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Glutathione-Depleting Nanoplatelets for Enhanced Sonodynamic Cancer Therapy

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MATERIALS AND METHODS

Materials

Cinnamaldehyde (CA) was purchased from Aladdin Reagent (Shanghai, China). IR780 iodide was purchased from Macklin Reagent (China). The MSNs used in this study were obtained from Shanghai Carboxyl Bio-pharmaceutical Technology Co., Ltd. (China). Reactive oxygen species assay kit, MTT Cell Proliferation and Cytotoxicity Assay Kit, 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI) were purchased from Beyotime Company (China). All of the aqueous solutions were prepared using purified deionized (DI) water purified with a purification system (Direct-Q3, Millipore, USA). The other solvents used in this work were purchased from Sinopharm Chemical Reagent (China) and Aladdin-Reagent (China).

Cell culture

4T1 mouse breast cancer cell line cells were obtained from the Cell Bank of the Chinese Academy of Sciences and incubated in RPMI-1640 medium supplemented with 10% FBS in a humidified atmosphere at 37℃ with 5% CO_2.

Animal Models

Female BALB/c aged 4-5 week were purchased from Vital River Company (Beijing, China). 100 ul of 4T1 cell suspension (5×10^6 cells per ml) were subcutaneous injected into each mouse to establish the tumor models.

Preparation and Characterization of PLT-Vesicles (PM)

Platelets (PLTs) from whole blood were isolated through gradient centrifugation. 10 mL mice whole blood was centrifuged at 100 × g for 20 min with no brake. Afterward, the supernatant was centrifuged at 800 × g for 20 min. The PLTs were washed by PBS and centrifuged repeatedly. The preparation processes were monitored using a conventional microscope (IX71, Olympus, Japan). PLT membranes were derived by a repeated freeze–thaw process. Aliquots of PLT suspensions were first frozen at −80 °C, thawed
at room temperature, and pelleted by centrifugation at 4000 × g for 3 min. After three repeated washes with PBS mixed with protease inhibitor tablets, the pelleted PLT membranes were suspended in water, sonicated in a capped glass vial for 5 min using a bath sonicator at a frequency of 53 kHz and a power of 100 W, and then extruded sequentially through 400 and 200 nm polycarbonate porous membranes on a mini extruder (Avanti Polar Lipids, USA).

Preparation of SCI and PSCI

To prepare the CA and IR-780 loaded MSN (SCI), the CA solution (5 mg dispersed in DMSO) and IR780 (5 mg dispersed in DMSO) dropwise added to 10 mL DMSO containing 10 mg MSN slowly, and stirred at room temperature overnight to reach the equilibrium state. The resulting solution was then centrifuged at 8000 rpm for 10 min, and washed with DMSO and distilled water to remove the physically adsorbed CA and

IR-780. The mixture of SCI and PM was sonicated for 0.5h. Thereafter, a polycarbonate membrane containing 200 nm pore size was used for extruding the mixture 10 times using an extruder. The PSI was prepared by the same method.

Characterization of the PSCI nanoparticles

The morphology structures of MSNs, MS and PMS nanoparticles were observed by the TEM (JEOL-2100). Uv-vis spectra of different samples were recorded by the Uv-vis spectrophotometry Lambda 35 (Perkin-Elmer). Hydrodynamic diameter and zeta potential were detected by the dynamic light scattering (Nano-ZS ZEN3600). Drug loading efficiency (DLE) = (weight of loaded drug/weight of feeding drug) × 100%.[1]

Western blotting for the key proteins in PM and PSCI

The total cellular protein in PM and PSCI were extracted using a protein extraction kit (Dingguo, China). The extracted proteins were separated using SDS-PAGE electrophoresis. After electrophoresis, the gel was treated with Coomassie blue staining. Extraction of protein for western blot was performed as described above. The proteins were then transferred onto polyvinylidene fluoride (PVDF) membranes (Bio-Rad). This was followed by a blocking step for 1 h with 5% skim milk, and then the membrane was incubated with the primary antibody against P-selectin (Proteintech) overnight at 4 °C using Na,K-ATPase as the control. Finally, the membrane was incubated with the secondary antibody for 1 h at room temperature.[2]

Drug release studies

1mg of PSCI were dispersed in 1ml PBS solution. The solution was exposed to ultrasound irradiation (US power density = 0.75 W/cm^2, transducer frequency = 1 MHz, 30% duty cycle) for 5min. At the given time points, the released CA was measured by Uv-vis spectra. No ultrasound irradiation was used as a control group.

