Clearable black phosphorus nanoconjugate for targeted cancer phototheranostics

Jana, Deblin; Jia, Shaorui; Bindra, Anivind Kaur; Xing, Pengyao; Ding, Dan; Zhao, Yanli

2020

Jana, D., Jia, S., Bindra, A. K., Xing, P., Ding, D., & Zhao, Y. (2020). Clearable black phosphorus nanoconjugate for targeted cancer phototheranostics. *ACS Applied Materials and Interfaces*, 12(16), 18342–18351. doi:10.1021/acsami.0c02718

<https://hdl.handle.net/10356/146314>

<https://doi.org/10.1021/acsami.0c02718>

This document is the Accepted Manuscript version of a Published Work that appeared in final form in *ACS Applied Materials and Interfaces*, copyright © American Chemical Society after peer review and technical editing by the publisher. To access the final edited and published work see <https://doi.org/10.1021/acsami.0c02718>

Downloaded on 26 Apr 2023 15:16:40 SGT

Clearable Black Phosphorus Nanoconjugate for Targeted Cancer Phototheranostics

Deblin Jana,^{†,¶} Shaorui Jia,^{‡,¶} Anivind Kaur Bindra,[†] Pengyao Xing,[†] Dan Ding,^{*,‡} Yanli Zhao^{*†,§}

[†]Division of Chemistry and Biological Chemistry, School of Physical and Mathematical Sciences, Nanyang Technological University, 21 Nanyang Link, Singapore 637371, Singapore. E-mail: zhaoyanli@ntu.edu.sg

[‡]State Key Laboratory of Medicinal Chemical Biology, Key Laboratory of Bioactive Materials, Ministry of Education, College of Life Sciences, Nankai University, 94 Weijin Road, Tianjin 300071, China. Email: dingd@nankai.edu.cn

[§]School of Materials Science and Engineering, Nanyang Technological University, 50 Nanyang Avenue, Singapore 639798, Singapore

ABSTRACT: Therapeutic efficacy of synergistic photodynamic therapy (PDT) and photothermal therapy (PTT) is limited by complex conjugation chemistry, absorption wavelength mismatch and inadequate biodegradability of the PDT-PTT agents. Herein, we designed biocompatible copper sulfide nanodot anchored folic acid-modified black phosphorus nanosheets (BP-CuS-FA) to overcome these limitations, consequently enhancing the therapeutic efficiency of PDT-PTT. *In vitro* and *in vivo* assays reveal good biocompatibility and commendable tumor inhibition efficacy of the BP-CuS-FA nanoconjugate owing to synergistic PTT-PDT mediated by near-infrared laser irradiation. Importantly, folic acid unit could target folate receptor overexpressed cancer cells, leading to enhanced cellular uptake of BP-CuS-FA. BP-CuS-FA also exhibits significant contrast

effect for photoacoustic imaging, permitting its *in vivo* tracking. The photodegradable character of BP-CuS-FA is associated with better renal clearance after the antitumor therapy *in vivo*. The present research may facilitate further development on straightforward approaches for targeted and imaging-guided synergistic PDT-PTT of cancer.

KEYWORDS: black phosphorus, photoacoustic imaging, renal clearance, synergistic therapy, targeted therapy

Introduction

Near-infrared (NIR) light facilitated phototherapeutic approaches, especially photodynamic therapy (PDT) and photothermal therapy (PTT), have shown quite a few advantages including enhanced spatial resolution, low side effects, high selectivity, minimal invasiveness and cost effectiveness over conventional cancer treatment methods.¹⁻³ PDT comprises light induced activation of photosensitizers (PS) to produce cytotoxic reactive oxygen species (ROS) from normal tissue oxygen. However, clinical applications of PDT are often obstructed by poor stability and low aqueous solubility of organic PS molecules as well as their incapability to be activated by NIR window (700-1100 nm) with high and tolerable tissue penetration depth.^{4,5} On the other hand, PTT involves mainly plasmonic nanomaterials inducing hyperthermia upon exposure to pulsed or continuous wave laser.⁶⁻⁸ Nevertheless, very high temperature (>70 °C) induced by photothermal heating may cause collateral damage toward healthy tissues. Thereby, the development of integrated PDT-PTT nanoplatfoms should allow the usage of moderate photothermal heating along with ROS-mediated cellular damage to ensure sufficient cancer treatment without obvious side effects. In this context, some contributions have been made toward conjugating PTT-active plasmonic nanomaterials with PDT-active organic PS molecules.^{3,9,10} However, complicated

conjugation chemistry, the incongruity between the absorption wavelengths of PTT and PDT agents, and the toxicity of nanoconjugates are still critical downsides restricting full utilization of hybrid nanostructures.¹¹

Black phosphorus (BP) nanosheets have been extensively used as a next generation water-dispersible inorganic PS to overcome the difficulties associated with organic PS.¹² Tunable layer-dependent energy bandgap and anisotropic property direct BP to achieve fascinating electronic and optoelectronic applications.¹³⁻¹⁵ BP nanosheets have a high surface to volume ratio, allowing it to act as a delivery platform with high loading capacity.¹⁶ Taking advantage from the excellent biocompatibility and biodegradation properties, BP nanosheets and nanodots are suitable as effective PDT, PTT and imaging agents.¹⁷⁻²² While BP nanosheets have high extinction coefficient per volume, few-layer two-dimensional nanomaterials often cannot effectively harvest incident light for the activation.²³ A synergy between BP nanosheets and plasmonic nanoparticles would thereby be inferred to adjust the electronic properties for enhanced PTT and PDT.²⁴ Thus, plasmonic copper sulfide (CuS) nanoparticles, a well-reported PTT agent, are considered here for their broad absorption in the NIR window.^{25,26} As compared with gold-based plasmonic nanostructures, CuS nanoparticles only require cheaper starting materials and a straightforward synthesis procedure to ensure morphological consistency. CuS nanoparticles with size of <10 nm, preferably called CuS nanodots, indicate efficient renal clearance for better biocompatibility.^{27,28} However, renal clearance of these small nanodots often compromises their considerable tumor accumulation for therapy.²⁹ Thereby, a smart nanoplatform designed by a conjugation of BP nanosheets and CuS nanodots could complement by each other for effective tumor accumulation and subsequent renal clearance.

