Supplementary Material for

Biomimetic phototherapeutic nanoagent based on bacterial double-layered membrane vesicles for comprehensive treatment of oral squamous cell carcinoma

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S1. Experimental methods

S1.1 *In vitro* evaluation of photodynamic performance

SOSG was used as a fluorescent probe to evaluate the photodynamic performance of PBAE/IR780@DMV through detecting the generation of ROS. Briefly, the solutions of PBS, free IR780, PBAE/IR780 and PBAE/IR780@DMV were added separately with 50 μM of SOSG, and then exposed to an 808 nm laser at an intensity of 2 W/cm^2 for 5 min. Here, the IR780 concentrations of free IR780, PBAE/IR780 and PBAE/IR780@DMV were 60 μg/mL. After that, the fluorescence intensities of SOSG in these sample solutions were detected with a fluorescence spectrophotometer (F7000, Hitachi, Japan) at the excitation and emission wavelengths of 504 nm and 525 nm, respectively.

Next, DCFH-DA was used as a fluorescent probe to evaluate the generation of ROS inside SCC-7 cells. Typically, the cells were seeded into the 12-well culture plates at a density of 1.5×10^5 cells each well and cultured for 24 h. These cells were incubated with free IR780, PBAE/IR780 and PBAE/IR780@DMV at the IR780 concentration of 1 μg/mL for 4 h, and then exposed to an 808 nm laser at a power density of 2 W/cm^2 for 5 min. After incubation for another 20 h, 20 mM of DCFH-DA and added into each well and incubated for 30 min. Finally, the cells were observed and imaged with a FV-1000 confocal microscopy (Olympus, Tokyo, Japan) after staining with DAPI. Furthermore, the intracellular DCF produced from oxidized DCFH-DA were also quantitatively measured using a flow cytometer (Beckman Coulter, CA, USA).

S1.2 *In vitro* evaluation of photothermal performance

Photothermal performance of PBAE/IR780@DMV was evaluated by monitoring the temperature change during 5 min of laser irradiation. Briefly, the solutions containing free IR780, PBAE/IR780, and PBAE/IR780@DMV all with the IR780 concentration of 60 μg/mL were exposed to an 808 nm laser for 5 min at a power density of 2 W/cm^2. Within 5 min of

laser irradiation, the thermal images and solution temperatures were recorded every 20 s with an IR thermal imaging camera (FLIR Corporation, USA). In the meantime, PBS was also monitored using the same method as the control. Subsequently, the temperature-time curves were drawn to compare he photothermal performance of PBAE/IR780@DMV with those of free IR780 and PBAE/IR780.

S1.3 Cytotoxicity assay

CCK-8 assay was used to evaluate the cytotoxicity of PBAE/IR780@DMV with 808 nm laser irradiation in SCC-7 cells. Briefly, the cells were seeded into the 96-well culture plates at a density of 5×10^3 cells per well and cultured for 24 h. Culture media were removed and meanwhile 200 μL of fresh culture media containing free IR780, PBAE/IR780 and PBAE/IR780@DMV were added at the concentration of IR780 ranged from 0.1 to 2.0 μg/mL. After 4 h of incubation, the cells were exposed to an 808 nm laser at a power density of 2 W/cm^2 for 5 min, followed by incubation for another 20 h. Here, the cells without any treatments were used as the control. Next, the cells were processed with the CCK-8 agent in accordance with the manufacture's protocol and the absorbance each well was measured at 450 nm with a microplate reader (Multiskan GO, Thermo Scientific, USA). The cell viabilities were calculated by comparing the absorbance of the samples to that of the control. Meanwhile, the cytotoxicity of DMVs derived from attenuated Pg and PBAE/IR780@DMV in NIH/3T3 cells were also determined by the same method to assess their biosafety.

S1.4 Cell apoptosis analysis

SCC-7 cells were seeded into the 12-well culture plates at a density of 1.0×10^5 cells per well and cultured for 24 h. Next, the cells were incubated with free IR780, PBAE/IR780 and PBAE/IR780@DMV for 4 h at the IR780 concentration of 1 μg/mL, followed by 808 nm laser irradiation with a power density of 2 W/cm^2 for 5 min. After incubation for another 24 h, the cells were collected after centrifugation at 1200 rpm for 5 min and stained with the

Annexin V-FITC/PI apoptosis kit in accordance with the manufacture's protocol. After that, the cells were detected with a flow cytometer for analyzing the apoptotic and necrotic cells.