***In vitro* cancer targeting study**

At first, 100μg/mL SCI and PSCI were incubated with 4T1 cells for 2 h at 37 °C. The cells were then washed with PBS several times, fixed with PFA for 30 min at room temperature, stained with Lyso-Tracker and then imaged by using a confocal laser scanning microscope (CLSM; IX81, Olympus, Japan). The fluorescence intensity of IR780 was measured by ImageJ software.

Extracellular GSH Depletion

A GSH and DTNB solution with the final concentration of 0.1 mM GSH and 0.2 mg/mL DTNB was prepared and maintained at 25 °C. The SCI, PSI and PSCI solutions (equivalent MSNs concentration: 200 μg/mL) were added to the above solution and reacted for 30 min. Every 5 min, the reaction solution was centrifuged at 10000 rpm to precipitate FM, CM and CFM, and UV−vis spectroscopy was applied to detect the absorbance of the supernatant.

***In vitro* anti-cancer effect of PSCI**

The anti-tumor effect was measured by MTT assay. 4T1 cells were seeded in 96-well plates at a density of 5 × 10^3 cells per well and incubated for 12 h under normoxia condition. Afterwards, cells were incubated for 6 different groups: (1) PBS; (2) US; (3) PSCI; (4) US+SCI; (5) US+PSI and (6) US+PSCI. The MSNs concentration was 200 μg/mL in group 3, 4, 5 and 6. Then, cells in group 2, 4, 5 and 6 were exposed to US radiation (US power density = 0.75 W/cm^2, transducer frequency = 1 MHz, 30% duty

cycle) for 5 min. At the end of the incubation, 5 mg/mL MTT PBS solution was added, and the plate was incubated for another 4 h. Finally, the absorbance values of the cells were determined by using a microplate reader (Emax Precision, USA) at 570 nm. The background absorbance of the well plate was measured and subtracted. The cytotoxicity was calculated by dividing the optical density (OD) values of treated groups (T) by the OD values of the control (C) (T/C × 100%).

***In vivo* pharmacokinetics, and distribution study**

4T1 tumor bearing BALB/c mice (n = 3) received an intravenous (i.v.) injection of 100 μL PBS containing SCI or PSCI (with equivalent MSNs dose of 10 mg/kg). At various time points after the injection (i.e., 1, 2, 4, 6, 12, and 24 h), 20 μL blood plasma was collected from the tail veins, and then centrifugation at 10000 rpm for 10 min. Finally the supernatants were collected and IR780 concentration was quantitatively analyzed by Uv-vis spectra.

4T1 tumor bearing BALB/c mice (n = 3) received an intravenous (i.v.) injection of 100 μL PBS containing SCI or PSCI (with equivalent MSNs dose of 10 mg/kg). All of the mice were euthanized at 12h, and then we collected their major organs to determine the biological distribution of the particles. As mentioned above, the IR780 content was measured using Uv-vis spectra.

Evaluation of intratumoral oxidative stress

When tumor size of BALB/c mice reached approximately 200 mm^3, the mice were divided randomly into 6 groups (each group included 3 mice): (1) PBS; (2) US; (3) PSCI; (4) US+SCI; (5) US+PSI; and (6) US+PSCI. The MSNs dose was 10 mg/kg in group 3, 4, 5 and 6. Then the fluorescent dye, DCFH-DA (10 μmol/L, 50 μL) was injected intratumorally 12 h after intravenous injection in all groups. Next, US (power density = 0.75 W/cm^2, transducer frequency = 1 MHz, 30% duty cycle, 10min) was performed in group 2, 4, 5 and 6. Subsequently, tumors from each group were dissected. The cryosections were observed by a confocal laser scanning microscope (CLSM; IX81, Olympus, Japan).

