Supporting Information

Synthesis of Monodispersed Single-Crystalline Mesoporous TiO_2 as Sonosensitizers for Efficient Sonodynamic Therapy of Tumor

Xi Wang,[1,2] Wenping Wang,[1,] Luodan Yu,[3] Yang Tang,[2] Jiaying Cao[1] and Yu Chen[3,]**

[*] Prof. W. Wang, Dr. Y. Chen, Corresponding-Author.
[1]Zhongshan Hospital Fudan University, Department of ultrasound, Shanghai, 200032, P. R. China.

[2]Shanghai Institute of Medical Imaging, Shanghai, 200032, P. R. China.

[3]State Key Laboratory of High Performance Ceramics and Superfine Microstructure, Shanghai Institute of Ceramics, Chinese Academy of Sciences, Shanghai, 200050, P. R. China.

A: Experimental Section

1.1 Materials

Tetrabutyl titanate (TBT), 2'-7'-dichloroufluorescein diacetate (DCFH-DA) and acetic acid (HAc) were purchased from Sigma-Aldrich Co. Ltd. Amino-polyethylene glycol (NH_2-PEG_{5000}) was obtained from Jemkem Technology Co. Ltd. Ethanol and dimethyl sulfoxide (DMSO) were bought from Sinopharm Chemical Reagent Co. Phosphate buffer solution (PBS) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Shanghai Ruicheng Bio-Tech Co. Ltd. The Annexin V-FITC/PI apoptosis detection kit was purchased from Beyotime Co., Ltd.. These chemical agents were directly used without further purification steps. Deionized water was used in all experiments for material synthesis.

1.2 Measurements and characterization

Transmission electron microscopy (TEM) images were recorded on a JEM-2100F electron microscope operated at 200 kV. X-ray diffraction patterns were recorded on a Rigaku D/Max-2200 PC X-ray diffractometer with Cu target (40 kV, 40mA). N_2 adsorption-desorption isotherm and corresponding pore size distribution were acquired on a Micrometitics Tristar 3000 system. Dynamic light scattering (DLS) of aqueous SC-MTNs were assessed on a Zetasizer Nanoseries (Nano ZS90). Raman spectrum was recorded on a Thermofisher spectrometer (DXR) with the excitation wavelength of 633 nm. Confocal laser scanning microscopy (CLSM) images were acquired on FV1000, Olympus Co.

1.3 Synthesis of single-crystalline mesoporous TiO_2 nano-crystals (MTNs)

Ellipsoidal MTNs were synthesized by a PH-ST soft-templating strategy. Typically, tetrabutyl titanate (TBT, 1 mL) was dropped into acetic acid (HAc, 15 mL) at room temperature. Then, deionized water (0.5 mL) was quickly added to initiate the hydrolysis of TBT. The mixture was stirred at room temperature and the reaction was

lasted for 10 min, which was then transferred into Teflon-lined stainless-steel autoclave with a capacity of 50 mL. The autoclave was put into an oven and heated at 150 ℃ for 12 h. The formed MTNs were obtained by centrifugation (13000 rpm, 10min) after cooling the autoclave to room temperature. Finally, the MTNs were washed thoroughly with ethanol and deionized water for three times. The obtained MTNs were re-dispersed into deionized water for further use. To investigate the formation mechanism, MTNs were collected after 2 h hydrothermal treatment. Other procedures were the same as aforementioned process.

1.4 PEGylation of MTNs

PEGylation process was used to improve the colloidal stability of MTNs in physiological condition. Typically, the as-synthesized MTNs solution (5mL) was slowly added into the PEG_{5000}-NH_2 aqueous solution (0.5 mg mL^{-1}, 100 mL) under magnetic stirring for 48 hours. The PEG-MTNs were obtained by centrifugation (13000 rpm, 10min) and were washed thoroughly within deionized water for several times with the assistance of ultrasound treatment. Finally, the PEG-MTNs were re-dispersed into deionized water (5 mL) and stored at 4 ℃ for further use.

1.5 Cell culture

Human hepatoma HepG2 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; GIBCO^{TM}, NY, USA) supplemented with 10% fetal bovine serum (FBS; GIBCO^{TM}), 100 U mL^{-1} of penicillin and 100 μg mL^{-1} of streptomycin (SIGMA$^{®}$, MO, USA). The cells were maintained at 37 ℃ in an atmosphere of 5% CO_2.

