The fluorescence images were captured using an inverted fluorescence microscope (Nikon Ti-U). And, quantitative analysis for CD31-positive areas was performed using an Image J software (Figure S1).

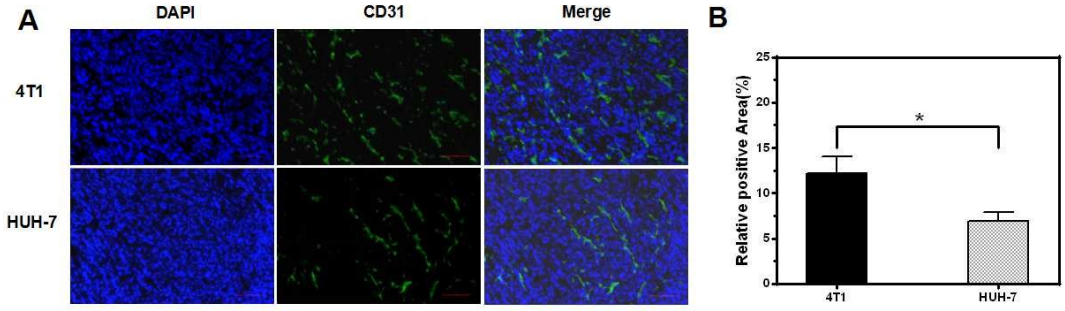

Figure S1. CD31 immunofluorescence staining of 4T1 and HUH-7 tumor tissues. (A) Representative immunofluorescence staining results for CD31 (green) and DAPI (blue). Images were captured using a fluorescence microscope at 200×. (B) Quantitative analysis based on an Image J software for CD31 positive areas. * $P < 0.05$. Scale bars, 100 μm.

Photoacoustic tomography imaging

The *in vitro* PATI for (please added the concentrations) WS_2-PEG was performed at 808 nm excitation in 200 μL eppendorf tubes. And the *in vivo* PATI was carried out in Balb/c and nude mice. Before PATI, tested mice were anesthetized with 2.5% isoflurane/oxygen mixture using a vaporized-isoflurane system. 400 μL 1 mg/mL WS_2-PEG was delivered to a mouse *via* intravenous (i.v.) injection. After 2, 4, 8, 12, and 24 h, the tumor areas were imaged at each time point. For all PATI experiments, optical parametric oscillator (OPO) pumped by a Nd:YAG laser was used as an excitation source for pluses generation. Excited PA waves were detected by 128 ultrasonic transducers. Data acquisition and reconstruction were controlled by a multi-core processor PC. In a continuous model, acquiring one data set needs 12 seconds. Following a previously reported protocol [2], PATI image reconstruction was conducted off-line using data acquired from all 128 transducers based on a back-projection algorithm. The algorithm can correct for pulse-to-pulse variations in the laser intensity and small changes in the temperature that may affect acoustic velocity in the water. The reconstructed raw data were analyzed using OSiriX software. The PATI signal

intensities of tested tumors were measured from the images using a regions-of-interest (ROI) method. The signal intensity-time curves were plotted by calculating mean signal intensity of the whole tumor.

MRI-monitored Photothermal Therapy

In this section, all MR evaluation was conducted on a 9.4 T small animal MR scanner (Bruker 94/20, Germany) using standard big body coils. An 808 nm NIR Laser was employed to irradiate the tumor during real-time MR temperature imaging. The laser power was delivered to the tumor through a 6-cm fiber. After i.v. injection of WS_2-PEG (1 mg/mL, 400 μL/mouse) for 4 h, the tumor-bearing mice were subjected to anesthetization and placed in a stretched prone position of the MR bed. At such a concentration of the WS_2-PEG, the photothermal effect was verified in our previous work [1]. A fast low angle shot (FLASH) MRI sequence was used to acquire data before and during the laser irradiation according to the following settings: TR/TE,150/4.1 ms; field of view, 4 cm×4 cm; Matrix,128×128; Slice thickness, 2 mm. Before the irradiation, T_2-weighted images were captured to determine the location and scope of the tumor. The irradiation treatment lasted for 10 min with the fiber orientation parallel to the main magnetic field B0. The laser power density used 2.0 W/cm^2. After irradiation, temperature distribution maps were constructed by importing the MR data to the MATLAB software (MathWorks, Natick, MA). B0 corrected phase information was acquired from the arctangent of the ratio of the imaginary and real parts of the raw MRI data, and using Goldstein's algorithm [3, 4] to solve the phase wraps dilemma. Then temperature change values for each pixel were computed based on Eq: $\Delta T = \Delta\Phi/\gamma B0\, \alpha\, TE$ [5], where ΔT is the change in temperature; $\Delta\Phi$ is the change in phase; γ [rad/T] is the gyromagnetic ratio, B0 is the MRI magnetic field which is independent of temperature; α is the temperature-dependent water chemical shift in ppm/^{o}C, which is constant within measurement range. TE is the time of echo formation of the field echo pulse sequences; The images were represented using a color-coded lookup tables scheme to show the temperature change.