***In vivo* antitumor study**

When tumor size of BALB/c mice reached approximately 200 mm^3, the mice were divided randomly into 6 groups (each group included 5 mice): (1) PBS; (2) US; (3) PSCI; (4) US+SCI; (5) US+PSI; and (6) US+PSCI. (equivalent MSNs concentration 10 mg/kg). The US (power density = 0.75 W/cm2, transducer frequency = 1 MHz, 30% duty cycle, 10min) was performed 12 h after intravenous injection. The treatment was conducted every 2 days for 14 days. Mice body weight and tumor volume in all groups were monitored every 2 days. A caliper was employed to measure the tumor length and tumor width and the tumor volume was calculated according to following formula. Tumor volume = tumor length × tumor width ^{2} / 2. After 14 days treatment, all the mice were sacrificed. the blood samples from these mice (≈1 mL) were collected for blood biochemistry analysis. Five main organs (heart, liver, spleen, lung and kidney) and tumors of all mice were harvested, washed with PBS, and fixed with paraformaldehyde for histology analysis. And the tumor tissues were weighed, and fixed in 4% neutral buffered formalin, processed routinely into paraffin, and sectioned at 4 μm. Then the sections were stained with hematoxylin and eosin (H&E) and finally examined by using

an optical microscope (BX51, Olympus, Japan).

Statistical analysis

Data analyses were conducted using the GraphPad Prism 5.0 software. Significance between every two groups was calculated by the Student's t-test. *P < 0.01, **P < 0.005, ***P < 0.001.

Supplementary figures

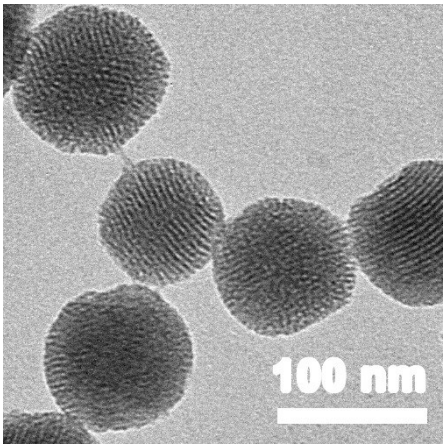

Figure S1. TEM image of MSNs.

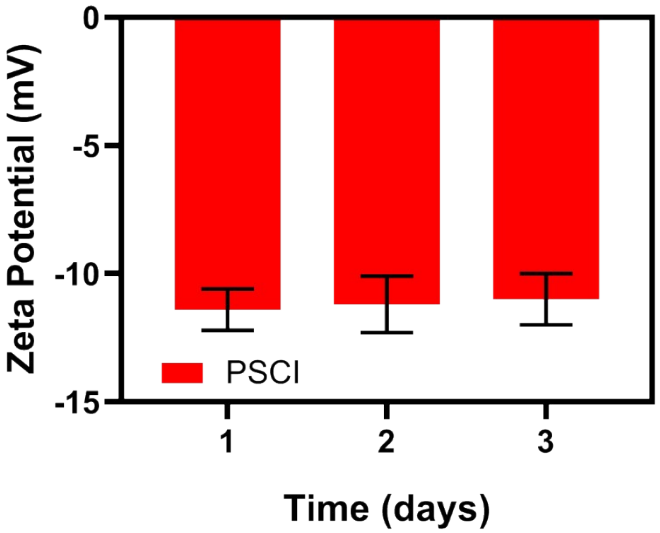

Figure S2. The zeta potential of PSCI suspended in PBS was assessed after 1, 2, and 3 days.

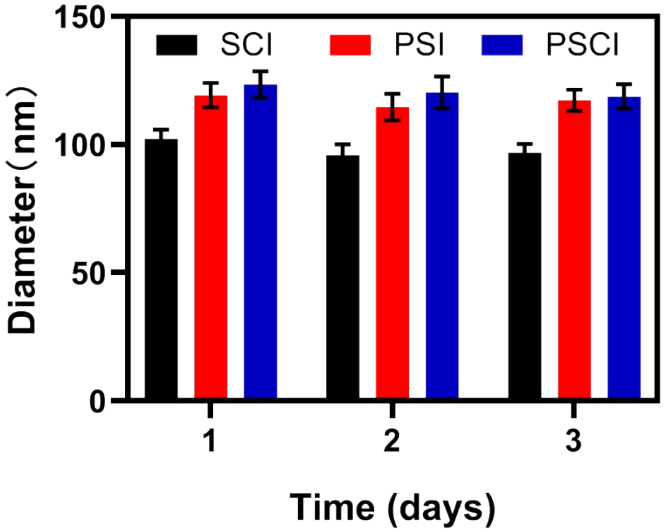

Figure S3. DLS was used to measure the hydrodynamic diameter of SCI, PSI and PSCI.

