Supplemental Material

A Novel Combination Strategy of High Intensity Focused Ultrasound (HIFU) and Checkpoint Blockade Boosted by Bio-inspired and Oxygen-supplied Nanoprobe for Multimodal Imaging-Guided Cancer Therapy

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METHODS

Detailed synthesis process of M@P-SOP

Firstly, the nanoprobe encapsulating SPIO and PFH (P-SP) was fabricated by using a typical double-emulsion process. Briefly, 50 μL SPIO was added into 2 mL of PLGA (50 mg) dissolved in dichloromethane, and 200 μL PFH was added. Next, the mixture was emulsified by using an ultrasonic probe (Sonics & Materials, Inc., USA) at 60 W for 3 min. The emulsion was then poured into 8 mL of 4% w/v poly (vinyl alcohol) solution and emulsified for 3 min at the same power. Afterward, 10 mL of 2% v/v of isopropyl alcohol solution was added to the as-prepared emulsion, and which was magnetically stirred for 4 h to evaporate organic solvent. Finally, the P-SP were collected after centrifugation at 10000 rpm for 8 min (4℃).

Secondly, 4T1 cell membranes were extracted using a membrane protein extraction kit (Beyotime Biotechnology, China) according to the instructions. Briefly, routinely cultured 4T1 cells were collected by a cell scraper and centrifuged at 3000 rpm for 5 min. Then the cell pellets were resuspended in membrane protein extraction solution (3 mL) added with phenylmethanesulfonyl fluoride (PMSF, 1×10^{-3} M) and incubated in an ice bath for 15 min. The 4T1 cell suspension was frozen-thawed 3 times repeatedly, then centrifuged at 2000 rpm for 10 min (4 ℃). The supernatant was further centrifuged at 14000 rpm for 30 min (4 ℃). Finally, the collected CCM (precipitate) was lyophilized for further use. Lastly, P-SP and CCM were fully mixed and being saturated with oxygen as illustrated in **Preparation and characterization of M@P-SOP** section (Main document) to obtain the final M@P-SOP.

In vivo therapeutic effect and antitumor immunity of M@P-SOP plus HIFU

Unilateral 4T1 tumor-bearing (about 0.6-0.8 cm in diameter) mice were randomized into 6 groups: Control, M@P-SP, M@P-SOP, HIFU, HIFU+M@P-SP, HIFU+M@P-SOP. Then the mice were

intravenously injected with 200 μL of saline, M@P-SP or M@P-SOP (5 mg/ml) with or without HIFU irradiation (120 W, 3 s) 24 h post injection. The tumor weights were measured on day 15. On day 3 post treatment, with another batch of mice, the immunofluorescence was used to determine Calreticulin (CRT) expression and $CD8^+$ T cells in tumor tissues. And Tumor-draining lymph nodes and spleen were collected to assess the mature DCs (defined as $CD11c^+$ $CD80^+$ $CD86^+$) by flow cytometry.

***In vitro* and *in vivo* toxicity of M@P-SOP**

4T1 cells (5×10^3) were seeded into 96-well plates and incubated overnight, then were treated with P-SOP or M@P-SOP for 24 h. 10 μL of cell counting kit-8 (CCK-8) was added and incubated for 1-4 h. The cell viability was tested by a microplate reader at 450 nm. Kunming mice were randomized into control and experimental groups (n=3). Control mice were sacrificed at 28d after saline injection, and experimental mice sacrificed at 1, 3, 5, 7, 14 or 28 d after M@P-SOP (5 mg/mL, 200 μL) injection. The major organs were collected for hematoxylin and eosin (H&E) staining. Blood samples were collected from orbit for blood examination and biochemical assay.