MRI monitor of therapy responsiveness

To investigate the therapy responsiveness of tumor subject to NIR laser irradiation, T_2-weighted imaging and diffusion-weighted magnetic resonance imaging (DWI-MRI) were carried out at different time-points including 1 d before laser irradiation, 1, 2, 3, 5, 7, and 10 d after laser irradiation. In the whole process of monitoring, all animals were anesthetized with 2.5% isoflurane/O_2 mixture. The sequences for T_2-weighted imaging and DWI-MRI were shown as follows. (1) T_2-weighted imaging: TR/TE=2500/33 ms; Field of view=4×4 cm; Matrix =256×256; Slice thickness=1 mm; (2) DWI-MRI: TR/TE=3000/27 ms; Fieldofview=4×4 cm; Matrix=128×128; Slice thickness=2 mm. The captured T_2-weighted images were used to find the tumor position and to monitor the change of tumor size. And, the DWI-MRI was supposed for early detection of the tumor change in molecular level. ADC maps were reconstructed on a pixel-by-pixel basis through a built-in software (Paravision 5.1, Bruker). The ADC values were measured from ADC maps by drawing ROI on the tumor. The volume of tumor was measured by a caliper tool and calculated as the method mentioned in the "Establishment of tumor model" section.

Hematoxylin-eosin (HE) staining

To further assess the tumor therapy responsiveness, 4T1 tumor-bearing mice (n=3) and HUH-7 tumor-bearing nude mice (n=3) were euthanized with a cervical dislocation after going through 3 day PTT. The tumors were removed from the animals, embedded with O.C.T. glue and placed at the -80℃ refrigerator for preservation. Before staining, the embedded tissue was taken out from the refrigerator and was sliced at the thickness of 20 μm using a frozen microtome (Germany, Leica CM1860). Then the slices were stained according to the conventional frozen section HE staining method shown as follows: Initially, the slides were fixed for 30 s in 4% formaldehyde solution before being stained for 3 min in hematoxylin solution. Subject to treatment with 1% hydrochloric acid alcohol mixture and repeated rinse with DI-H_2O, the slices were stained for 1 min in 1% Eosin staining solution, then washed with DI-H_2O and dehydrated with a gradient of alcohol. The tissue was finally treated

with xylene twice and sealed with a neutral gum. These slices were observed under an inverted fluorescence microscope (Nikon IU). Five fields of each slice were randomly selected for subsequent image acquisition.

Data processing and statistical analysis

Contrast-enhanced MRI data analysis was performed on a ParaVision 5.1 (Germany, Bruke) software. The mean signal intensity (SI) of tumor tissue, normal tissue and background noise were measured by drawing ROIs respectively in the tumor site, adjacent muscles, and the background. The contrast-to-noise ratio (CNR) was used to quantify tumor enhancement over time in the two groups. And it was calculated as fellows: CNR= (SItumor - SImuscle) / SDnoise, where SItumor is the mean signal intensity of the tumor, SImuscle is the mean signal intensity of the muscle, and SDnoise is the standard deviation of the background noise. Experiment results were expressed as Mean ± SD. Statistical analysis was performed using one-way ANOVA, followed by Bonferroni multiple comparison test. $P < 0.05$ was considered statistically significant.

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