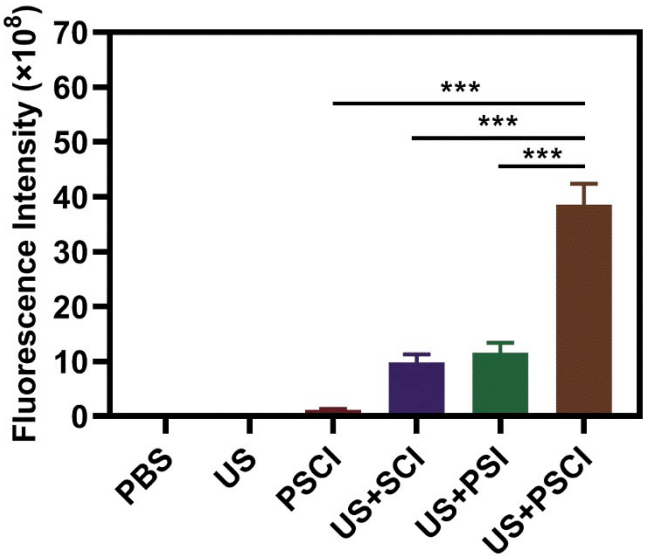

Figure S4. Average fluorescence intensity of DCFH-DA probe after different treatment.

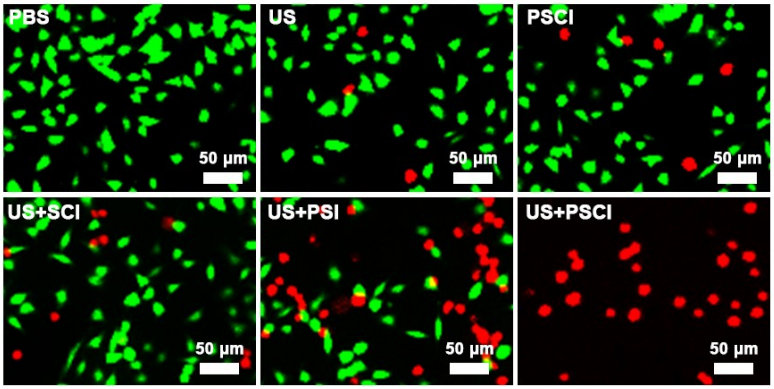

Figure S5. Fluorescence images of 4T1 cells stained with FDA (live cells, green fluorescence) and PI (dead cells, red fluorescence) after incubation with different formulations.

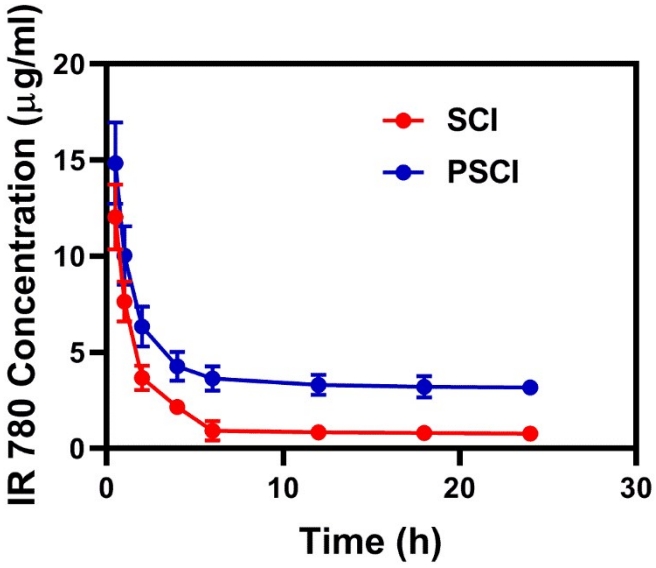

Figure S6. The pharmacokinetic behaviors of SCI and PSCI in mice after i.v. administration at MSNs dose of 10 mg/kg. Data were presented as mean ± SD (n = 3)