Scheme 1. Schematic illustration for the preparation of BP-CuS-FA nanoconjugate for PAI-guided and tumor-targeted synergistic PDT-PTT in cancer treatment.

To achieve the stated goal, we designed a nanoconjugate (designated as BP-CuS-FA) comprising three primary constituents, *i.e.*, BP nanosheets as the delivery platform, CuS nanodots as the NIR light harvester, and a folate receptor targeting polymer (Scheme 1). The components were premeditated for the conjugation through a physisorption process. The conjugation was expected to make the electronic properties of the inorganic components suitable for the biomedical application. Following this hypothesis, a synergistic PDT-PTT effect was observed as the nanoconjugate formed could be excited by a single wavelength of NIR light. Folic acid-modified polyethylene glycol amine (FA-PEG-NH₂) was utilized as the tumor targeting polymer, which specifically provided an increased physiological stability, biocompatibility and folate receptor mediated active targeting for enhanced tumor uptake.^{9,30} Moreover, the nanoconjugate could photothermally generate ultrasound signals to display strong contrast effect for photoacoustic imaging (PAI), permitting its *in vivo* tracing.³¹⁻³³ Optical imaging combined with deep tissue

infiltration of PAI afforded high contrast and high resolution for achieving efficient noninvasive imaging.^{34,35}

After intravenous injection, BP-CuS-FA was anticipated to accumulate in the tumor by the folate receptor facilitated active targeting. Upon cellular internalization, the nanoconjugate would produce thermal energy and ROS under NIR light irradiation, causing the disruption of endosomes and the induction of cancer cell apoptosis. Importantly, BP nanosheets would be degraded by the generated ROS through an oxidative process, leading to an efficient renal clearance of BP-CuS-FA to ensure minimal side effect throughout the therapeutic process.^{36,37} Thus, this strategy shows a promising potential to inspire more straightforward methodologies for targeted and synergistic PDT-PTT of cancer.

RESULTS AND DISCUSSION

To validate the design, BP nanosheets were first exfoliated from bulk BP following a modified literature procedure.¹⁹ Bulk BP was first immersed in liquid nitrogen prior to the immersion in water and probe sonication for green exfoliation. The process was less time-consuming than the reported procedure. The cryo-pretreatment weakens the interlayer van der Waals forces and forms cracks between layers to permit the solvent insertion for efficient exfoliation.³⁸ The as-prepared BP nanosheets were non-covalently conjugated with FA-PEG-NH₂ to avoid non-specific protein binding and enable targeting effect through folate-receptor mediated endocytosis. Glutathione (GSH) capped CuS nanodots were then conjugated with the as-prepared BP-FA to provide the final nanoconjugate BP-CuS-FA. The major rationale behind the conjugation on BP nanosheets is physisorption process. Oxygen and nitrogen atoms being comparatively more electronegative could accept electron density from the phosphorene surface.³⁹ This rationale

inspired us to choose functionalities (GSH and FA-PEG-NH₂) consisting mainly amine and carboxylic groups for the physisorption. Transmission electron microscopy (TEM) images of BP nanosheets, CuS nanodots, and BP-CuS-FA demonstrated good distribution of nanodots on BP nanosheets, supporting successful conjugation process (Figures 1A and S1). The energy dispersive spectroscopy (EDS) elemental mapping of BP-CuS-FA displayed the elemental distributions of P and S elements (Figure S2). Atomic force microscopy (AFM) images showed topographic morphology of the nanosheets, and corresponding thickness was deduced by the cross-sectional analysis (Figures 1B,C and S3). The measured heights of BP nanosheets and BP-CuS-FA were compared, and a significant difference of ~7-10 nm was confirmative of the conjugation process. The loading of CuS nanodots on the BP nanosheet surface was calculated to be 39 wt% by inductively coupled plasma - optical emission spectrometry.

Figure 1. Characterizations of BP-CuS-FA. (A) TEM image of BP-CuS-FA. (B) AFM image of BP-CuS-FA. Scale bar: 200 nm. (C) AFM height profile of the indicated lines in the image (B). (D) UV–vis–NIR absorption spectra of BP-CuS-FA, BP and CuS. (E) Size distributions of BP-CuS-FA, BP and CuS determined by DLS. (F) Zeta potential of BP-CuS-FA, BP-FA, BP and CuS. (G) Survey XPS spectrum of BP-CuS-FA. (H) FTIR spectra of BP-CuS-FA, BP-FA and CuS. (I) Raman spectra of BP-CuS-FA, BP-FA and BP.

The stepwise conjugation process was further confirmed by UV-vis spectra, dynamic light scattering (DLS), ζ -potential measurements, X-ray photoelectron spectroscopy (XPS), Fourier transform infrared (FT-IR) spectra, and Raman spectra. UV-vis spectra revealed broad absorption peak of the BP-CuS-FA nanoconjugate in the NIR range, supporting its suitability for NIR laser activated therapy (Figure 1D). Different CuS loading percentage was indicated through the structural changes of absorbance spectra (Figure S4). The bandgap analysis showed a bandgap value of 2.31 eV for the nanoconjugate, also indicating the formation of a heterojunction (Figure S5). The hydrodynamic size increased gradually upon stepwise conjugation, with a final size of 250 nm for BP-CuS-FA (Figure 1E). BP-CuS-FA showed a negative ζ -potential value after the preparation (Figure 1F). XPS measurements depicted the presence of phosphorus, copper, sulfur and nitrogen elements in BP-CuS-FA, confirming successful conjugation of individual components (Figures 1G and S6). The stepwise conjugation was also traced by FT-IR spectra (Figure 1H). BP-FA was comprised of an absorption band at $\sim 2900\text{ cm}^{-1}$ ascribing to the C–H vibration in the PEG segment and a characteristic stretching vibration band at $\sim 1637\text{--}1653\text{ cm}^{-1}$ from the amide unit within the FA structure, thereby confirming the coating of FA-PEG-NH₂ on BP. Broad N–H stretching vibration signal at $\sim 3250\text{ cm}^{-1}$, the C–N vibration signal at 1600 cm^{-1} ,

