Supporting Information

Two Dimensional Semiconductor for Ultrasound-Mediated Cancer Therapy: The Case of Black Phosphorus Nanosheets

Jiang Ouyang a, Liu Deng a, Wansong Chen,* a, b Jianping Sheng a, Zhenjun Liu a, Liqiang Wang a, and You-Nian Liu*a, b

a College of Chemistry and Chemical Engineering, Central South University, Changsha, Hunan 410083, P. R. China. E-mail: chenws@csu.edu.cn; liuyounian@csu.edu.cn
b State Key Laboratory for Powder Metallurgy, Central South University, Changsha, Hunan 410083, P. R. China.

Table of Contents

1. Experimental procedures
   1.1 Materials
   1.2 Preparation of BP nanosheets
   1.3 Synthesis of Au@BP nanohybrids
   1.4 Characterization
   1.5 Sonodynamic activity study
   1.6 Sonodynamic activity of Au@BP nanohybrids in deep tissue
   1.7 Au@BP nanohybrids stability under ultrasound irradiation
   1.8 Cell culture
   1.9 Biocompatibility study
   1.10 Intracellular oxidative stress
   1.11 Cellular internalization study
   1.12 In vitro antitumor study
   1.13 In vivo antitumor study

2. Supplementary Figures and Table
1. Experimental procedures

1.1. Materials. Black phosphorus crystals were obtained from HWRK Chemicals (Beijing, China) and stored under a N₂ atmosphere in the dark. All the cell lines were obtained from Xiangya Hospital of Central South University (Changsha, China). DMEM high glucose culture medium, fetal bovine serum (FBS), trypsin, penicillin-streptomycin and singlet oxygen sensor green (SOSG) were provided by Thermo Fisher (Beijing, China). N-Methyl-2-pyrrolidone (NMP), 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), chlorpromazine, methyl-β-cyclodextrin (MβCD), amiloride and Hoechst 33342 were bought from Heowns Biochemical Technology (Tianjin, China). Rhodamine B conjugated polyethylene glycol thiol was supplied by Yare Biotech (Shanghai, China). Calcein acetoxymethyl ester (calcein-AM), propidium iodide (PI), CCK-8, and Annexin V-FITC/PI apoptosis detection kit were bought from Beyotime Inst. Biotech. (Haimen, China). Au NPs were provided by Ruixi Biotech (Xi'an, China). Other reagents were of analytical purity and used without further purification.

1.2. Preparation of BP nanosheets. BP nanosheets were prepared according to our previously reported method[1]. Briefly, BP crystals (10 mg) were dispersed in 30 mL of NMP with saturated NaOH. The mixture was sonicated at room temperature for 10 h. Then the mixture solution was centrifuged (3 000 rpm, 10 min) to remove the unexfoliated BP crystals. The supernatant was collected and centrifuged at 10 000 rpm for 10 min to obtain BP nanosheets as dark brown pellets. BP nanosheets were dispersed in NMP and stored at −20 °C.

1.3. Synthesis of Au@BP nanohybrids. The Au@BP nanohybrids were prepared with the assistance of sonication. In detail, HAuCl₄ aqueous solution (10 mM) at different volumes (6, 13, 28, 53 or 106 μL) was then added into 0.5 mL of BP nanosheets in water (0.10 mg mL⁻¹) under sonication, and the mixture solution was sonicated for another 10 min. After centrifugation (12 000 rpm, 10 min) and washing with water twice, Au@BP nanohybrids with Au at different atomic ratios (3.75%, 7.5%, 15%, 25% and 40%) were obtained.

1.4. Characterization. The morphology of BP nanosheet and Au@BP nanohybrids was observed on a transmission electron microscopy (TEM; JEM-2100F, JEOL, Japan) with the accelerating voltage at 200 kV. The thickness of BP nanosheets was analyzed on an atomic force microscopy (AFM; Veeco, NanoMan, USA). Raman spectra were measured on a Raman microspectrometer (HR 800, Jobin Yvon LabRam-010, France). XPS spectra were recorded on an X-ray photoelectron spectroscopy (ESCALAB 250, Thermo Fisher, USA). XRD analysis was carried out on an X-ray automatic diffractometer (SIMENS D500, Switzerland). UV-Vis absorbance spectra were recorded on a UV-Vis spectrophotometer (UV-2450, Shimadzu, Japan). The size distributions and zeta potential of BP nanosheets and Au@BP nanohybrids were determined on a Malvern Zetasizer Nano-ZS instrument (ZEN3600, Malvern Instruments, US) at 25 °C.