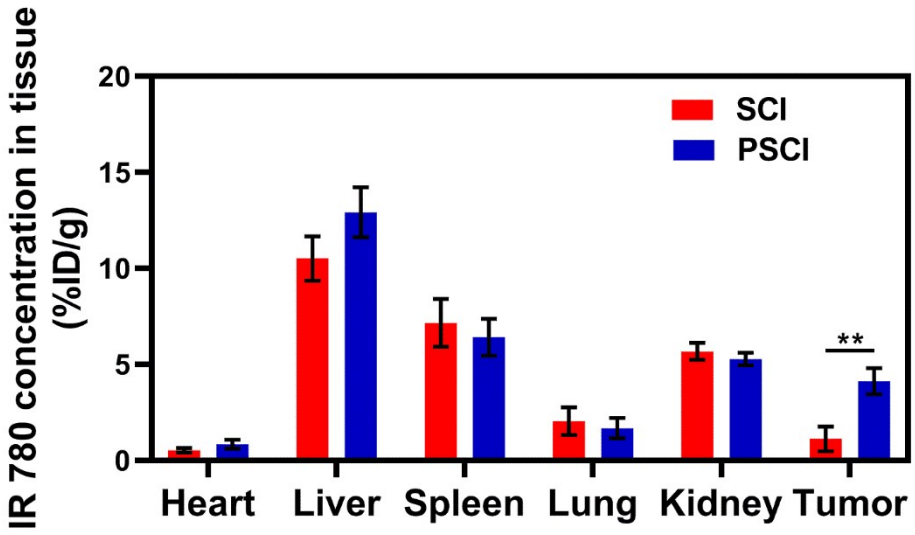

Figure S7. Quantitative analysis of Si biodistribution in tissues and tumors of tumor-bearing mice injected with SCI and PSCI at MSNs dose of 10 mg/kg.

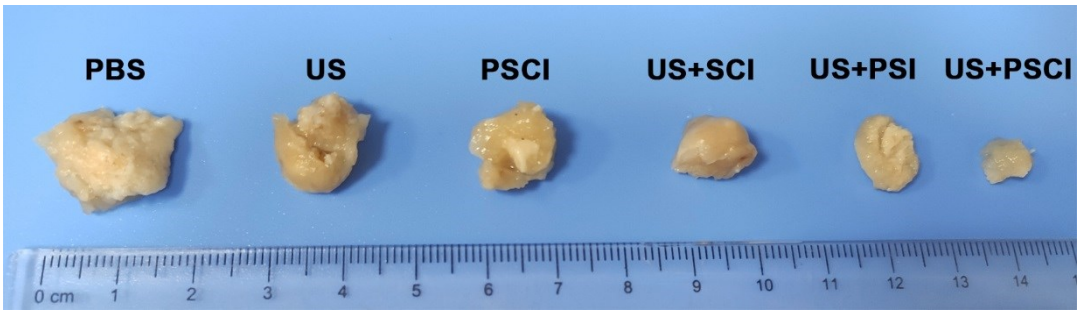

Figure S8. Images of lymph node at 20 d post-administration PBS, US, PSCI, US + SCI, US + PSI and US + PSCI treatment for 5 times.

1. D. Zhu, Y. Duo, S. Meng, Y. Zhao, L. Xia, Z. Zheng, Y. Li and B. Z. Tang, *Angewandte Chemie*, 2020, **59, 2-10**.

2. H. Ye, K. Wang, M. Wang, R. Liu, H. Song, N. Li, Q. Lu, W. Zhang, Y. Du, W. Yang, L. Zhong, Y. Wang, B. Yu, H. Wang, Q. Kan, H. Zhang, Y. Wang, Z. He and J. Sun, *Biomaterials*, 2019, **206**, 1-12.