and the N–H vibration signal at 1394 cm^{-1} were observed from the FT-IR spectrum of CuS nanodots, indicating the presence of GSH molecule on the surface. Moreover, the intensity of the S–H stretching vibration at $\sim 2500\text{ cm}^{-1}$ was noticeably diminished, which could be attributed to the formation of the Cu–S bonding. The FT-IR spectrum of BP-CuS-FA retained all characteristic peaks of the individual components. Raman scattering analysis of BP revealed three prominent peaks at 361.9 , 439.1 and 467.0 cm^{-1} , assigned to A^1_g , B^2_g and A^2_g modes, respectively.⁴⁰ These peaks are characteristic to the bulk counterpart and comparable to BP-CuS-FA, suggesting the fact that the structural features were unchanged throughout the exfoliation and fabrication process (Figure 1I).

Figure 2. Photothermal and Photodynamic performance. (A) Photothermal conversion behavior of BP-CuS-FA at different concentrations (0-200 $\mu\text{g/mL}$) under laser irradiation (808 nm, 1.50 W cm^{-2}). (B) Heating and cooling curves of BP-CuS-FA aqueous solution under 808 nm along with the linear time data obtained from the cooling period. (C) Decay curves of DPBF absorption at 426 nm corresponding to different solutions as a function of irradiation time by 808 nm laser at 1.0 W cm^{-2} . (D) ESR spectra demonstrating $^1\text{O}_2$ generation under 808 nm for different groups. (E)

ROS generation evaluated by DCFDA in 4T1 cells treated by PBS, BP, CuS and BP-CuS-FA under laser irradiation (808 nm, 1.0 W cm⁻²). Scale bar: 100 μm.

To determine the photothermal efficiency, BP-CuS-FA was dissolved in phosphate-buffered saline (PBS, pH = 7.4) at different concentrations and irradiated by 808 nm laser (1.50 W cm⁻²) over a period of 10 min. The solutions containing BP-CuS-FA showed a substantial increase in temperature (ΔT of up to $\sim 30.8^{\circ}\text{C}$) as compared to the control group (PBS), confirming its efficiency as a photothermal agent (Figure 2A). According to the fitting cooling curve, the photothermal conversion efficiency of BP-CuS-FA was calculated to be 62.6% for 808 nm (Figure 2B). Four laser on/off cycles of laser irradiation indicated good photothermal stability of the nanoconjugate (Figure S7). Interestingly, control studies corroborated that the BP-CuS-FA nanoconjugate presented enhanced efficiency as compared to individual components, *i.e.*, BP and CuS, under the same conditions (Figure S8A). This result suggested that, when these two semiconductor type nanostructures were combined, there was a modification of electronic energy levels to further enhance the property of laser-mediated photothermal heating.^{41,42} Finally, a laser power dependent study was performed to optimize the laser intensity of 1.0 W cm⁻² for achieving substantial photothermal heating to ablate cancer cells (Figure S8B).

In order to evaluate the singlet oxygen generation capacity, 1,3-diphenylisobenzofuran (DPBF) was used, which could undergo Diels–Alder 1,4-cycloaddition with ¹O₂ to exhibit decreased intensity of the absorption maxima. Under 808 nm laser irradiation, both BP and BP-CuS-FA showed prominent ROS generation over time as compared to the control groups (Figures 2C and S9). ABDA was also used as a specific ¹O₂ generation indicator to further support the photodynamic performance of BP-CuS-FA under NIR laser irradiation (Figure S10A,B). Addition

of an ROS specific scavenger NaN_3 resulted in less prominent absorbance intensity decrement of ABDA over time, which further proved the $^1\text{O}_2$ generation ability of BP-CuS-FA under 808 nm laser irradiation (Figure S10C,D). Electron spin resonance (ESR) was also employed to detect the generation of $^1\text{O}_2$ (Figure 2D). Among the various groups, the peak intensity of the BP-CuS-FA and BP groups increased more significantly compared to the CuS group, further signifying that BP-CuS-FA could serve as an efficient photosensitizer for $^1\text{O}_2$ generation under 808 nm. To further confirm the singlet oxygen generation capacity at cellular level, intracellular ROS generation was assessed using a 2',7'-dichlorofluorescein diacetate (DCFDA) cellular ROS detection assay kit. Upon the administration and laser irradiation, the as-produced ROS could convert DCFDA to green fluorescent DCF that could be imaged by confocal laser scanning microscopy (CLSM). Under 808 nm laser irradiation, CLSM investigations revealed that BP and BP-CuS-FA could elevate the ROS level of the cells, whereas CuS showed negligible singlet oxygen generation ability (Figure 2E). A control experiment revealed that BP-CuS-FA was unable to produce ROS under *in vitro* hypoxic condition (Figure S11), further demonstrating that the said process is an oxygen dependent process and $^1\text{O}_2$ generation under NIR light irradiation is the main source of ROS elevation. Control experiments with an ROS scavenger *N*-acetylcysteine further indicated the ROS generation capability of BP-CuS-FA (Figure S12). We also inspected the biocompatibility of BP-CuS-FA through hemolysis assay (Figure S13). No significant hemolysis (<12%) can be found when BP-CuS-FA was incubated with red blood cells for 4 h at 37 °C.

