Catalase-like pleated niobium carbide MXene loaded with polythiophene for oxygenated sonodynamic therapy in solid tumor

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EXPERIMENTAL SECTION

Materials and Instrutments

Poly(vinylpyrrolidone) (PVP) and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich Trading Co., Ltd. 9,10-Anthracenediyl-bis(methylene)dimalonic Acid (ABDA) was purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. Dihydrorhodamine 123 (DHR123) was purchased from Dalian Meilun Biotech Co., Ltd. 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA), calcein-AM, and propidium iodide (PI) is purchased from Heowns Biochem Technologies, LLC, Tianjin. The hydrogen peroxide kit was purchased from Beijing Solarbio Science & Technology Co., Ltd. The EdU kit was purchased from Shanghai Bioscience Co., Ltd.

The UV-vis-NIR absorption and fluorescence spectra were recorded on Shimadzu UV2600 and RF6000 spectrophotometers, respectively. Scanning electron microscope (SEM) images were obtained from JSM-7610F, JEOL Ltd. Fluorescence imaging of the cells was performed under an inverted fluorescence microscope (Leica DMIL LED). MTT cell viability experiments were performed on an enzyme-labeled instrument (Varioskan LUX). The Zeta potential experiment was obtained on Particle Size and zeta potential Analyzer (Zetasizer Nano ZS). Animal fluorescence imaging was obtained on a multifunctional imaging analysis system (Perkin Elmer, IVIS Lumina III). The dissolved oxygen test was obtained on dissolved oxygen analyzer (Rex Electric, JPBJ-609L). The ultrasonic treatment of mice was performed under an ultrasonic therapy equipment (Welld, WED-100).

Synthesis of PT2

The synthesis of PT2 is described in the previous work of our group [Ref: *J. Am. Chem. Soc.*, 2012, **134**, 6685-6694].

Preparation of Nb_2C-PVP-PT2 nanosheets

Nb_2C was prepared according to our previous work. Two milliliters of aqueous Nb_2C (1 mg/mL) and 10 mL of aqueous PVP (1 mg/mL) were mixed and stirred at room temperature for 6 h. After 2 mL of PT2 in methanol (1 mg/mL) was added dropwise, the mixture was stirred overnight. After 12 h, methanol was removed by centrifugation (9000 rpm), and the precipitate was dissolved in water to obtain Nb_2C-PVP-PT2 nanosheets, which were stored in a refrigerator (4 °C).

Calculation of PT2 load efficiency

The loading capacity (*LC*, w/w%) of PT2 is calculated using the following equation:

$$LC = \frac{Weight\ of\ loaded\ PT2}{Weight\ of\ Nb_2C\ nanosheets}$$

The standard curves for PT2 and Nb_2C were calculated according to FigureS2.

Determination of oxygen content *in vitro*

First, the anaerobic state of a dissolved oxygen electrode was calibrated in a newly prepared sodium sulfite solution, and the full oxygen state was then calibrated in air. After calibration, the electrode was placed in a 50 mL centrifuge tube filled

with 9 mL of water until the reading was stable, and 1 mL of hydrogen peroxide solution (1 mM) was then added to water. After 10 min, 1 mL of Nb_2C solution (1 mg/mL) was added to the solution, and the amount of oxygen in the mixture was recorded.

Determination of $^1O_2$ and $O_2^{\bullet-}$ in aqueous solution

$^1O_2$ generated by Nb_2C-PVP-PT2 under ultrasonic conditions was detected using ABDA sodium salt (ABDA-Na). ABDA-Na (50 μL) was added into Nb_2C-PVP-PT2 aqueous solution (2 mL, 200 μg/mL), and the mixture was then irradiated with ultrasound using an ultrasonic therapy instrument (1.0 MHz, 1.5 W/cm^2) for 5 cycles, each for 2 min. The UV-vis-NIR absorption spectra of $^1O_2$ captured by ADBA was measured.

Commercial DHR123 was used to assay the $O_2^{\bullet-}$ generation capability of Nb_2C-PVP-PT2. The assay was conducted by first adding 5 μL of DHR123 probe to 2 mL of Nb_2C-PVP-PT2 (200 μg/mL). The fluorescence spectra of the mixture were then scanned every 2 min under ultrasound irradiation (λ_{ex}=500 nm). The $O_2^{\bullet-}$ generation capability of H_2O was determined using the same procedure described above.