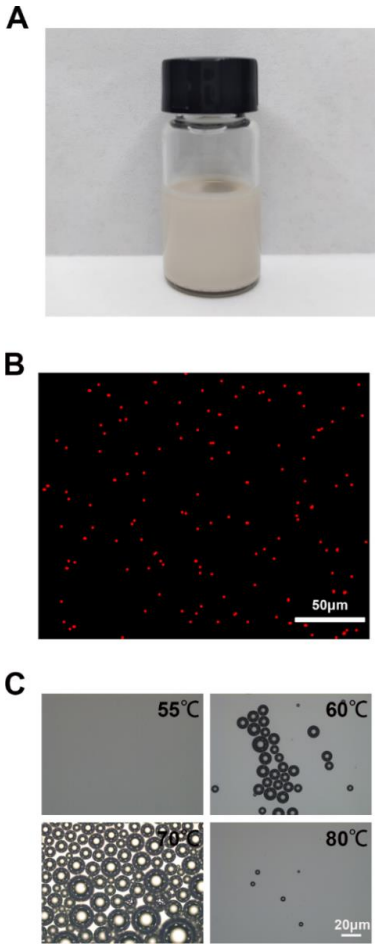

Figure S1 Characterization of M@P-SOP nanoparticles. (A) The digital photo of M@P-SOP dispersed in saline. (B) CLSM image of DiI-labeled M@P-SOP. Scale bars: 50 μm. (C) Light-microscopy images of M@P-SOP at different temperatures. Scale bar: 20 μm.

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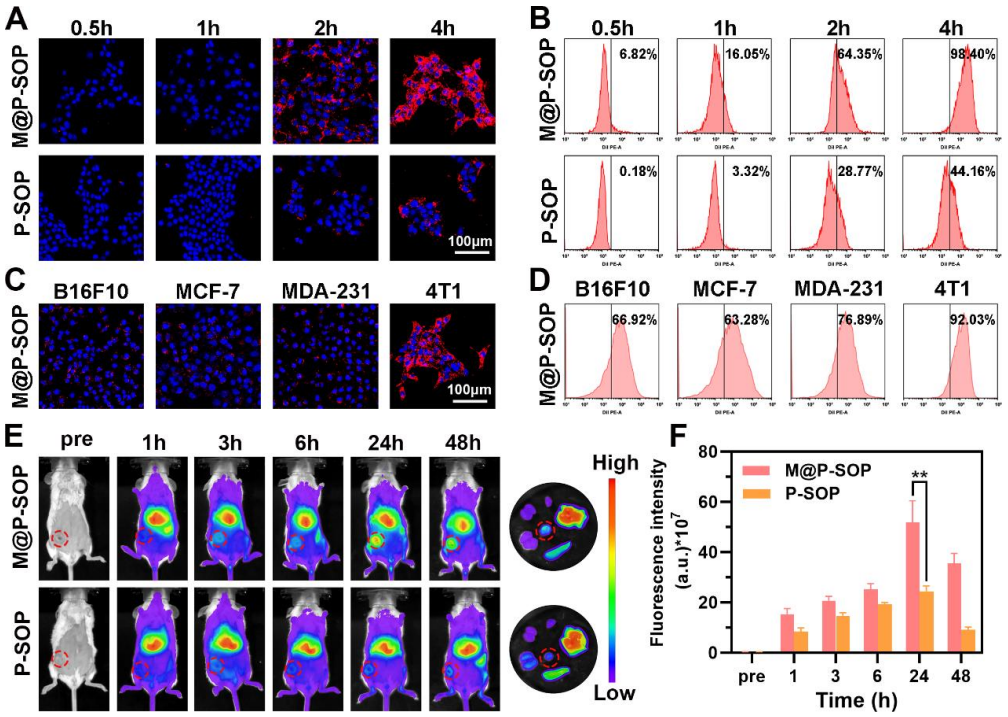

Figure S2 *In vitro* and *in vivo* behaviors of nanoparticles. (A) Confocal fluorescence images and (B) flow cytometry analyses of 4T1 cells after incubation with DiI-labeled P-SOP and M@P-SOP for 0.5, 1, 2, 4 h, respectively. (C) Confocal images and (D) flow cytometry analyses of B16F10, MCF-7, MDA-231 and 4T1 cells after 4 h incubation with DiI-labeled M@P-SOP. Cell nuclei was labeled by DAPI. All scale bars were 100 μm. (E) *In vivo* fluorescence imaging of mice at pre, 1, 3, 6, 24 and 48 h after intravenous injection of DiR-labeled P-SOP and M@P-SOP. Tumors of mice were highlighted by the dotted circles. (F) The quantitative fluorescence intensity analysis of E (n=3, t-test, $^{**}P < 0.01$).