S1.5 *In vitro* evaluation of the ICD

We further investigated the surface exposure of calreticulin (CRT) and the expression change of heat-shock protein 70 (HSP70) in the cells at 24 h after receiving the above treatments at the IR780 concentration of 1 μg/mL. For analysis of the surface exposure of CRT, the cells fixed with 4% paraformaldehyde were processed with the primary anti-CRT antibody (1:200; Bioss, Beijing, China) and the secondary Alexa Fluor 488 goat anti-rabbit IgG antibody (1:200; sparkjade, Shandong, China) successively according to the manufactures' instructions. For detection of the expression of HSP70, the treated cells receiving the above treatments were fixed with 4% paraformaldehyde and then punched with 0.2% Triton X-100 for 10 min at room temperature. Next, the cells were processed with the primary rabbit anti-HSP70 antibody (1:200; Bioss, Beijing, China) and the secondary Alexa-488 goat anti-rabbit antibody (1:200) successively according to the manufactures' instructions. All the above cells were further stained with DAPI (1:900) and finally observed under a confocal fluorescence microscope. In addition, we also quantitatively analyzed the surface exposure of CRT in the above treated cells using the flow cytometry after staining with the rabbit anti-calreticulin/AF488 antibody (Bioss, Beijing, China).

S1.6 *In vitro* evaluation of antitumor immunity

We firstly prepared the lysates from SCC-7 cells with various treatments to be used as the tumor antigens for evaluating the antitumor immunity induced by these treatments. Briefly, the cells were seeded into the 12-well culture plates at a density of 1.0×10^5 cells per well and cultured for 24 h. Next, the cells were incubated with PBS, free IR780, PBAE/IR780 and PBAE/IR780@DMV at the IR780 concentrations of 1 μg/mL. After further incubation for 4 h, the cells were irradiated with an 808 nm laser at a power density of 2

W/cm^2 for 5 min and then cultured for another 24 h. Afterwards, the cell culture media were collected and centrifuged at 300 g for 10 min to remove the debris, and the supernatants thus obtained were used for the following experiments

Bone marrow-derived dendritic cells (BMDCs) were harvested from the bone marrow of C57BL/6 and cultured in the RPMI-1640 complete medium supplemented with 10 ng/mL of IL-4 and 20 ng/mL of GM-CSF for 6 d. Next, BMDCs were seeded in the 12-well plates at a density of 6×10^5 cells per well and incubated with the tumor antigens as above prepared for 48 h. All the culture media were collected and centrifuged at 1500 rpm for 5 min. The cell precipitates thus obtained were resuspended in 100 μL of PBS and processed with anti-mouse-PE-CD11c antibody (1:100; Biolegend, San Diego, CA, USA), anti-mouse-APC-CD80 antibody (1:1000; Invitrogen, Carlsbad, CA, USA), and anti-mouse-FITC-CD86 antibody (1:700; Biolegend, San Diego, CA, USA) according to the manufactures' instructions. Finally, the expressions of CD11c, CD80, and CD86 on BMDCs were analyzed with a flow cytometer.

We also evaluated the M1 polarization of macrophages induced by the above treatments. Briefly, RAW264.7 cells were seeded into the 12-well plates at a density of 1×10^5 cells per well and incubated with the tumor antigens as above prepared for 48 h. After that, the cells were collected and resuspended in 100 μL of PBS and processed with anti-mouse-PE-CD80 antibody (1:300; Invitrogen, Carlsbad, CA, USA) in accordance with the manufacture's protocol, and finally detected with a flow cytometry.