Determination of intracellular ROS

4T1 cells were divided into four groups (PBS, PBS + US, Nb_2C-PVP-PT2, and Nb_2C-PVP-PT2 + US). The cells in each group were first incubated in a 37 ℃ cell incubator for 24 h. Subsequently, the medium used to culture cells in PBS and US groups was replaced with a new 1640 culture medium, while that used to culture cells in the Nb_2C-PVP-PT2 and Nb_2C-PVP-PT2 + US groups was replaced with 1640 culture medium containing Nb_2C-PVP-PT2. After incubation for 4 h, DCFH-DA was added, and the incubation was continued for additional 30 min. After that, all cells were washed three times with PBS, and cells in the US and Nb_2C-PVP-PT2 + US groups were treated with ultrasonic therapeutic apparatus (1.0 MHz, 1.5W /cm^2). Finally, cells in all groups were imaged using an inverted fluorescence microscope.

Determination of intracellular H_2O_2

4T1 cells were uniformly inoculated in 24-well plates. When the cell growth reached 80-90%, the cells were divided into two groups, namely non-Nb_2C and Nb_2C.

Each well was added with 500 μL medium or 400 μL medium mixed with Nb_2C. 4 h later, the cells were collected into the centrifuge tube and the supernatant was discarded. Then 2 mL acetone solution was added to the two groups of cells and treated with ultrasonic cell disruptor (power 20%, ultrasonic 3 s, 10 s interval, repeat 30 times). After the ultrasound, centrifuge at 4 ℃ at 8000 r for 10 minutes, take the supernatant liquid ice to be measured, and prepare the sample to be measured according to the proportion. Finally, the prepared samples were transferred to 96-well plates, and the prepared reagents were used as blank group to eliminate errors, and tested under the enzyme label instrument.

MTT cell experiment

The 4T1 cells were evenly planted in 96-well plates and cultured at 37 ℃ for 24 h in a cell incubator containing 5% CO_2. The 1640 culture group containing different concentrations of Nb_2C-PVP-PT2 was replaced for further incubation. The cells in the ultrasound group were incubated for 4 h, and ultrasonic therapy instrument was used for ultrasound (1.0 MHz, 1.5 W/cm^2, 10 min). After 24 h, the medium was discarded and 200 μL cell medium containing MTT (10%) was added to each well. After 4 h, the medium was discarded and 200 μL DMSO was added to each well. After 10 min, DMSO was tested under an enzyme-labeled instrument.

AM/PI staining

The 4T1 cells were divided into four groups, *i.e.* PBS, PBS + US, Nb_2C-PVP-PT2, Nb_2C-PVP-PT2 + US groups, and evenly grown in 96-well plates. After 24 hours of incubation in the cell incubator, the Nb_2C-PVP-PT2 and Nb_2C-PVP-PT2 + US groups were replaced with fresh 1640 medium containing Nb_2C-PVP-PT2 (200 μg/mL), and the PBS and PBS + US groups were replaced with a mixture of PBS and 1640 medium and incubation continued for 4 hours. The cells in each group were then stained with calcein-AM (2 μM, 10 μL)-PI solution (2 μM, 10 μL) for 10 min (calcein-AM shows green fluorescence for live cells; PI stains for dead cells, showing red fluorescence). Subsequently, ultrasound treatment (1.0 MHz, 1.5 W/cm^2，5 min) was performed on the US group and the Nb_2C-PVP-PT2 + US group using an ultrasonic therapy instrument. After washing three times with PBS, the staining of the

cells in each group was observed with a fluorescent inverted microscope.

EdU staining of proliferating cells

4T1 cells divided into 4 groups described above were inoculated into confocal dishes for 24 h, and each group was treated accordingly. 4 h later, the cells in laser groups were exposed to ultrasound (1.5 W/cm^2) for 5 min. The cells were then incubated with a mixture of 10 μM EdU and culture medium for 1 h. After incubation was complete, the medium was removed and 50 μL of 4% paraformaldehyde fixative was added and incubated for 15 min. The fixative was then removed after incubation was complete. Next, 50 μL of glycine (2 mg/mL) was added to each group of cells and incubated for 5 min to neutralize any residual fixative. Then 100 μL of 0.5% Triton X-100 was added and incubated for 20 min.

For preparing Click-iT working solution, 860 μL of Click-iT EdU reaction buffer, 40 μL of copper sulfate and 2 μL of Azide-488 were mixed. 100 μL of Click-iT working solution was added to each well and incubated for 30 min at room temperature. The cells were washed twice with PBS before adding 100 μL of Hoechst 33342 solution and subsequent incubation for 30 min. Finally, cells were washed three times with PBS and imaged with a confocal scanning microscope.

***In Vivo* Fluorescence Imaging**

200 μL of Nb_2C-PVP-PT2 (200 μg/mL) in aqueous solution was administered to mice by intratumor injection. After Half an hour later, images were obtained in the multifunctional imaging analysis system (Ex=440 nm).

