Supplemental materials

ICG-Loaded PEG-Modified Black Phosphorus Nanosheets for Fluorescence Imaging-Guided Breast Cancer Therapy

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Materials and methods

Materials

N,N’-dimethylformamide (DMF), dimethyl sulfoxide (DMSO), N-Methyl pyrrolidone (NMP), tin (Sn 99.9%) powder and tin (IV) iodide (99.9%) were purchased from Aldrich Chemical Co. Ltd. (U.S.) and used without further purification. Red phosphorus powder (100 mesh, 98.9%) was purchased from Alfa Aesar (China) Chemical Co. Ltd. Fetal bovine serum (FBS), Dulbecco’s Modified Eagle Medium (DMEM), phosphate-buffered saline (PBS), trypsin fluorescein diacetate (FDA), and propidium iodide (PI) were purchased from Beyotime Company (China). Indocyanine green (ICG) was purchased from Shanghai Aladdin Company (China). The amino PEG (mPEG-NH₂, Mw 5000) and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (USA). A Zeta sizer (Nano ZS) instrument, which was equipped with a He-Ne ion laser (λ = 633 nm) was used to test the sizes, size distributions, and zeta potentials of the nanocomposites at 25 °C, and the measured scattering angle was fixed at 173 °C. An FP-9600 (Hitachi) spectrofluorometer was employed to obtain the fluorescence spectra. A Fourier infrared spectrometer (TENSOR 27) was used to obtain the infrared peak of the nanocomposites. A Hitachi H-7650 electron microscope was used to obtain transmission electron microscopy (TEM) images at an acceleration voltage of 100 kV. 808nm Laser (MDL-Ⅲ-808-2W, China) was used for photothermal experiments and Infrared Camera (ICI7320, China) was used to detect temperature. Multiskan GO Microplate Reader (Thermo, USA) was used to analysis the results of MTT. Flow Cytometry (Beckman Coulter, USA) was used to evaluate the intracellular fluorescence. The result of FDA/PI co-staining was observed under a fluorescence microscope (OLYMPUS, USA).
Fluorescence imaging in vivo was detected by IVIS Spectrum (Perkin Elmer, USA).

**Preparation of few-layered BP**
Few-layered BP was synthesized from red phosphorous (RP) using a previously reported method, with modifications. In brief, RP (0.5 mg), tin powder (30 mg), and tin (IV) iodide (120 mg) were mixed in a sealed ampule at a pressure lower than $10^{-3}$ mbar. After heating at $220^\circ$C for 36 h, crystalline BP was obtained. In the next step, the obtained bulk BP with biological purity (100 mg) was dispersed into 500 mL NMP followed by sonication in an ice bath (400 W) under N$_2$ atmosphere to prevent oxidation for 24 h. The as-prepared BP suspension was centrifuged at 3000 rpm three times to remove sediment. Then, the supernatant was further centrifuged at 5000 rpm to harvest suitable nanosized few-layered BPNSs. Finally, the collected sediment was re-dispersed into deionized water three times to remove the organic solvent and then re-dispersed into PBS for the following steps.

**In vitro ICG loading behavior of BP**
Briefly, BP (5 mg) and ICG with various mass ratios (ICG/BP: 0, 0.2, 0.4, 0.8, and 1.0) were dissolved in deionized water and mixed for 12 h. Then, through centrifugation (3000 rpm) for 5 min in a dark environment, the as-prepared products were harvested, followed by centrifugation in deionized water five times to remove residual ICG. In order to enhance their stability in PBS, PEG-NH$_2$ (Mw 2000 Da) was dissolved in PBS (pH 7.4) with dispersed ICG@BPNS and stirred overnight. The purified ICG@BPNS-PEG was obtained after removing excess PEG-NH$_2$ by centrifugation (3000 rpm), followed by centrifugation in deionized water three times. To determine the ICG loading content in BP, 1 mL of the ICG@BPNS solution was diluted in 4 mL water/DMSO (1:9, v/v) and sonicated for 10 min to extract the ICG completely. ICG concentration was determined by UV-vis absorption spectra according to the standard
curve of ICG and the calculation methods are shown as follow. All measurements were performed in triplicate.

Where the ICG-loading amount was detected by UV-vis absorption spectra according to standard curve of ICG.

**Cell Culture**

RPE normal cells, MCF-7 and 4T1 breast cancer cells were cultured in DMEM supplemented with 10% FBS, 1% penicillin, and 1% streptomycin in a humidified environment of 5% CO$_2$ at 37 °C. RPE, MCF-7, and 4T1 cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China).