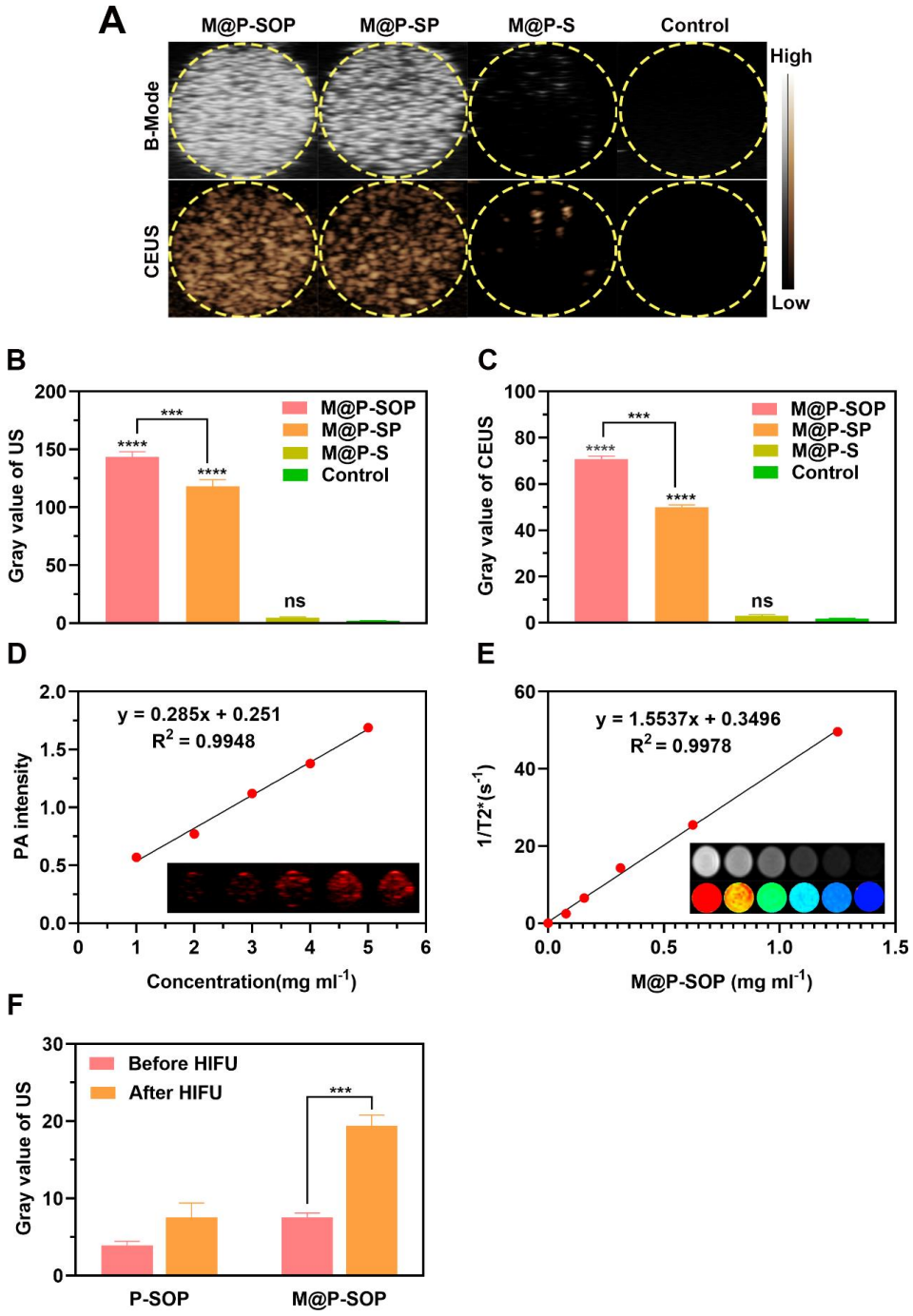

Figure S3 The multimodal imaging of nanoparticles *in vitro* and *in vivo*. (A) *In vitro* B-mode and CEUS ultrasound imaging of PBS, M@P-S, M@P-SP and M@P-SOP NPs after HIFU irradiation.

(B, C) The quantitative analyses of A (n=3, one-way ANOVA, ****P < 0.0001, ***P < 0.001). (D) *In vitro* PA images of M@P-SOP NPs at different concentrations. (E) *In vitro* T2-weighted MR images of M@P-SOP NPs. (F) The quantitative analyses of Figure 3A (n=3, t-test, ***P < 0.001).