S1.7 *In vivo* evaluation of PDT and PTT performances

An OSCC animal model was constructed through subcutaneous inoculation of SCC-7 cells into the right groins of the mice for evaluating the *in vivo* photothermal and photodynamic performances of PBAE/IR780@DMV. In brief, the mice were administered separately with PBS (the control), free IR780, PBAE/IR780, and PBAE/IR780@DMV via

intratumoral injection at the IR780 dose of 1 mg/kg. After 4 h, the mice were given 808 nm laser irradiation at the tumor site at a power density of 1 W/cm^2 for 5 min. Within this period, the mice were imaged with an IR thermal camera (FLIR Corporation, USA) and the temperatures of their tumors were recorded at the same time. After 48 h, the mice were sacrificed and their tumors were collected for further frozen section. By using the same method as described above, all tumor sections were processed with immunofluorescence staining of HSP70 and DAPI, and then observed under a confocal microscope.

SOSG was used as a fluorescence probe to detect the generation of intratumoral ROS to evaluate the *in vivo* photodynamic performance. Briefly, SCC-7 tumor-bearing mice were administered intratumorally with PBS (the control), free IR780, PBAE/IR780, and PBAE/IR780@DMV at the IR780 dose of 1 mg/kg. In the meantime, SOSG was injected together with the above treatment agents into the tumors. After 4 h, the mice were given 808 nm laser irradiation at a power density of 1 W/cm^2 for 5 min. Next, the mice were sacrificed and their tumors were isolated for frozen section. Tumor sections thus obtained were stained with DAPI and finally observed under a confocal fluorescence microscope.

S1.8 *In vivo* assessment of antitumor effects at 48 h after treatments

SCC-7 tumor-bearing mice were divided randomly into 8 groups with 5 mice each group and received separately the treatments of PBS (the control), free IR780, PBAE/IR780, and PBAE/IR780@DMV all with and without 808 nm laser irradiation. Here, free IR780, PBAE/IR780 and PBAE/IR780@DMV were injected into the tumors of the mice at the IR780 dose of 1 mg/kg. After 4 h, the mice in the laser irradiation groups were given 808 nm laser irradiation at the tumor site at a power density of 1 W/cm^2 for 5 min. At 48 h after various treatments, all the mice were sacrificed and their tumors, spleens and blood were collected for further examinations.

The tumors and spleens sourced from 3 mice each group were homogenated and

digested for 5 h in the serum-free RPMI 1640 medium supplemented with type-IV collagenase, hyaluronidase and DNase I. The cell suspensions were filtered through a 200 μm nylon filter mesh and then processed with centrifugation. The thus-obtained cell precipitates were resuspended in 3 mL of ACK lysing buffer at 4°C for 10 min, and the collected cells were then resuspended in PBS to obtain the single-cell suspensions. For evaluating the activation of DCs, the cells were stained with anti-mouse-PE-CD11c antibody (1:100; Biolegend, San Diego, CA, USA), anti-mouse-APC-CD80 antibody (1:1000; Invitrogen, Carlsbad, CA, USA) and anti-mouse-FITC-CD86 antibody (1:700; Invitrogen, Carlsbad, CA, USA) meanwhile according to the manufactures' instructions. For evaluating the activation of T cells, the single cell suspensions were double-stained with anti-mouse-FITC-CD3 antibody (1:500; Invitrogen, Carlsbad, CA, USA) and anti-mouse-APC-CD8 antibody (1:400; eBioscience, San Diego, CA, USA) meanwhile according to the manufactures' instructions. Finally, these stained cells were detected with a flow cytometer to analyze the proportions of $CD80^+$ $CD86^+$ DCs gated on $CD11c^+$ and effector $CD8^+$ T cells gated on $CD3^+$.

Blood samples extracted from 3 mice each group were centrifuged at 3000 rpm at 4°C for 10 min and then the supernatants were collected in the tubes. The plasma levels of TNF-α in these samples were measured by using the ELISA kit according to the manufacturer's protocol. In addition, the tumors sourced from the reminder 2 mice each group were paraffin-embedded and cut into the 5 μm-thick sections. After that, these tumor sections were processed separately with hematoxylin and eosin (H&E) staining and immumohistochemical staining with rabbit anti-Ki67 antibody (Abcam, Cambridge, MA, USA) according to the manufactures' instructions, and finally observed under a microscope.