1.6 Bio-TEM observation of cell endocytosis

For determining the intracellular uptake and location, Bio-TEM was conducted after the co-incubation of HepG2 cells with PEG-MTNs. PEG-MTNs were initially dispersed into culture medium at the concentration of 100 μg mL^{-1}. The culture medium of HepG2 cells was replaced with PEG-MTNs-containing culture medium, which was subsequently co-incubated for 12 h. After washing by PBS for three times,

HepG2 cells were then detached by 0.25% trypsin and collected by centrifugation at 3000 rpm for 2 min. The cells were fixed in 2.5% glutaraldehyde, rinsed with propylene oxide, which was then embedded in EPOMB12. The cells were then polymerized in the oven at 37 ℃ for 12 h, 45 ℃ for 12 h and 60 ℃ for 48 h. Sections of approximately 70 nm were cut with a Leica UC6 ultra microtome and were moved to a copper grid. The TEM images were obtained on a JEM-1230 electron microscopy.

1.7 Cytotoxicity investigation

Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method to evaluate the cytotoxicity of as-synthesized PEG-MTNs. HepG2 cells were seeded into 96-well plates with the density of 5×10^3 cells/well suspended in 100 μL culture medium. After a 24 h incubation, the medium was substituted with fresh culture medium containing PEG-MTNs at different concentrations (25, 50, 100, 200, 400 μg mL^{-1}). The cells were then incubated for another 24 h, the culture medium was replaced with the MTT culture medium solution at the concentration of 0.8 mg mL^{-1} (100 μL per well). The MTT solution was dropped after 4 h incubation. Then, dimethyl sulfoxide (DMSO) was added with the volume of 100 μL per well. The absorbance at the wavelength of 490 nm of each well was acquired using a microplate reader(Bio-TekELx800). The cytotoxicity was expressed as the percentage of cell viability compared to control cell group without PEG-MTNs.

1.8 *In vitro* MTNs-sonosensitized sonodynamic therapy against HepG2 cells

To evaluate the *in vitro* MTNs-sonosensitized SDT effect, HepG2 cells were initially seeded into 96-well plates with 5×10^3 cells per well and incubated for 24 hours, allowing cells to be attached into the well. The cells were then incubated with culture medium containing PEG-MTNs at different concentrations (0, 25, 50, 100, 200, 400 μg mL^{-1}) at 37 ℃. After 8 h co-incubation, the cells were treated with ultrasound at different intensities (1.0 and 1.5 W cm^{-2}) for 0 min (as the control), 30 s,

60 s, and 90 s, respectively. Then, the cells were further incubated for 12 h. Cell viability in the presence of PEG-MTNs in conjunction with ultrasound was detected by the standard MTT protocol.

1.9 Flow cytometry analysis

HepG2 cells were divided into four groups: control group, PEG-MTNs group, US group and PEG-MTNs + US group. After the incubation with culture medium (control group and US group), culture medium containing PEG-MTNs at the concentration of 100 μg mL^{-1} (PEG-MTNs group and PEG-MTNs + US group) at 37℃ for 12 h. Then, the cells in US group and PEG-MTNs + US group were irradiated under ultrasound for 1 min at the intensity of 1.5 W cm^{-2}, which were then incubated for another 6 h. Apoptosis and necrosis of HepG2 cells were analyzed using a standard Annexin V-FITC apoptosis kit by flow cytometry.

1.10 Detection of the generation of intracellular reactive oxygen species (ROS)

The intracellular ROS generation by MTNs-sonosensitized SDT was determined *via* the probe 2,7-dichlorofluorescein (DCF). After incubation with PEG-MTNs (100 μg mL^{-1}) in PEG-MTNs group and PEG-MTNs + US group for 4 h, the cells were then gently washed with PBS twice. All four groups were then incubated with 10 μM 2'-7'-dichloroufluorescein diacetate (DCFH-DA) for 20 min. Then US group and PEG-MTNs group were treated with US irradiation for 30 s at the power intensity of 1.5 W cm^{-2}. The intracellular fluorescence images were taken on CLSM, FV1000, Olympus Co.