1.5. Sonodynamic activity study. The production of ¹⁸O₂ was measured using SOSG as a fluorescent probe.[2] The experiment was divided into five groups: (1) blank control, (2) ultrasound, (3) BP nanosheets with ultrasound, (4) Au NPs with ultrasound, (5) Au@BP nanohybrids with ultrasound. For group (3) or (5), 0.1 mL of BP nanosheets (100 μg mL⁻¹) or Au@BP nanohybrids (with BP concentration at 100 μg mL⁻¹ and 15 atomic% Au) were added into 0.9 mL of HEPES buffer (10 mM, pH 6.6). For group (4), 0.1 mL of Au NPs (95 μg mL⁻¹) were added into 0.9 mL of HEPES buffer. Then, 1 μL of SOSG (2.5 mM) in methanol was added into each group. Afterwards, the solution in group (2)–(5) was irradiated under ultrasound (1 MHz, 1 W cm⁻², 40% duty cycle) using an ultrasound transducer (WED-100, WELLD Medical Electronics, China) for 3 min. The fluorescence spectra of the solution
before and after ultrasound irradiation were measured on a fluorescence spectroscopy with excitation wavelength at 488 nm. The sonodynamic activity of Au@BP nanohybrids with Au at different atomic ratios (3.75%, 7.5%, 15%, 25% and 40%) was measured following the similar procedure.

To further confirm \(^1\text{O}_2\) generation from Au@BP nanohybrids during ultrasound irradiation, NaN\(_3\) was used as the specific \(^1\text{O}_2\) scavenger. The experiment was divided into three groups: (1) Au@BP nanohybrids, (2) Au@BP nanohybrids + ultrasound, (3) Au@BP nanohybrids + NaN\(_3\) + ultrasound. Firstly, 1 µL of SOSG (2.5 mM) in methanol was mixed with 1.0 mL of Au@BP nanohybrids (with BP concentration at 10 µg mL\(^{-1}\) and 15 atomic% Au) in HEPES buffer (10 mM, pH 6.6). For group (3), NaN\(_3\) was also added with the final concentration at 10 mM. Then the mixture solution was irradiated under ultrasound (1 MHz, 1 W cm\(^{-2}\), 40% duty cycle) for 3 min. The fluorescence spectra of the solution before and after ultrasound irradiation were recorded on a fluorescence spectroscopy with excitation wavelength at 488 nm. The UV-Vis and fluorescence spectra of SOSG or Au@BP nanohybrids before and after NaN\(_3\) addition were also measured.

1.6. Sonodynamic activity of Au@BP nanohybrids in deep tissue.

1 mL of Au@BP nanohybrids (with BP concentration of 10 µg mL\(^{-1}\)) in HEPES buffer (10 mM, pH 6.6) were placed in a transparent quartz cell. Then SOSG (1 µL, 2.5 mM) in methanol was added into the solution. The experiment was divided into four groups: (1) PDT, (2) PDT under skin, (3) SDT, (4) SDT under skin. For group (2) and (4), pork skin (~ 3 mm in thickness) was coated at the outside of the quartz cell. The mixture was irradiated under 660 nm laser (1 W cm\(^{-2}\), Q-LINE Electronics & Technology Co., Ltd., China) or ultrasound (1 MHz, 1 W cm\(^{-2}\), 40% duty cycle) for 5 min. The fluorescence intensities of SOSG before and after irradiations were measured on the fluorescence spectroscopy. The relative \(^1\text{O}_2\) production efficiency was calculated according to equation 1:

\[
\text{Relative } ^1\text{O}_2 \text{ production efficiency} = \frac{\Delta F}{\Delta F_1} \times 100\% \tag{1}
\]

where \(\Delta F\) is the fluorescence intensity increment in each group, and \(\Delta F_1\) is fluorescence intensity increment in group (1).

1.7. Au@BP nanohybrids stability under ultrasound irradiation. Au@BP nanohybrids (with BP concentration at 15 µg mL\(^{-1}\)) and ICG (15 µg mL\(^{-1}\)) were irradiated under ultrasound (1 MHz, 2.5 W cm\(^{-2}\), 40% duty cycle) for 5 min. Their UV-Vis absorbance spectra before and after ultrasound irradiation were recorded on a UV-Vis spectrophotometer.