S1.9 *In vivo* evaluation of antitumor effects at 21 d after treatments

SCC-7 tumor-bearing mice were randomly divided into 8 group with 5 mice each group and given the same treatments as described above. After various treatments, the tumor

volumes and the body weights of these mice were monitored continuously for 21 d. Next, all the mice were sacrificed and their tumors and major organs (heart, liver, spleen, kidney, and lung) were collected for further examinations. Firstly, the tumors were imaged and weighed for comparison. Secondly, the spleens chosen from 3 mice each group were dissected according to the method described above for detecting the proportion of memory T cells. The single-cell suspensions thus obtained were stained with anti-mouse-FITC-CD3 antibody (1:500), anti-mouse-PE-CD44 antibody (1:600; Biolegend, San Diego, CA, USA), and anti-mouse-APC -CD62L antibody (1:1000; eBioscience, San Diego, CA, USA). Finally, the stained cells were analyzed with a flow cytometry. Thirdly, the lungs were freeze-sectioned and then respectively stained with the primary anti-CD86 antibody (Bioss, Beijing, China), anti-CD4 antibody (Bioss, Beijing, China), and anti-CD8 antibody (Bioss, Beijing, China), followed by processing with the secondary Alexa-488 goat anti-rabbit antibody (1:200). After further staining with DAPI, these stained sections were observed under a confocal fluorescence microscope. Finally, the major organs were processed with paraffin-embedding and section, and then stained with H&E. These stained sections were observed under a microscope for evaluating the *in vivo* biosafety and the lung metastases.

Supporting Figures

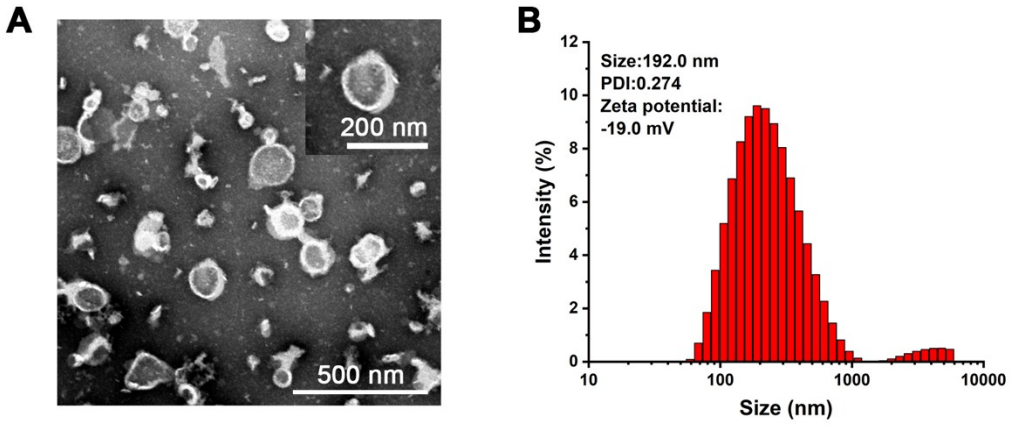

Figure S1. TEM image and size distribution of DMVs.

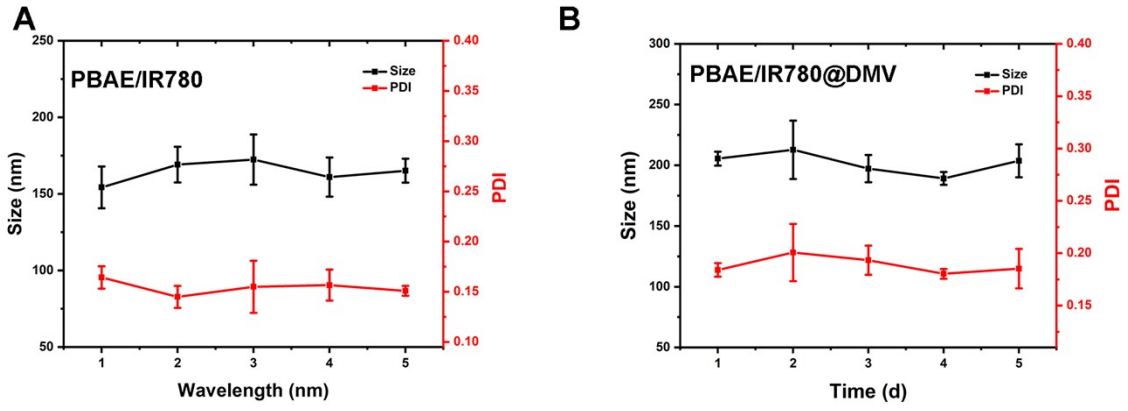

Figure S2. Sizes and polydispersity indexes (PDIs) of PBAE/IR780 (A) and PBAE/IR780@DMV (B) within 5 d of storage in deionized water at 4 °C.