Animal Model

Animal experiments were performed under the approval of the ethics committee of Hunan Normal University (No. 430727221100498257). Balb/c female mice (8 weeks) were purchased from Hunan SJA Laboratory Animal Co., Ltd. To establish a mouse tumour model, we evenly grew 8 boxes of 4T1 cells in a 90 mm^2 culture dish. When the cells grew to 80-90%, the cells were digested and centrifuged, 2 mL of culture medium was added and blown well with a pipette, and the 4T1 cells were injected under the skin of the back of two mice (1mL/each). After about 2 weeks, when the tumour grew to 800 mm^3, the tumour was peeled and divided into 2 mm × 2

mm size and transplanted to the subcutaneous dorsal surface of healthy mice. After about 10 days, the tumours grew to 80mm^3 size and the mouse experiment was started.

Mice were randomly divided into 4 groups, and Nb_2C-PVP-PT2 group and Nb_2C-PVP-PT2 + US group were injected intratumorally with Nb_2C-PVP-PT2 solution (200 μg/mL, 200μL). After waiting for 10 minutes, coupling agent was spread on the ultrasound probe and then ultrasound treatment (1.0 MHz, 1.5 W/cm^2 for 10 minutes) was applied to the tumours of mice requiring ultrasound treatment. Changes in tumour growth and weight changes in mice were then recorded over the next 14 days.

H&E and Ki67 section experiments

Four 4T1 tumour-bearing mice were treated with PBS, PBS + US Nb_2C-PVP-PT2, Nb_2C-PVP-PT2 + US (200 μL, 200 μg/mL of Nb_2C-PVP-PT2 or 200 μL of PBS) and then immediately peeled of tumour tissue and fixed with 4% paraformaldehyde. Next H&E and Ki67 stained sections were performed at Wuhan Sevier Biotechnology Co. Ltd.

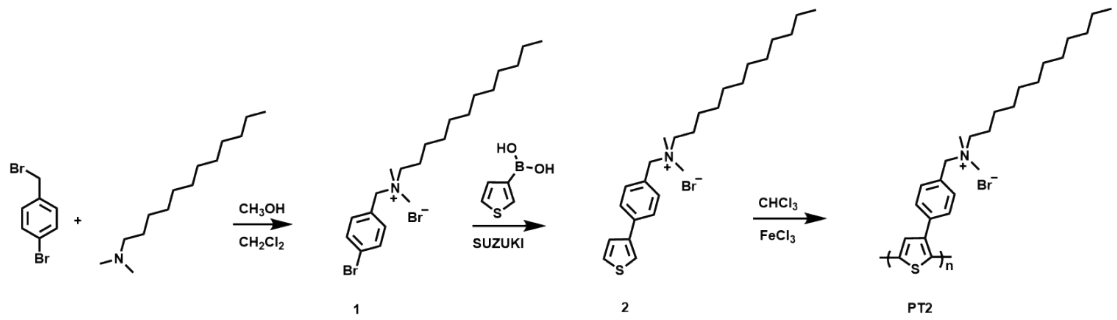

Figure S1 Synthetic route of PT2.

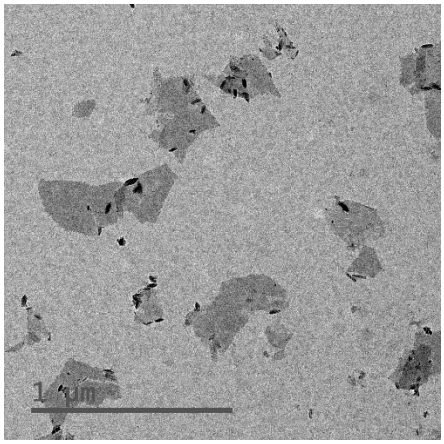

Figure S2 TEM image of Nb_2C MXene.

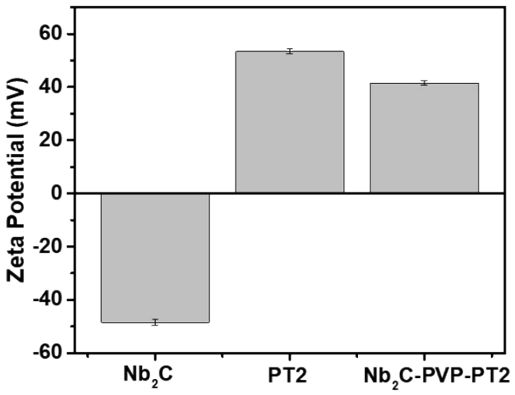

Figure S3 The Zeta potential of Nb_2C MXene, PT2 and Nb_2C-PVP-PT2 NSs aqueous solutions.