1.11 *In vivo* tumor xenograft

Balb/c mice (about six weeks old, female) were purchased from the Shanghai Laboratory Animal Center. The studies were conducted under protocols approved by the Shanghai Medical College, Laboratory Animal Center of Fudan University. The HepG2 xenografts were generated by subcutaneous injection of 5×10^6 cells in unilateral shoulder.

1.12 *In vivo* sonodynamic therapy sonosensitized by PEG-MTNs

After the establishment of tumor xenograft, the HepG2 tumor-bearing mice were divided into four groups (n = 5 per group) when the average diameter of tumor reached approximately 8 - 12 mm (about two weeks) to receive normal saline (100 μL), saline (100 μL) + US, PEG-MTNs saline solution (100 μL, 20 mg kg^{-1}) or PEG-MTNs saline solution (100 μL, 20 mg kg^{-1}) + US. After tail vein injection for 24 h, the tumors were irradiated by ultrasound at the power intensity of 1.5 W cm^{-2} for 90 s. The treatment was conducted every 4 days and repeated 3 times. A caliper was used to measure the tumor sizes every other day and calculated according to the following formula: tumor volume = (longest tumor diameter) × $(\text{shortest tumor diameter})^2$ / 2. After 14 days, the mice were sacrificed, and the tumor tissue was excised and weighed. Part of tumor was fixed in buffered formalin for further histological and immunohistochemical TUNEL analysis.

1.13 Coagulation evaluation

Human fresh blood plasma was kindly donated from Shanghai Blood Center, which (450 μL) was further mixed with PEG-MTNs PBS solution (50 μL) at elevated concentrations (25, 50, 100, 200, 300, 500 μg mL^{-1}). The free plasma without the co-incubation with PEG-MTNs was used as the control group for comparison. The mixture was mildly votexed and then left to stand for 5 min at room temperature. After the centrifugation (5000 rpm, 5 min), the supernatants were collected and tested on a ACL™ 7000 blood coagulation analyzer by using HemosIL™ kit (Instrumentation Laboratory Company, Lexingtion, MA 02421-325, USA). Calcium chloride, SynthASil and PT-Fibrinogen HS Plus were put into the corresponding reagent tanks based on the manufacture's guidance. Finally, calibration plasma and the tested samples after co-incubation with PEG-MTNs were then put into the sample tanks. PT, APTT, FIB were evaluated for coagulation assay. Each test was performed in triplicate.

1.14 Biocompatibility assay of PEG-MTNs by a single-dose intravenous administration

The KM mice (four to six-weeks old, female) were obtained from Shanghai Laboratory Animal Center. The toxicity evaluation of PEG-MTNs was conducted under protocols approved by the Shanghai Medical College, Laboratory Animal Center of Fudan University. A series doses (10, 50, 150 mg kg^{-1}, n = 5 per group) of PEG-MTNs suspension in saline were injected through tail vein. Intravenous administration of saline was set as control group. Mortality was recorded and clinic manifestation was observed. Blood samples were collected for testing after 2 weeks feeding.

1.15 Biocompatibility assay of PEG-MTNs by a repeated-dose intravenous administration

For repeated-dose toxicity evaluation, KM mice received different levels of PEG-MTNs suspension in saline (10, 50 and 100 mg kg^{-1}, n = 5 per group) *via* intravenous injection every three days for 4 times (total doses: 40, 200 and 400 mg kg^{-1}). Tail vein injection of sterile saline was set the control group. Mortality and behavior setting were recorded. All animals were sent for blood test and histocompatibility assay 2 weeks after the last intravenous administration.

1.16 Hematology analysis and blood biochemical assay

Blood samples of animals in single-dose and repeated-dose groups were collected using potassium EDTA tubes for hematology analysis through saphenous vein. The standard hematology parameters selected for analysis were as follows: red blood cell count (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet count (PLT), mean platelet volume (MPV), platelet distribution width (PDW) and white blood cell count (WBC). Blood samples collected *via* the ocular vein were centrifuged to separate the serum. In this study, liver function was determined with serum levels of alanine aminotransferase (ALT), aspartate

aminotransferase (AST) and alkaline phosphatase (ALP). Nephrotoxicity was evaluated by blood urea nitrogen (BUN) and creatinine (CREA). These parameters were all obtained using a Biochemical Autoanalyzer (Type 7170, Hitachi, Japan).