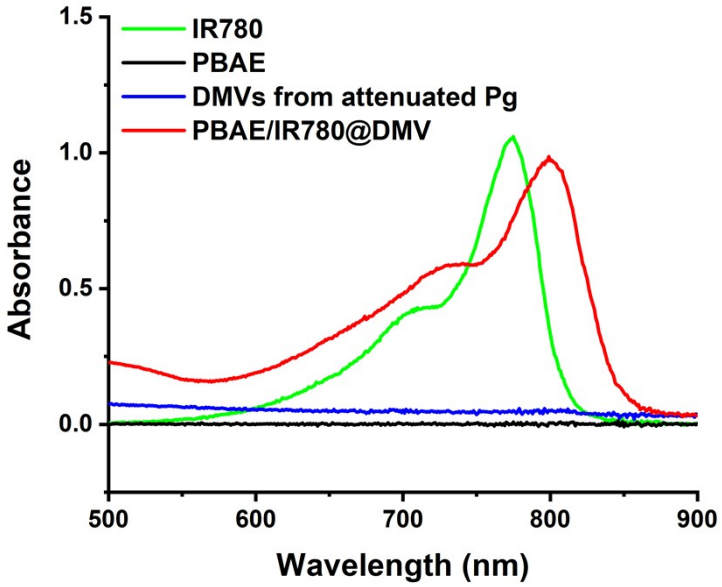

Figure S3. UV-Vis-NIR absorption spectra of IR780, PBAE, DMVs from attenuated Pg, and PBAE/IR780@DMV.

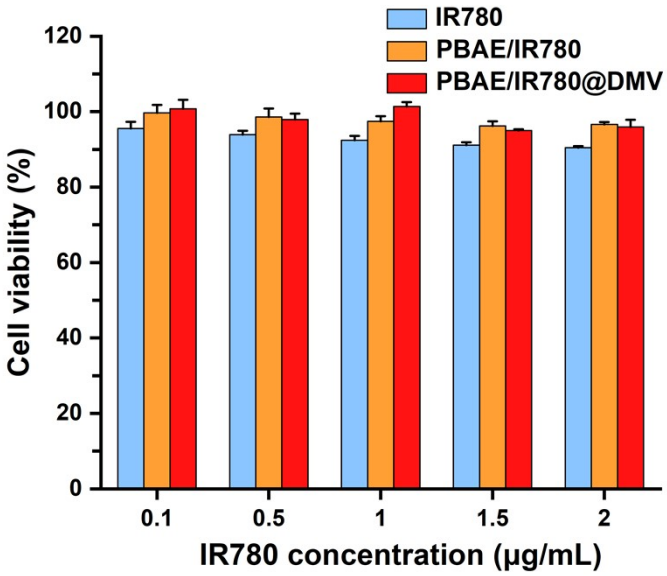

Figure S4. Cytotoxicity of free IR780, PBAE/IR780 and PBAE/IR780@DMV in NIH/3T3 cells after incubation for 24 h.