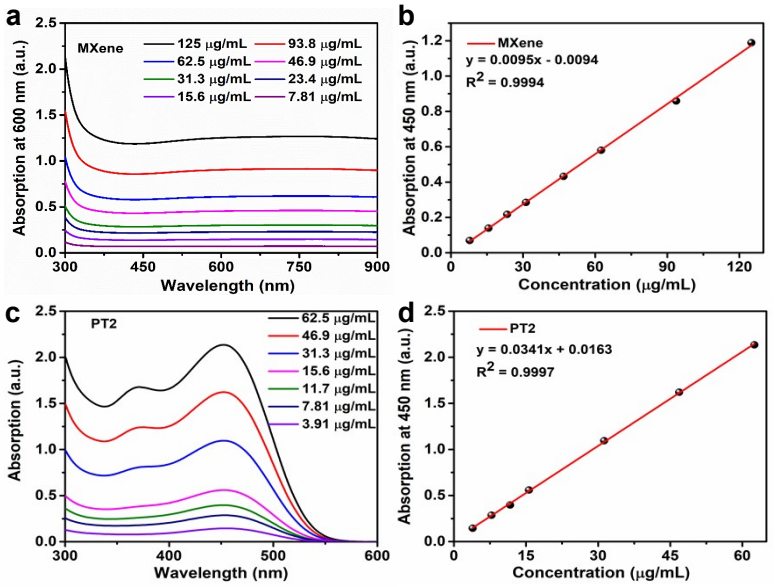

Figure S4 Standard curves of (A, B) MXene and (C, D) PT2, and its linearity plots.

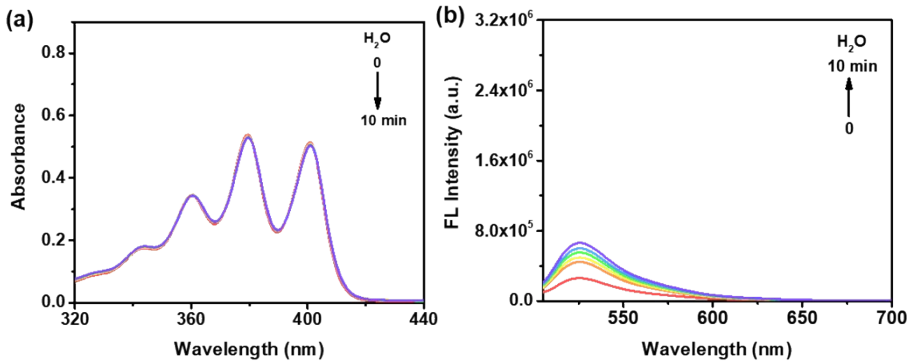

Figure S5 US irradiation time-dependent (a) UV-vis absorption spectra of ABDA, and (b) fluorescence spectra of DHR 123 (λ_{ex}=500 nm) in the absence of Nb_2C-PVP-PT2 NSs.

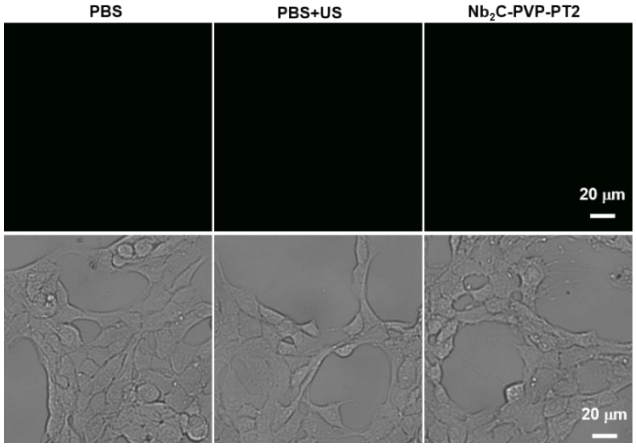

Figure S6 Images of 4T1 cells from different treatment groups after incubation with O22 probe. (1.0 MHz, 1.5 W/cm^2, 10 min)

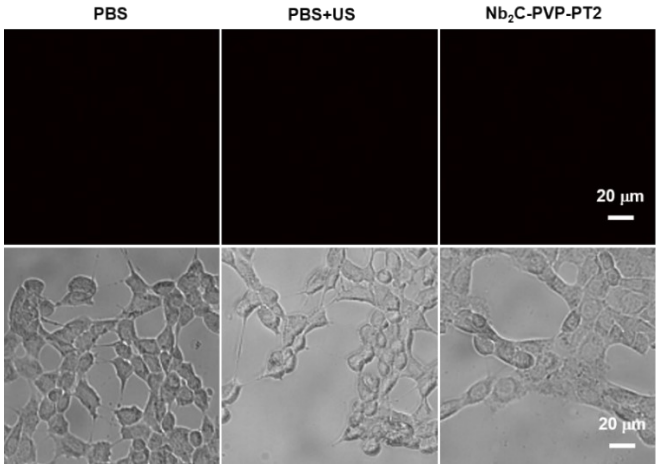

Figure S7 Images of 4T1 cells from different treatment groups after incubation with DHE probe. (1.0 MHz, 1.5 W/cm^2, 10 min).