1.17 Histopathological examinations

At the end of the single-dose and repeated-dose toxicity feeding, the mice were sacrificed and the major organs including heart, lung, liver, spleen and kidney were excised for histopathological analysis using the typical hematoxylin and eosin (H&E) staining assay. Histological results of tumors in the *in vivo* sonodynamic therapy were observed using the typical H&E staining and a terminal deoxynucleotidyl transferasemediated dUTP nick-end labeling (TUNEL) assay kit (Roche) according to the manufacturer's instructions.

B: Supplementary figures

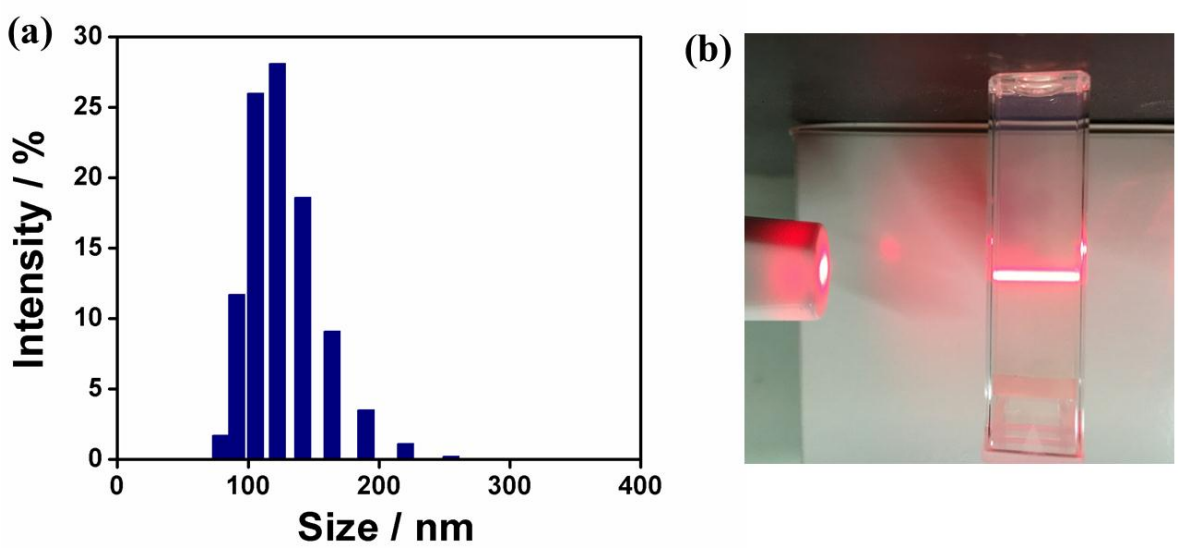

Figure S1. (a) Dynamic light scattering (DLS) of PEG-MTNs in aqueous solution and (b) the corresponding photographic image of PEG-MTNs in aqueous solution, showing the high dispersity as the presence of Tyndall effect.