1.8. Cell culture. 4T1, HeLa and L929 cells were cultured in DMEM high glucose medium containing 10% FBS and 1% penicillin-streptomycin and incubated at 37 °C with 5% CO\(_2\).

1.9. Biocompatibility study. Cytotoxicity of Au@BP nanohybrids to three different cell lines (4T1, HeLa and L929 cells) was measured using CCK-8 assay kit. Cells were seeded into 96 well plates with density of 7 × 10\(^4\) cells per milliliter and incubated overnight. Then Au@BP nanohybrids in cell culture medium at different concentrations (0, 20, 50, 100 and 200 µg mL\(^{-1}\)) were added. After incubation for 24 h, the cell viability was measured using CCK-8 assay kit.

The in vivo biocompatibility of Au@BP nanohybrids was studied in BALB/c mice. Animal experiments were carried out under regulation approved by the Laboratory Animal Center of the Xiangya School of Medicine, Central South University (Changsha, China). Male BALB/c mice (6 weeks old) were purchased from Hunan
Silaike Experimental Animal Co. Ltd. (Changsha, China). Mice (n = 4) were received the intravenous injection of Au@BP nanohybrids at the dosage of 1 mg kg\(^{-1}\). Healthy mice without injection were taken as control. After a week, the bloods were collected and the blood cell density was measured on an automated blood cell counter (BC-2800 Vet Analyzers, Mindray, China). The liver/spleen function markers (including aspartate transaminase, total bilirubin, blood urea nitrogen and creatinine) were determined using the blood biochemistry analysis kits (JCBIO, Nanjing, China).

1.10. Intracellular oxidative stress. The experiment was divided into six groups: (1) blank control, (2) BP, (3) Au@BP, (4) SDT, (5) BP + SDT, (6) Au@BP + SDT, (7) Au@BP + PDT. 4T1 cells were seeded into a 96-well plate at density of \(7 \times 10^4\) cells per milliliter and incubated overnight. Then the supernatants were removed and 100 \(\mu\)L of BP nanosheets (25 \(\mu\)g mL\(^{-1}\)) or Au@BP nanohybrids (with BP concentration at 25 \(\mu\)g mL\(^{-1}\)) in DMEM medium were added. After 3 h of incubation, DCFH-DA in DMSO was added into each well with the final concentration of 50 \(\mu\)M. After cultured for another 30 min, cells were irradiated with ultrasound (1 MHz, 1 W cm\(^{-2}\), 40% duty cycle) or 660 nm light (1 W cm\(^{-2}\)) for 5 min. It is noteworthy that the space between the wells in the plate should be filled with PBS to mediate the ultrasound transmission. After washed with PBS twice, the cells were imaged under an inverted fluorescence microscope (IX 83, Olympus, Japan). The fluorescence intensity was quantified using CellSens software (Olympus, Japan).

1.11. Cellular internalization study. Au@BP nanohybrids were labled by rhodamine B conjugated polyethylene glycol thiol. Briefly, rhodamine B conjugated polyethylene glycol thiol (1 mg mL\(^{-1}\)) was sonicated with Au@BP nanohybrids (with BP concentration at 100 \(\mu\)g mL\(^{-1}\)) for 5 min. Then the mixture was kept in dark for 1 h. Afterwards, RhB-Au@BP nanohybrids were collected by centrifugation and washed with water for four times. To study the cellular internalization of the nanohybrids, cells were planted into a 96-well plate at density of \(7 \times 10^4\) cells per milliliter and incubated overnight. Then the cell culture medium was replaced by fresh medium containing three different endocytosis inhibitors: a) chlorpromazine (10 \(\mu\)g mL\(^{-1}\)); b) M\(\beta\)CD (13 mg mL\(^{-1}\)); or c) amiloride (0.53 mg mL\(^{-1}\)). After 30 min of incubation, RhB-Au@BP nanohybrids were added with the final BP concentration at 30 \(\mu\)g mL\(^{-1}\). To investigate the influence of ultrasound or NIR light on the cellular internalization of the nanohybrids, cells were irradiated under ultrasound (1 MHz, 1 W cm\(^{-2}\), 40% duty cycle) or 660 nm light (1 W cm\(^{-2}\)) for 5 min. Afterwards, cells were washed with pre-cold PBS twice and the nuclei were stained by Hoechst 33342. Finally, cells were washed with PBS twice and imaged under the inverted fluorescence microscope.