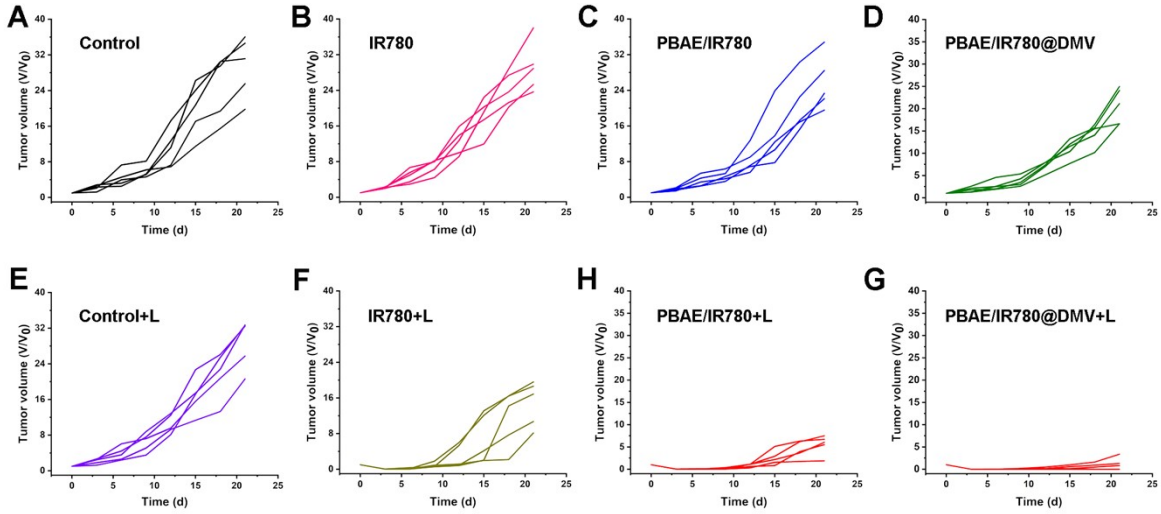

Figure S5. Tumor growth curves of SCC-7 tumor-bearing mice with treatments of PBS (the control), free IR780, PBAE/IR780, and PBAE/IR780@DMV as well as their combined with 808 nm laser irradiation at a power density of 1 W/cm^2 for 5 min (n=5).

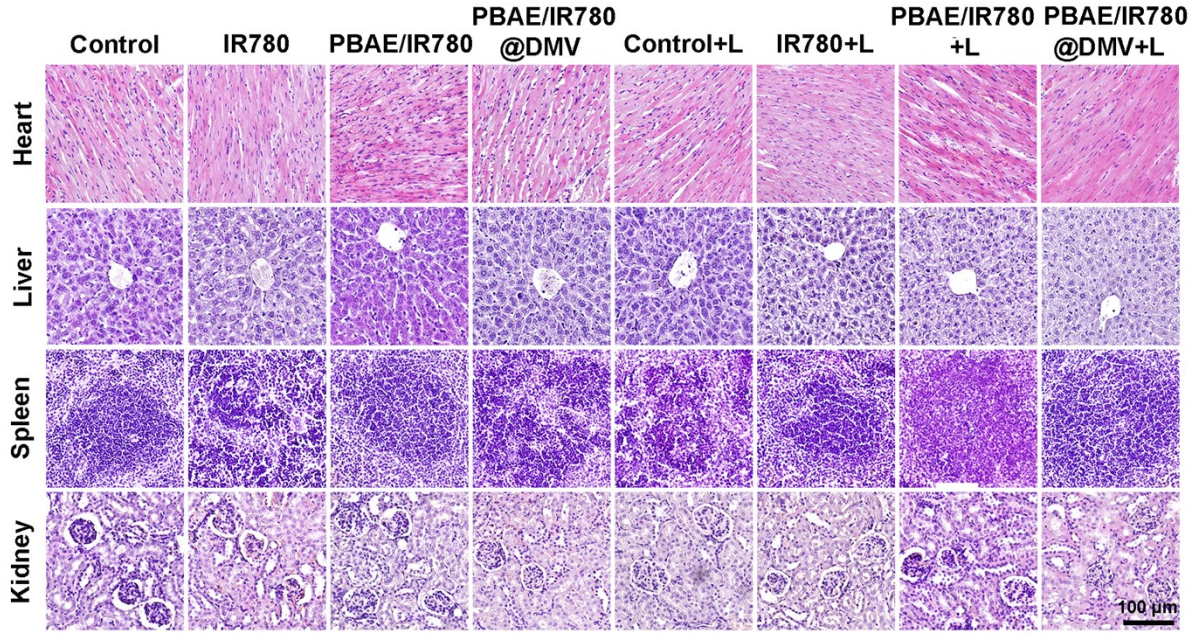

Figure S6. Microscope images of H&E-stained sections of major organs (heart, liver, spleen and kidney) sourced from mice at the end of various treatments.