**In vitro cytotoxicity assay**

RPE, MCF-7 and 4T1 cells were plated in 96-well plates at $4 \times 10^4$ cells per well and incubated overnight. The cells were treated with different concentrations of ICG@BPNS-PEG for 4 h and then incubated with fresh medium for 24 h. Then, 10 µL of MTT solution was added into the medium and incubated for 4 h, and then 100 µL of MTT solubilized solution was added into each well and incubated overnight. The next day, the absorbance was measured at 570 nm using a Multiskan GO Microplate Reader. The cell viability standard without any treatment was used as a blank control.

**In vitro cellular uptake assays**

Quantitative analysis was performed by Flow Cytometry (FCM). 4T1 cells were seeded in a six-well plate and cultured overnight. The cells were then treated with free ICG and ICG@BPNS-PEG for 0 h, 0.5 h, and 1 h. The medium was then removed, and the cells were washed with PBS and collected. Intracellular fluorescence was detected by FCM at a wavelength of 780 nm.

**In vitro PTT**
The PTT effects of ICG@BPNS-PEG were verified by MTT kit and FDA/PI co-staining. MCF-7 and 4T1 cells were seeded in 96-well plates and incubated overnight, and then different concentrations of BPNS-PEG, ICG and ICG@BPNS-PEG were added to the culture medium. Then, each group was treated with 808nm NIR light for 5 minutes. Further, 10 µL of MTT solution was added to the medium and incubated for 4 h, and then 100 µL of MTT solubilized solution was added into each well and incubated overnight. The next day, the Multiskan GO Microplate Reader was used to evaluate the cell viability at 570 nm. Meanwhile, 4T1 cells were seeded in 6-well plates and incubated overnight. Subsequently, cells were cultured with the same process as above and incubated with FDA and PI for 6 h. Finally, dead and live cells were observed under a fluorescence microscope.

**Animal and tumor model**
The animal experiments were performed according to the Regulations for the Administration of Affairs Concerning Experimental Animals (Hefei, revised in June 2013). Protocols for animal assays were approved by the Administrative Committee of Animal Research at the University of Science and Technology of China. Four- and five-week-old female BALB/C mice were purchased from the Shanghai SLAC Laboratory Animal Co. Ltd (Shanghai, China). To induce tumors, 4T1 cells suspended in PBS (1 × 10^5 cells) was injected subcutaneously onto the back of the mice.

**In vivo fluorescence imaging**
When the tumor volume reached approximately 150 mm³, eight BALB/C mice were randomly divided into two groups: ICG@BPNS-PEG group and ICG group, with four mice in each group. At 0.5, 4, 12, 18 and 24 h after tail vein injection, fluorescence imaging of the tumor site in mice was observed with the small-animal live imaging
system IVIS Spectrum. The live imaging system was used to analyze and compare the fluorescence intensity and duration of each group of tumor sites. Fluorescence imaging was completed at the Core Facility Center for Life Sciences, University of Science and Technology of China (USTC).

**In vivo PTT**

The tumor-bearing mice were randomly divided into five groups with four mice in each group and treated with PBS, ICG@BPNS-PEG, NIR, BPNS-PEG + NIR and ICG@BPNS-PEG + NIR respectively. When the tumor volume reached approximately 150 mm³, the tumor-bearing mice were separately injected with PBS, BPNS-PEG and ICG@BPNS-PEG in PBS, ICG@BPNS-PEG, BPNS-PEG + NIR and ICG@BPNS-PEG + NIR groups. Twenty-four hours after vein injection, the tumor site was irradiated with an 808 nm laser at a power density of 1.65 W/cm² for 5 min. The photothermal image of tumor was collected by Infrared Camera. Tumor volume and body weight were measured every day, for 14 days in total. After 14 days, all mice were sacrificed. Hearts, livers, spleens, kidneys, lungs, and tumors were collected, fixed with 4% paraformaldehyde, embedded in paraffin, and stained with HE to observe changes in tissue structure. H&E staining was performed at the First Affiliated Hospital of the University of Science and Technology of China.
Figure S1. (A) TEM image of bare BP nanosheets; (B) Raman spectroscopy; (C) XRD spectroscopy of bulk black phosphorus (BP) and BPNS-PEG.

Figure S2. (A) XPS survey spectra of ICG@BPNS-PEG NCs; (B) High resolution P 2p spectra; (C) high resolution C 1s XPS spectra; (D) high resolution O 1s XPS spectra of ICG@BPNS-PEG NCs.
Figure S3. (A) TEM image of bare BP nanosheets after 7 days; (B) Raman spectroscopy of various materials after 7 days; (C) High resolution P 2p spectra of bare BPNS after 7 days.