1.12. In vitro antitumor study. 4T1 cells were seeded into 96-well plate (8 \(\times\) 10\(^3\)/well) and cultured overnight. Then cells were exposed to BP nanosheets (25 \(\mu\)g mL\(^{-1}\)) or Au@BP nanohybrids (with BP concentration at 25 \(\mu\)g mL\(^{-1}\)) in DMEM medium for 3 h. Afterwards, the cells were irradiated under ultrasound (1 MHz, 1 W cm\(^{-2}\), 40% duty cycle) or 660 nm light (1 W cm\(^{-2}\)) for 5 min. It is noteworthy that the space between the wells in the plate should be filled with PBS to mediate the ultrasound transmission. After 12 h of incubation, the cell viability was determined using CCK-8 cytotoxicity assay kit.

1.13. In vivo antitumor study. Animal experiments were carried out under regulation approved by the Laboratory Animal Center of the Xiangya School of Medicine, Central South University (Changsha, China). Male BALB/c mice (6 weeks old) were purchased from Hunan Silaike experimental animal Co. Ltd (Changsha, China). To construct the tumor model, mice received subcutaneous injection of \(5 \times 10^6\) 4T1 cells on the back. After the tumor
volume reached 100 mm³, the mice were randomly divided into eight groups (n = 4 in each group): (1) saline as control, (2) BP, (3) Au@BP, (4) NIR light, (5) ultrasound, (6) BP + ultrasound, (7) Au@BP + NIR, (8) Au@BP + ultrasound. 20 μL of saline containing BP nanosheets (0.5 mg kg⁻¹) or Au@BP nanohybrids (with BP concentration at 0.5 mg kg⁻¹) were intratumorally injected. After 1 h, all the mice received intratumoral injection of 20 μL of SOSP (100 μM) in saline. After another 0.5 h, mice in group (5), (6) and (8) were irradiated under ultrasound (1 MHz, 2 W cm⁻², 40% duty cycle) for 5 min (after irradiation for 2.5 min, the ultrasound transducer was cooled down for 30 s using ice pack to prevent skin burn). Then mice were euthanized, the tumors were excised and cryosectioned with 6 μm thickness. The slices were fixed in polyformaldehyde, stained by DAPI and imaged under the inverted fluorescence microscope.

For in vivo antitumor study, mice were intratumorally injected with 20 μL of BP nanosheets (0.5 mg kg⁻¹) or Au@BP nanohybrids (with BP concentration at 0.5 mg kg⁻¹). After 2 h, the mice were irradiated under ultrasound (1 MHz, 2 W cm⁻², 40% duty cycle) or 660 nm NIR light (1 W cm⁻²) for 5 min. All the treatments were repeated on day 5. The tumor volume as well as body weight was recorded every other day. Twelve hours after the second treatment, tumors were harvested from the mice and homogenized to obtain cell suspensions. Then cells were stained with Annexin V-FITC/PI apoptosis detection kit and analyzed on flow cytometer (FACS-Calibur, Becton Dickinson, USA). On day 15, all the mice were euthanized, and the major organs (heart, liver, spleen, lung and kidney) as well as tumors were excised for histopathologic study. The tumor growth inhibition (TGI) rates were calculated according to following equation.

\[
\text{TGI} = \frac{V_C - V_T}{V_C} \times 100\% \quad (2)
\]

where \(V_C\) and \(V_T\) represent the tumor volume before and after the treatment, respectively.
2. Supplementary Figures and Table

**Fig. S1** a) Size distribution analysis and zeta potential of BP nanosheets and Au@BP nanohybrids, b) AFM image of BP nanosheets, and c) the thickness analysis along the white line is shown in b).

**Fig. S2** Raman spectra of bulk BP and BP nanosheets.

**Fig. S3** UV-Vis spectra of Au@BP nanohybrids with atomic ratio of Au increased from 0 to 40%. Insert: the digital photo of BP nanosheets and Au@BP nanohybrids in water.
Fig. S4 XRD analysis of Au@BP nanohybrids.

Fig. S5 a) NaN₃ (10 mM) as a singlet oxygen scavenger inhibits the fluorescence increase of SOSG. b) UV-Vis absorbance spectra of Au@BP nanohybrids and c) SOSG reveal that NaN₃ has little influence on both Au@BP nanohybrids and SOSG, excluding the destruction of NaN₃ to the chemical structures of Au@BP nanohybrids and SOSG.

Fig. S6 TEM image of Au NPs.
Fig. S7 UV-Vis absorption spectra of Au@BP nanohybrids and ICG before and after ultrasound irradiation.