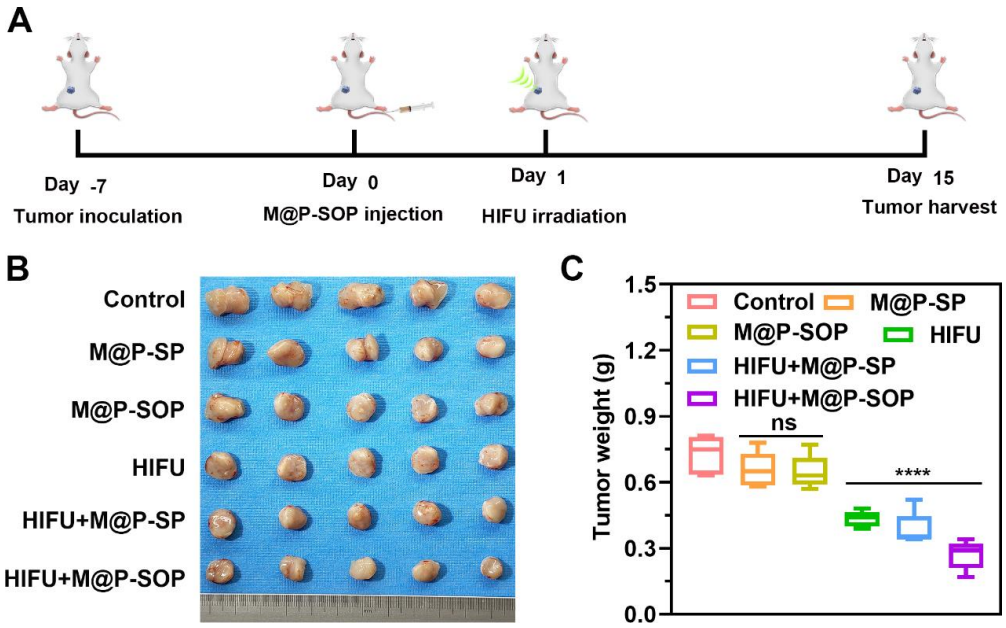

Figure S4 *In vivo* therapeutic efficiency of M@P-SOP with HIFU. (A) Schematic illustration of tumor treatment experiment design. (B) Representative pictures of the tumors of mice after different treatments. (C) Weights of tumors with various treatments (n=5, one-way ANOVA, ****P < 0.0001).