Figure 3. *In vitro* targeted and synergistic PDT-PTT. (A) Time dependent CLSM images showing green fluorescence of FITC in 4T1 cells treated with FITC-stained BP-CuS-FA. For blocking the folate receptor on tumor cells, the cells were pre-treated with FA at 1 h before the nanoconjugate treatment. Scale bar: 100 μ m. (B) Concentration dependent cytotoxicity of BP-CuS-FA on HeLa, HepG2 and 4T1 cells for 24 h determined by MTT assay (n = 3). (C) Cell viability assay of 4T1 cells after the incubation with PBS, BP, CuS and BP-CuS-FA at different concentrations under laser irradiation (808 nm, 1.0 W cm⁻², 8 min). (D) CI of PDT and PTT by BP-CuS-FA over 4T1 cells. (E) Live/dead cell assay for 4T1 cells treated with PBS, BP, CuS and BP-CuS-FA with or without laser irradiation (808 nm, 1.0 W cm⁻²). Scale bar: 200 μ m. ***p < 0.001, **p < 0.01, or *p < 0.05.

The photothermal property of BP having semiconductor like features with low density of carriers can be attributed to phonon emission.²⁰ On the other hand, localized surface plasmon resonance is a well-established cause of the photothermal induction from CuS based nanomaterials.²⁵ Upon the formation of a heterojunction in BP-CuS-FA, the valance band (VB) of

CuS situates between the conduction band (CB) and VB of BP.^{43,44} During light illumination, the electrons in the VB of the BP jump to its CB, most of which tend to recombine through the radiative recombination.⁴⁵ However, the VB of CuS gives an additional pathway for the electrons to come back to the ground state through nonradiative recombination. CuS being a *p*-type nanomaterial and BP being an *n*-type semiconductor, the carrier populations of electrons and holes in the CB of BP and the VB of CuS remain very high, respectively. This in turn increases more with the entry of the photoexcited carriers during the illumination, which further accelerates this nonradiative recombination. The increase in nonradiative recombination leads to the release of the energy through phonons or thermal energy, ultimately causing the enhancement of photothermal performance. On the other hand, singlet oxygen generation for photodynamic property is postulated to undergo nonradiative energy transfer from long-lived triplet excitons to ground state oxygen molecule. BP nanosheets possessing high excitonic effects are thereby proposed to be effective in generating ¹O₂ under visible and NIR light irradiation.^{46,47} Moreover, BP nanosheets can exhibit robust many-body effects, which may contribute additionally to the photocatalytic behavior. The absorption behavior of BP-CuS-FA suggests a similar light harvesting ability across the NIR region in comparison to BP. The photodynamic performance of the nanoconjugate remains similar under both 660 nm and 808 nm light irradiation, unlike that of BP (Figure S14). This result leads to synergistic and dual-modal phototherapeutic performance of BP-CuS-FA under 808 nm laser illumination.

We also inspected the stability of BP-CuS-FA in ambient conditions as a function of time to confirm the surface passiveness imparted by the conjugation process. The absorbance of BP-CuS-FA did not change significantly over a course of 10 days, whereas unmodified BP showed a significant decrement in absorbance intensity (Figure S15). This phenomenon could be explained

by more surface availability of pristine BP, which gets oxidized in the presence of water and oxygen as compared to a passive surface of BP-CuS-FA. The ability of BP to produce $^1\text{O}_2$ also significantly decreased owing to the said process, whereas BP-CuS-FA exhibited minimal photodynamic activity decrement (Figure S16). When irradiated by laser, an insignificant change in absorbance for BP-CuS-FA could be observed (Figure S17). However, when the nanoconjugate was kept for 24 h in ambient conditions after laser irradiation, a significant decrement of absorbance spectra was observed. The generated $^1\text{O}_2$ upon laser irradiation on BP-CuS-FA could oxidize the surface phosphorus species over a course of time to promote the degradation of the said nanoconjugate. In addition, the degradation mechanism of BP inside cells or organisms often depends on the pH and activities of lysosomal enzymes amongst other factors, and thereby it is less comparable to solution studies.⁴⁸

We then investigated the cellular uptake of BP-CuS-FA stained with a fluorescent dye fluorescein isothiocyanate (FITC) in order to confirm its targeted efficacy through the folate receptor mediated endocytosis. Folate receptor overexpressed cells could be preferentially targeted by FA conjugated nanosystems. Therefore, a pre-treatment of the cells with FA to bind to the folate receptor would restrict efficient uptake of these nanosystems.⁴⁹ CLSM images confirmed efficient uptake of FITC-labeled BP-CuS-FA by FR-positive 4T1 cells (Figure 3A), where green fluorescence was clearly observed. For the cells pre-treated by FA, very weak fluorescence was detected. Comparative time-dependent uptake of FITC-labeled BP-CuS-FA by FR-positive HeLa cells and FR-negative HepG2 cells further demonstrated the targeting efficacy of the nanoconjugate (Figure S18).⁵⁰ After confirming the ROS generation and targeting efficacy of the nanocomposite, its *in vitro* cytotoxicity with and without laser irradiation was investigated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay. Cell viability tests on

4T1, HeLa and HepG2 cells did not show any significant toxicity when incubated with BP-CuS-FA at concentrations up to 200 $\mu\text{g/mL}$ over the course of 24 h without any laser irradiation (Figure 3B). Human embryonic kidney (HEK293) cells also did not show any significant cytotoxicity upon the incubation with BP-CuS-FA over a period of 24 h (Figure S19). When irradiated with 808 nm laser (1.0 W cm^{-2}) for 8 min, the cell viability of $\sim 6\%$ for 100 $\mu\text{g/mL}$ BP-CuS-FA indicated an effective cell-killing effect by synergistic PDT-PTT (Figure 3C). The concentration dependent study revealed the therapeutic efficacy of BP-CuS-FA over individual counterparts, *e.g.*, BP and CuS. In addition, BP-CuS-FA exhibited improved cell killing efficiency as compared to the control system BP-CuS without the FA targeting ligand. Moreover, we conjugated FA onto BP and CuS to synthesize BP-FA and CuS-FA respectively, in order to further study the targeting ability. When conjugated with fluorescent FITC dye, both BP-FA and CuS-FA could show targeted accumulation over time as compared to the control studies (Figure S20A,B). Both BP-FA and CuS-FA exhibited higher cytotoxicity in 4T1 cells when irradiated with laser as compared to their BP and CuS counterparts, respectively (Figure S20C,D). The folate receptor mediated targeting efficacy was further demonstrated by a concentration-dependent cytotoxicity study of BP-CuS-FA in HepG2 cells under laser irradiation (Figure S21).