Fig. S8 Photos of Au@BP nanohybrids dispersed in (i) water and (ii) cell culture medium at (a) 0 h and (b) 24 h.

Fig. S9 Fluorescence spectra of Au@BP nanohybrids and RhB-Au@BP nanohybrids. The excitation wavelength was 530 nm.
**Fig. S10** Endocytosis of RhB-Au@BP nanohybrids under different inhibitors: (1) blank control, (2) amiloride, (3) chlorpromazine, (4) MβCD (scale bar = 100 μm). (b) Quantification of intracellular fluorescence intensity in (a).

**Fig. S11** Endocytosis of RhB-Au@BP nanohybrids under ultrasound in the presence of MβCD (scale bar = 50 μm).

**Fig. S12** Cell viability of 4T1 cells treated by Au@BP nanohybrids with or without NIR light irradiation.
**Fig. S13** a) Cellular uptake of RhB-Au@BP nanohybrids with or without 660 nm NIR irradiation. The nuclei were stained by Hoechst33342. b) Intracellular oxidative stress after the treatment with Au@BP nanohybrids under NIR light or ultrasound irradiation was imaged with DCFH-DA as probe.

**Fig. S14** Intratumoral $^1$O$_2$ generation after different treatments using SOSG as probe: (1) saline as control, (2) BP nanosheets, (3) Au@BP nanohybrids, (4) ultrasound, (5) BP nanosheets with ultrasound, (6) Au@BP nanohybrids with ultrasound. The nuclei were stained by Hoechst 33342.
Fig. S15 Cells isolated from tumor-bearing mice were stained by PI and Annexin V-FITC, and analyzed by flow cytometry after different treatments: (1) saline as control, (2) BP nanosheets, (3) Au@BP nanohybrids, (4) NIR light, (5) ultrasound, (6) BP nanosheets with ultrasound, (7) Au@BP nanohybrids with NIR light, (8) Au@BP nanohybrids with ultrasound.

Fig. S16 Change of body weight during the treatments.
Fig. S17  H&E staining of slices from the main organs (heart, liver, spleen, lung and kidney) after different treatments: (1) saline as control, (2) BP nanosheets, (3) Au@BP nanohybrids, (4) NIR light, (5) ultrasound, (6) BP nanosheets with ultrasound, (7) Au@BP nanohybrids with NIR light, (8) Au@BP nanohybrids with ultrasound.

Fig. S18  Cytotoxicity of Au@BP nanohybrids to three different cell lines (4T1 cells, L929 cells and HeLa cells).
### Table S1. Blood biochemistry analysis of mice injected with Au@BP nanohybrids.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal Range*</th>
<th>Control</th>
<th>Au@BP</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC (× 10^{12})</td>
<td>6.93 – 12.24</td>
<td>11.02 ± 0.40</td>
<td>10.94 ± 0.36</td>
</tr>
<tr>
<td>WBC (× 10^9)</td>
<td>8.62 – 14.03</td>
<td>10.09 ± 0.74</td>
<td>10.55 ± 0.95</td>
</tr>
<tr>
<td>PLT (× 10^{12})</td>
<td>0.42 – 1.70</td>
<td>1.45 ± 0.07</td>
<td>1.57 ± 0.08</td>
</tr>
<tr>
<td>HGB (g L⁻¹)</td>
<td>126 – 205</td>
<td>161 ± 12.96</td>
<td>157 ± 13.35</td>
</tr>
<tr>
<td>BUN (mmol L⁻¹)</td>
<td>2.50 – 9.28</td>
<td>6.1 ± 0.22</td>
<td>5.5 ± 0.37</td>
</tr>
<tr>
<td>Cr (mmol L⁻¹)</td>
<td>17.68 – 35.36</td>
<td>29.80 ± 1.41</td>
<td>32.03 ± 0.87</td>
</tr>
<tr>
<td>AST (IU L⁻¹)</td>
<td>55 – 352</td>
<td>69 ± 6.79</td>
<td>82.92 ± 9.26</td>
</tr>
<tr>
<td>T-Bil (mmol L⁻¹)</td>
<td>3.42 – 8.55</td>
<td>6.0 ± 0.68</td>
<td>5.5 ± 0.79</td>
</tr>
</tbody>
</table>

* Normal ranges of healthy male Balb/c mice were referred to Charles River Laboratories (http://www.criver.com/) and the Jackson Laboratory (https://www.jax.org/).

### References