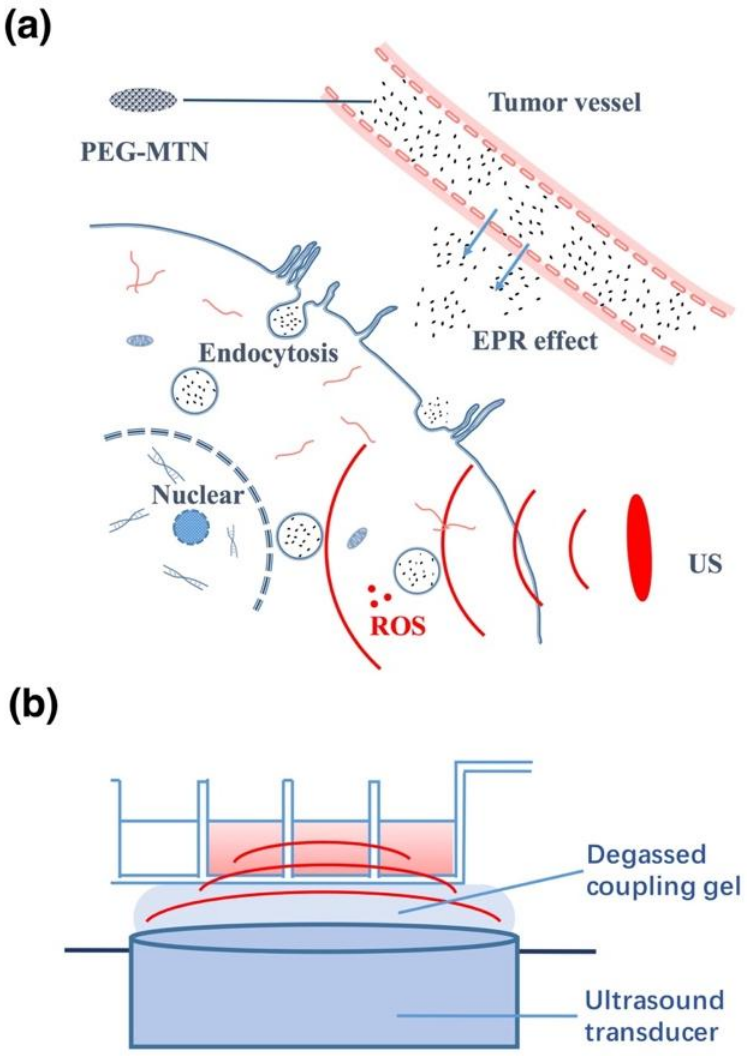

Figure S2. (a) Schematic diagram illustrating PEG-MTNs in intracellular sonodynamic therapy. (b) The scheme of ultrasound-irradiated SDT procedure during the cell experiment.

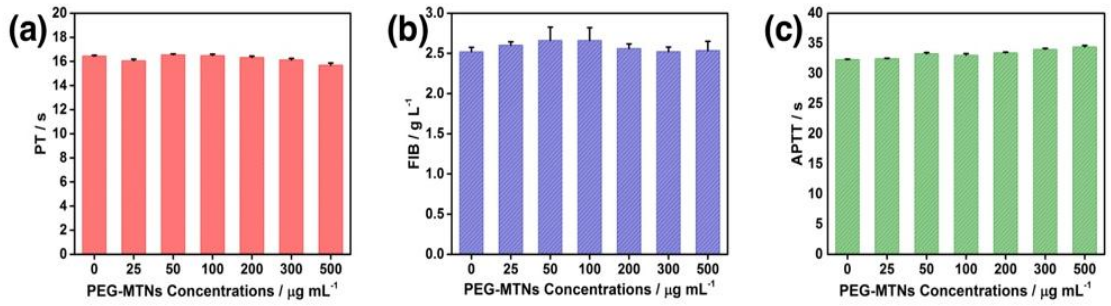

Figure S3. (a) PT, (b) FIB and (c) APTT values of the blood plasma after exposure to PEG-MTNs of different concentrations, showing the low coagulation effect of PEGylated MTNs (PET-MTNs).