The cytotoxicity of BP-CuS-FA for 4T1 cells under laser irradiation and hypoxic condition was studied. The low concentration of oxygen in hypoxic cells could only allow PTT to be effective compared to a synergistic PDT-PTT efficacy in normoxic condition, which could be inferred from the concentration dependent cell viability trend (Figure S22). The combination index ($\text{CI} < 1$) of PDT and PTT was calculated based on the MTT assay (Figure 3D).⁵¹⁻⁵³ The data points were collected after comparing the therapeutic efficacy of groups, namely BP, CuS and BP-CuS-FA, under comparable concentrations of the components. This study confirmed that two

components (BP and CuS) as well as therapeutic modalities (PDT and PTT) followed an excellent synergy to treat cancer cells. To further validate the observations by CLSM, calcein AM and propidium iodide (PI) were used to stain live and dead cells, respectively. In contrast to the control groups, BP-CuS-FA with effective synergistic therapy through PDT and PTT displayed enhanced cytotoxicity upon 808 nm laser irradiation, as indicated by very weak green fluorescence and bright red fluorescence (Figure 3E). An investigation of the said experiment on hypoxic cells resulted in faint red fluorescence compared to the normoxic condition, further confirming the efficacy of the synergistic PDT-PTT of BP-CuS-FA (Figure S23).

Concentration-dependent *in vitro* PAI of BP-CuS-FA was conducted to demonstrate the imaging ability of the nanoconjugate (Figure 4A). The photoacoustic amplitudes of the nanoconjugates at 850 nm over a series of concentrations indicated good linear relationship (Figure 4B). Moreover, the photoacoustic property of BP-CuS-FA was investigated by recording the photoacoustic intensity at different wavelengths from 680 to 900 nm (Figure S24). Similar investigations were carried out for a control group BP-CuS, which also showed comparable *in vitro* PAI performance as that of BP-CuS-FA (Figure S25). Photoacoustic signal of BP-CuS-FA in tumor microenvironment was shown under NIR pulsed laser irradiation, and the photoacoustic spectrum was found to be in alignment with the NIR absorption data. To investigate the tumor targeting ability of BP-CuS-FA *in vivo*, we used 4T1 tumor bearing mice as a xenograft model. After systematic administration of BP-CuS-FA and BP-CuS *via* tail vein injection, photoacoustic signals in the tumor region of the living mice were recorded and quantified. The photoacoustic intensity gradually enhanced for the case of BP-CuS-FA over time, reaching an appreciable signal at 7 h (Figure 4C,D). The observed photoacoustic intensity for BP-CuS at the same time point was

~4.2-fold lower than that of BP-CuS-FA. This result supported the folate-receptor mediated endocytosis pathway taken up by BP-CuS-FA for cellular accumulation.

Figure 4. PAI studies. (A) *In vitro* PAI of BP-CuS-FA with different concentrations upon the excitation at 850 nm. (B) Photoacoustic (PA) amplitudes of BP-CuS-FA at 850 nm as a function of concentrations. (C) PAI of tumor sites after intravenous administration of BP-CuS-FA and BP-CuS for selected time intervals. Scale bar: 2 mm. (D) Photoacoustic (PA) intensity at tumor sites against post-injection time. *** $p < 0.001$, ** $p < 0.01$, or * $p < 0.05$.

The *in vivo* photothermal effect of BP-CuS-FA was evaluated by monitoring the thermal image of the tumor region upon the irradiation of 808 nm laser (Figures 5A and S26). A time-dependent study demonstrated that BP-CuS-FA could induce a temperature difference of $\sim 26.3^\circ\text{C}$ within 8 min of 808 nm laser irradiation (1.0 W cm^{-2}) as compared to the PBS control group, which was significant for an efficient PTT. The BP and CuS control groups did not show considerable temperature increase, on account of their lower accumulation in the tumor. The BP-CuS control

group only presented a temperature difference of ~ 7.5 °C, again demonstrating the importance of tumor-targeting capacity by BP-CuS-FA. For the evaluation of *in vivo* therapeutic effect, 4T1 tumor bearing mice were divided into seven groups: (a) BP-CuS-FA + 808 nm (PDT/PTT/Targeting group), (b) BP-CuS + 808 nm (Targeting control group), (c) BP + 808 nm (laser control group), (d) CuS + 808 nm (laser control group), and (e) PBS + 808 nm (laser control group), (f) BP-CuS-FA (control group), and (g) PBS (control group). As compared with the PBS control group, no significant therapeutic effect was observed from BP-CuS-FA without laser irradiation. Amongst the laser irradiation groups, BP-CuS-FA showed comparatively better tumor inhibition over a course of 16 days than its control counterparts, owing to the targeting effect and synergistic PDT-PTT (Figure 5B). During the therapeutic experiments, body weights of the mice were monitored over the course of 16 days to assess long term toxicity of these therapeutic systems (Figure 5C). No noteworthy changes were observed from respective treated groups when compared to initial body weights of the mice.

To investigate the renal clearance property of BP-CuS-FA, we monitored the phosphorus content in urine of the mice at different time intervals after intravenous injection of the nanoconjugate. Laser irradiation in the tumor region after the nanoconjugate administration for 6 h led to enhanced nanoconjugate excretion overtime as compared to the control group without the laser irradiation (Figure S27). This observation can be attributed to the dissociation of BP into phosphorus oxides under laser irradiation, enabling the degraded species to be excreted efficiently. Moreover, the XPS analysis indicated that the degraded species collected from the urine sample consisted mostly of the PO_4^{3-} anion, in agreement with the oxidation of the BP solution in air (Figure 5D). The excreted form of phosphorus as PO_4^{3-} was also confirmed by the presence of a single prominent peak at 2.98 ppm in the ^{31}P -NMR spectrum (Figure 5E).⁵⁴ Additionally, the

haematoxylin and eosin (H&E) staining analysis of excised tumor further proved that the tumor tissues were more seriously damaged by targeted PDT-PTT of BP-CuS-FA than the control groups (Figure 5F). Other major organs, including kidney, liver, lung, heart and spleen, showed no major abnormality, demonstrating negligible side effect of BP-CuS-FA based treatment (Figure S28).