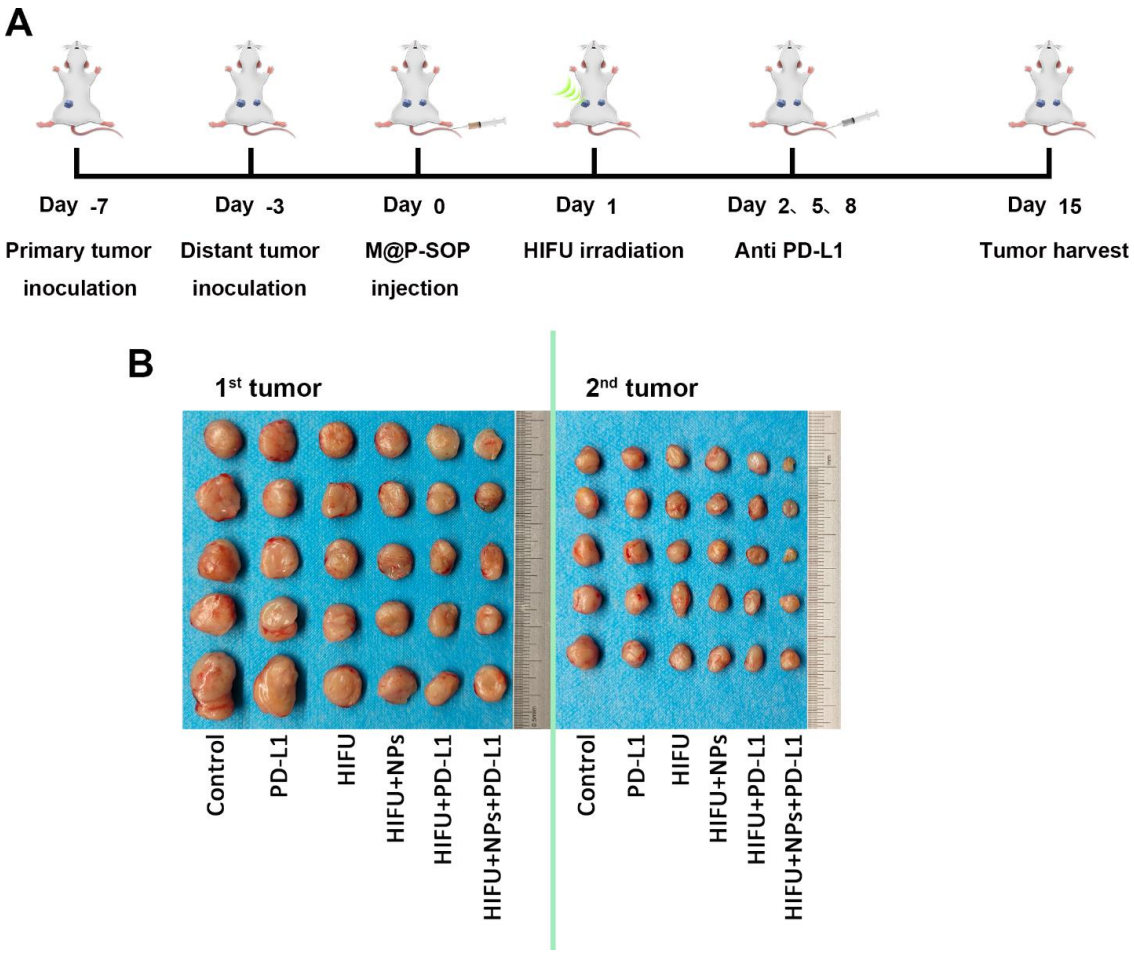

Figure S5 Representative pictures of the primary and metastatic tumors of mice after different treatments. (A) Schematic illustration of tumor treatment experiment design. (B) Representative pictures of the tumors of mice after different treatments.

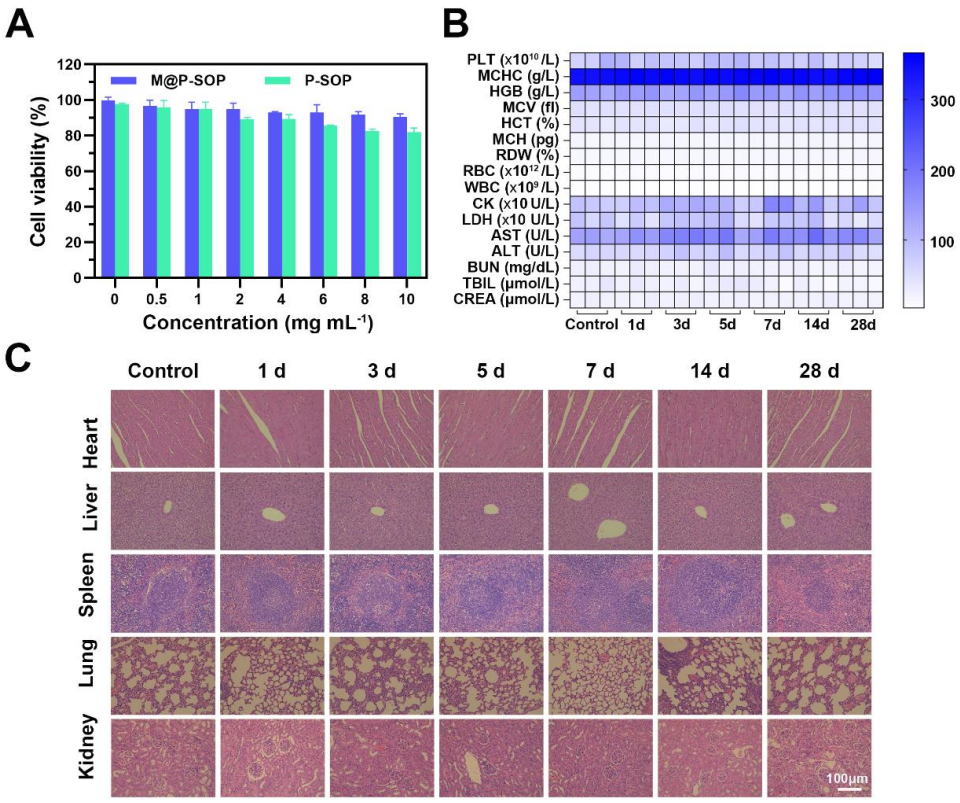

Figure S6 Biocompatibility and biosafety analyses of M@P-SOP. (A) Cell viability of 4T1 cells incubated with P-SOP and M@P-SOP at different concentrations for 24 h, as determined by CCK-8 assay. (B) Analyses of blood routine and serum biochemistry assay. (C) Representative histological examinations of the major organs stained with H&E in healthy mice with or without receiving M@P-SOP.