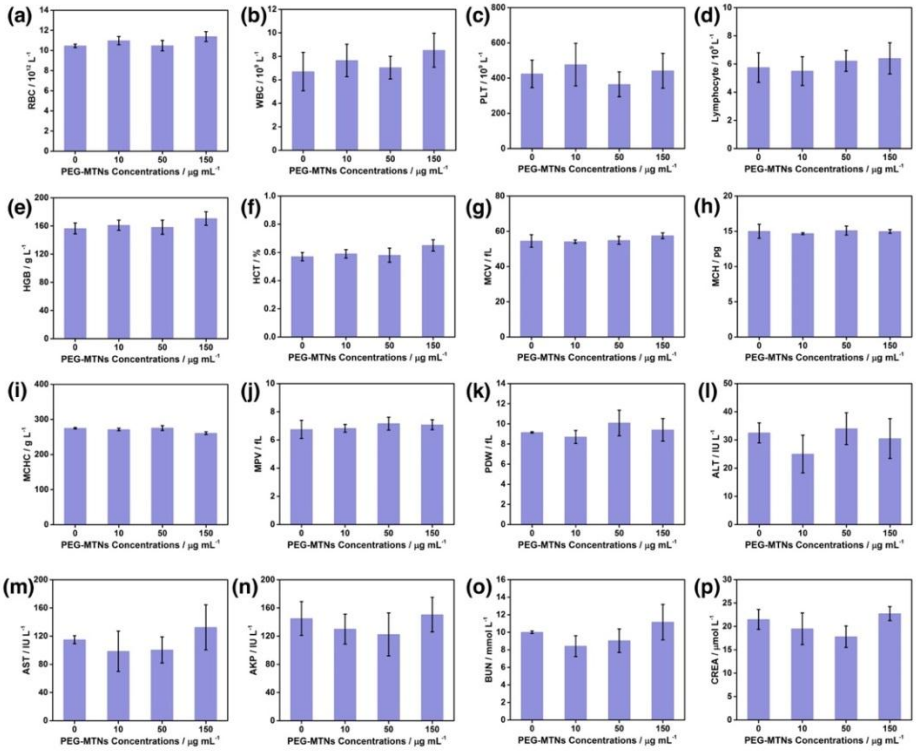

Figure S4. Hematology and blood biochemical changes after single-dose administration of PEG-MTNs. Selected hematology markers (RBC, HGB, HCT, MCV, MCH, MCHC, PLT, MPV, PDW and WBC) and blood biochemical parameters (ALT, AST, ALP, BUN and CREA) were assayed after single-dose

intravenous injection of PEG-MTNs at 10, 50, 150 mg kg^{-1}. No statistically significant changes were observed between groups (n = 5).

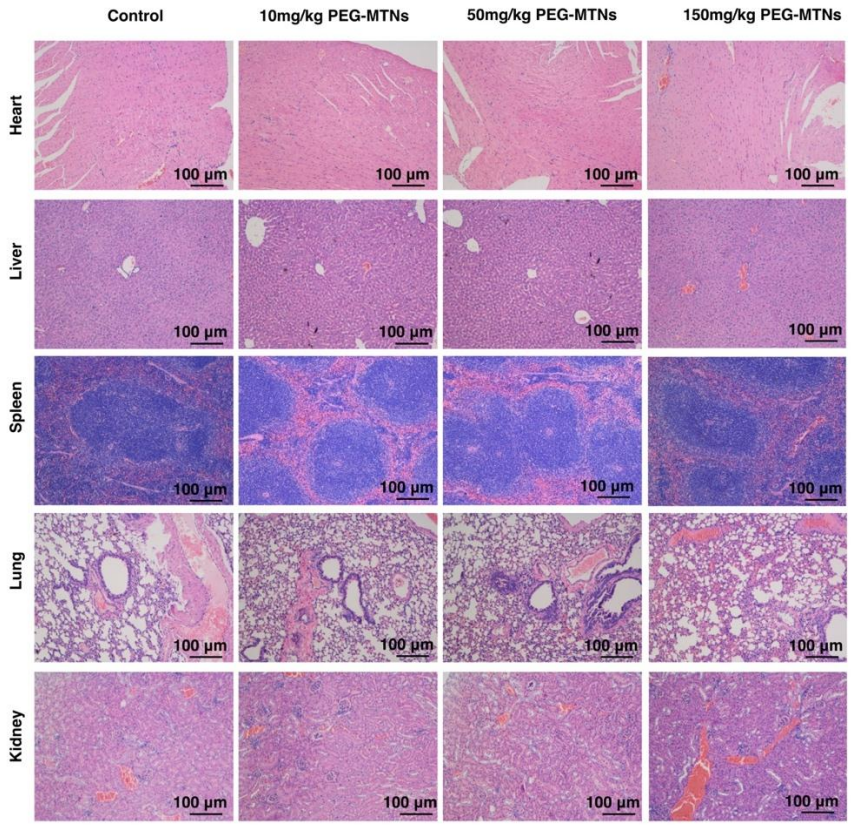

Figure S5. Histological analyses of main organs after single-dose administration of PEG-MTNs. The heart, liver, spleen, lung and kidney were observed using H&E staining after single-dose intravenous injection of PEG-MTNs at 10, 50, 150 mg kg^{-1}. Data are representative of all mice at the same dose group.