Figure 5. *In vivo* therapy and renal clearance. (A) IR thermal images of 4T1-bearing mice under 808 nm laser irradiation (1.0 W cm^{-2}) at 6 h after systemic administration of saline, CuS, BP, BP-FA and BP-CuS-FA ($n = 5$). (B) Relative tumor volume and (C) body weight of mice after different treatments at different time points ($n = 5$). (D) XPS spectra (P 2p) of BP-CuS-FA before the treatment and after renal excretion from mice. (E) ³¹P NMR spectrum of BP-CuS-FA after renal excretion from mice. (F) H&E staining of tumors from 4T1 tumor-bearing mice at day 16 after different treatments. Scale bar: 100 μm . *** $p < 0.001$, ** $p < 0.01$, or * $p < 0.05$.

CONCLUSION

In summary, we have developed a multifunctional nanoconjugate (BP-CuS-FA) by integrating BP nanosheets with plasmonic CuS nanodots and targeting moiety FA-PEG-NH₂ in a mild and straightforward process. Our present studies have demonstrated single wavelength NIR light activated synergistic PDT-PTT process by BP-CuS-FA with both biocompatible and photodegradable characters. Minimally invasive intravenous injection of the nanoconjugate leads to folate receptor mediated endocytosis in tumor cells for enhanced intracellular uptake. The tumor growth is significantly inhibited after synergistic PDT-PTT treatment by BP-CuS-FA *in vitro*. In addition, the BP-CuS-FA nanoconjugate displays intense PA signal for its tracking of tumor accumulation *in vivo*. This work would pave an avenue for developing a simple strategy of targeted PDT-PTT toward cancer theranostics.

EXPERIMENTAL METHODS

Synthesis of FA-PEG and PEG Coated BP Nanosheets. FA-PEG-NH₂ (25 mg) was dissolved in ultrapure water (2 mL) and transferred to BP solution (10 mL, 0.5 mg/mL) under constant ultrasonication in an ice-bath. After sonication for 30 min, the solution was further stirred at 500 rpm in ice-bath under inert atmosphere for 6 h. The obtained BP-PEG-FA solution was centrifuged at 8000 rpm at 4 °C for 45 min and washed 2 times with ultrapure water using a similar procedure to remove excess FA-PEG-NH₂. Finally, the precipitate was dissolved in ultrapure water and stored at 4 °C. BP-PEG was synthesized by following the same procedure, except PEG-NH₂ was used instead of FA-PEG-NH₂.

Synthesis of BP-CuS-FA Nanoconjugate. As prepared BP-PEG-FA (10 mL, 500 ppm) was mixed with CuS nanodot solution (5 mL, 1000 ppm), and the mixture was subsequently stirred in an ice-bath under inert atmosphere for 8 h for complete conjugation. The excess CuS nanodots were eliminated by centrifugation at 9000 rpm for 30 min. The precipitate was washed once with ultrapure water and then dispersed in ultrapure water for further experiments. A control compound BP-CuS was prepared following the same procedure while using BP-PEG instead of BP-PEG-FA.

Photodynamic Properties of Nanoconjugate. Typically, DMSO solution (10 mL) with BP-CuS-FA (0.1 mg/mL) and DPBF (0.2 mg/mL) was mixed in the dark for 30 min to achieve an equilibrium before the test. 808 nm laser (1.0 W cm^{-2}) was used as the illumination source. Absorption spectra of the irradiated samples were measured at different time points. BP and CuS with comparable concentrations were taken as the controls.

Photothermal Properties of Nanoconjugate. The photothermal heating trends were attained by monitoring the changes in temperature of different solutions upon the irradiation of 808 nm laser (1.50 W cm^{-2}). The temperature of the samples was recorded by an electronic thermometer. Control experiments were conducted using BP and CuS. Different laser power densities (1.5, 1.0, and 0.5 W cm^{-2}) were also used as the control.

***In Vitro* Antitumor Therapy.** To study the combination therapy of the nanoconjugate *in vitro*, the viability of 4T1 cells was tested by MTT assay. After culturing 4T1 cells in 96 well plates at 37°C for 24 h, different concentrations (0, 5, 25, 50, and $100 \mu\text{g/mL}$) of BP-CuS-FA were added and incubated in the dark. After 4h, cells were irradiated with 808 nm NIR laser at 1.0 W cm^{-2} for 8 min. BP and CuS were used for control groups for the same experiment. BP-CuS was employed as the control to evaluate the targeting property of the nanoconjugate. Untreated cells were accounted for 100% viability.

Intracellular ROS Assay: ROS generation inside cells under light irradiation was detected using the Image-IT LIVE green reactive oxygen species detection kit from Thermo Fisher. 4T1 cells (1×10^5 cells/well) were seeded on coverslips in a six-well plate and then incubated at 37 °C in the dark for 24 h. The medium was replaced with complete Dulbecco's modified eagle medium containing BP-CuS-FA. After the incubation for 4 h, DCFDA was added by following the standard protocol given by the supplier. After the incubation of 10 min, cells were washed thrice with PBS. Then, it was irradiated by 808 nm laser (1.0 W cm^{-2} , 5 min). PBS was used to wash the treated cells, and fluorescence images of the cells were attained under a confocal laser scanning microscope (Carl Zeiss LSM 800).

Animal Models: All methods of animal studies were carried out following the guidelines set by Tianjin Committee of Use and Care of Laboratory Animals. The overall project protocols were approved by the Animal Ethics Committee of Nankai University. Female BALB/c mice (6 weeks old) were acquired from the Laboratory Animal Center of the Academy of Military Medical Sciences (Beijing, China). To set up the xenograft mouse model bearing 4T1 tumor, RPMI-1640 medium (50 μL) was used to make a suspension of 1×10^6 4T1 cells. Right axillary space of mice was chosen for subcutaneous injection. Seven days later, the tumor volumes were measured to be about 80–120 mm^3 , and afterward the mice were treated.

***In Vitro* and *In Vivo* PAI:** Photoacoustic signals or images were acquired on a commercial Endra Nexus 128 photoacoustic tomography system (Endra, Inc., Ann Arbor, Michigan, USA). Photoacoustic images were then obtained at 850 nm at selected time periods after the injection. Concentration-dependent *in vitro* photoacoustic images were generated with BP-CuS-FA and BP-CuS upon the excitation at 850 nm ($n = 4$). The 4T1 tumor-bearing mice were anesthetized using

2% isoflurane in oxygen. Subsequently, BP-CuS-FA and BP-CuS (150 μ L, 200 μ g/mL) were intravenously injected into the mice through tail vein, respectively.

In Vivo Therapy: The xenograft mice bearing 4T1 tumor were randomly separated into seven groups (n = 5), namely (a) BP-CuS-FA + 808 nm, (b) BP-CuS + 808 nm, (c) BP + 808 nm, (d) CuS + 808 nm, (e) PBS + 808 nm, (f) BP-CuS-FA, and (g) PBS. For PBS and BP-CuS-FA groups, PBS and BP-CuS-FA (200 μ g/mL) were injected through tail vein of 4T1 tumor-bearing mice without subsequent laser irradiation. For the rest of the groups, after 6 h of intravenous injection of samples (200 μ g/mL), the tumors of the mice in each individual group were irradiated with 808 nm laser (1.0 W cm⁻²) for 8 min. Following the said treatments, the mouse body weights and tumor volumes were monitored every other day for a course of 16 days. The tumor volumes were calculated by the following equation: Volume = Width² $\times$ Length/2. Moreover, the changes in temperature for the laser irradiated groups in the tumor region upon 808 nm laser irradiation were checked and imaged every 2 min using an IR thermal camera (Fluke Shanghai Inc).

Histological Studies. After 16 days following the treatments, the abovementioned seven groups of mice were sacrificed. The important organs and tumors were excised and sliced. For H&E staining, corresponding tissues were stained and then fixed in formalin solution (4%). It was then processed into paraffin, and afterward sectioned into slices of 5 μ m thickness. The as-prepared slices were imaged using a digital microscope (Leica QWin).

Renal Excretion Studies: To investigate the fate of the samples after renal clearance, two groups, namely (a) BP-CuS-FA and (b) BP-CuS-FA + 808 nm laser, were intravenously injected to 4T1 tumor bearing mice (n = 4 per group). At different time points, urine was collected from mice by gentle abdominal pressure, and the phosphorus content in urine was investigated by XPS and ³¹P NMR.

Statistical Analysis. Quantitative data were generated as mean $\pm$ standard deviation (SD). Statistical comparisons were made by ANOVA analysis and two-sample Student's t-test. P value <0.05 was measured to be statistically significant.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI:

Additional experimental details, TEM and STEM images, AFM images, absorption spectra, XPS spectra, CLSM images, photoacoustic spectra, temperature change curves, cell viability study curves, hemolysis assay curves, cumulative clearance curves, and H&E staining images (PDF)

AUTHOR INFORMATION

Corresponding Author

*Email: zhaoyanli@ntu.edu.sg, dingd@nankai.edu.cn

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

[¶]These authors contributed equally to this work.

ACKNOWLEDGEMENTS

This research is supported by the Singapore Agency for Science, Technology and Research (A*STAR) AME IRG grant (No. A1883c0005), and the Singapore National Research Foundation Investigatorship (No. NRF-NRFI2018-03).

REFERENCES

1. Dolmans, D. E. J. G. J.; Fukumura, D.; Jain, R. K. Photodynamic Therapy for Cancer. *Nat. Rev. Cancer* **2003**, *3*, 380–387.
2. Agostinis, P.; Berg, K.; Cengel, K. A.; Foster, T. H.; Girotti, A. W.; Gollnick, S. O.; Hahn, S. M.; Hamblin, M. R.; Juzeniene, A.; Kessel, D.; Korbelik, M.; Moan, J.; Mroz, P.; Nowis, D.; Piette, J.; Wilson, B. C.; Golab, J. Photodynamic Therapy of Cancer: An Update. *Ca-Cancer J. Clin.* **2011**, *61*, 250–281.
3. Lin, J.; Wang, S.; Huang, P.; Wang, Z.; Chen, S.; Niu, G.; Li, W.; He, J.; Cui, D.; Lu, G.; Chen, X.; Nie, Z. Photosensitizer-Loaded Gold Vesicles with Strong Plasmonic Coupling Effect for Imaging-Guided Photothermal/Photodynamic Therapy. *ACS Nano* **2013**, *7*, 5320–5329.
4. Chen, C.; Wang, J.; Li, X.; Liu, X.; Han, X. Recent Advances in Developing Photosensitizers for Photodynamic Cancer Therapy. *Comb. Chem. High Throughput Screening* **2017**, *20*, 414–422.
5. Gao, J.; Li, J.; Geng, W.-C.; Chen, F.-Y.; Duan, X.; Zheng, Z.; Ding, D.; Guo, D.-S. Biomarker Displacement Activation: A General Host–Guest Strategy for Targeted Phototheranostics *in Vivo*. *J. Am. Chem. Soc.* **2018**, *140*, 4945–4953.
6. de Melo-Diogo, D.; Pais-Silva, C.; Dias, D. R.; Moreira, A. F.; Correia, I. J. Strategies to Improve Cancer Photothermal Therapy Mediated by Nanomaterials. *Adv. Healthcare Mater.* **2017**, *6*, 1700073.
7. Tan, C.; Zhao, L.; Yu, P.; Huang, Y.; Chen, B.; Lai, Z.; Qi, X.; Goh, M. H.; Zhang, X.; Han, S.;

- Wu, X.-J.; Liu, Z.; Zhao, Y.; Zhang, H. Preparation of Ultrathin Two-Dimensional $\text{Ti}_x\text{Ta}_{1-x}\text{S}_y\text{O}_z$ Nanosheets as Highly Efficient Photothermal Agents. *Angew. Chem., Int. Ed.* **2017**, *56*, 7842–7846.
8. Huang, X.; El-Sayed, I. H.; Qian, W.; El-Sayed, M. A. Cancer Cell Imaging and Photothermal Therapy in the Near-Infrared Region by Using Gold Nanorods. *J. Am. Chem. Soc.* **2006**, *128*, 2115–2120.
9. Carron, P. M.; Crowley, A.; O'Shea, D.; McCann, M.; Howe, O.; Hunt, M.; Devereux, M. Targeting the Folate Receptor: Improving Efficacy in Inorganic Medicinal Chemistry. *Curr. Med. Chem.* **2018**, *25*, 2675–2708.
10. Tham, H. P.; Chen, H.; Tan, Y. H.; Qu, Q.; Sreejith, S.; Zhao, L.; Venkatraman, S. S.; Zhao, Y. Photosensitizer Anchored Gold Nanorods for Targeted Combinational Photothermal and Photodynamic Therapy. *Chem. Commun.* **2016**, *52*, 8854–8857.
11. Tian, B.; Wang, C.; Zhang, S.; Feng, L.; Liu, Z. Photothermally Enhanced Photodynamic Therapy Delivered by Nano-Graphene Oxide. *ACS Nano* **2011**, *5*, 7000–7009.
12. Wang, H.; Yang, X. Z.; Shao, W.; Chen, S. C.; Xie, J. F.; Zhang, X. D.; Wang, J.; Xie, Y. Ultrathin Black Phosphorus Nanosheets for Efficient Singlet Oxygen Generation. *J. Am. Chem. Soc.* **2015**, *137*, 11376–11382.
13. Liu, H.; Neal, A. T.; Zhu, Z.; Luo, Z.; Xu, X.; Tománek, D.; Ye, P. D. Phosphorene: An Unexplored 2D Semiconductor with a High Hole Mobility. *ACS Nano* **2014**, *8*, 4033–4041.
14. Wang, X.; Jones, A. M.; Seyler, K. L.; Tran, V.; Jia, Y.; Zhao, H.; Wang, H.; Yang, L.; Xu, X.; Xia, F. Highly Anisotropic and Robust Excitons in Monolayer Black Phosphorus. *Nat. Nanotechnol.* **2015**, *10*, 517–521.
15. Li, L.; Yu, Y.; Ye, G. J.; Ge, Q.; Ou, X.; Wu, H.; Feng, D.; Chen, X. H.; Zhang, Y. Black

Phosphorus Field-Effect Transistors. *Nat. Nanotechnol.* **2014**, *9*, 372–377.

16. Chen, W.; Ouyang, J.; Liu, H.; Chen, M.; Zeng, K.; Sheng, J.; Liu, Z.; Han, Y.; Wang, L.; Li, J.; Deng, L.; Liu, Y.-N.; Guo, S. Black Phosphorus Nanosheet-Based Drug Delivery System for Synergistic Photodynamic/Photothermal/Chemotherapy of Cancer. *Adv. Mater.* **2017**, *29*, 1603864.

17. Lv, R. C.; Yang, D.; Yang, P. P.; Xu, J. T.; He, F.; Gai, S. L.; Li, C. X.; Dai, Y. L.; Yang, G. X.; Lin, J. Integration of Upconversion Nanoparticles and Ultrathin Black Phosphorus for Efficient Photodynamic Theranostics under 808 nm Near-Infrared Light Irradiation. *Chem. Mater.* **2016**, *28*, 4724–4734.

18. Sun, Z.; Xie, H.; Tang, S.; Yu, X. F.; Guo, Z.; Shao, J.; Zhang, H.; Huang, H.; Wang, H.; Chu, P. K. Ultrasmall Black Phosphorus Quantum Dots: Synthesis and Use as Photothermal Agents. *Angew. Chem., Int. Ed.* **2015**, *54*, 11526–11530.

19. Tao, W.; Zhu, X.; Yu, X.; Zeng, X.; Xiao, Q.; Zhang, X.; Ji, X.; Wang, X.; Shi, J.; Zhang, H., Mei, L. Black Phosphorus Nanosheets as a Robust Delivery Platform for Cancer Theranostics. *Adv. Mater.* **2017**, *29*, 1603276.

20. Qian, X.; Gu, Z.; Chen, Y. Two-Dimensional Black Phosphorus Nanosheets for Theranostic Nanomedicine. *Mater. Horiz.* **2017**, *4*, 800–816.

21. Qiu, M.; Ren, W. X.; Jeong, T.; Won, M.; Park, G. Y.; Sang, D. K.; Liu, L. P.; Zhang, H.; Kim, J. S. Omnipotent Phosphorene: A Next-Generation, Two-Dimensional Nanoplatfom for Multidisciplinary Biomedical Applications. *Chem. Soc. Rev.* **2018**, *47*, 5588–5601.

22. Qiu, M.; Wang, D.; Liang, W.; Liu, L.; Zhang, Y.; Chen, X.; Sang, D. K.; Xing, C.; Li, Z.; Dong, B.; Xing, F.; Fan, D.; Bao, S.; Zhang, H.; Cao, Y. Novel Concept of the Smart NIR-Light-Controlled Drug Release of Black Phosphorus Nanostructure for Cancer Therapy. *Proc. Natl.*

Acad. Sci. U.S.A. **2018**, *115*, 501–506.

23. Qian, Z.; Ginger, D. S. Reversibly Reconfigurable Colloidal Plasmonic Nanomaterials. *J. Am. Chem. Soc.* **2017**, *139*, 5266–5276.

24. Yang, D.; Yang, G.; Yang, P.; Lv, R.; Gai, S.; Li, C.; He, F.; Lin, J. Assembly of Au Plasmonic Photothermal Agent and Iron Oxide Nanoparticles on Ultrathin Black Phosphorus for Targeted Photothermal and Photodynamic Cancer Therapy. *Adv. Funct. Mater.* **2017**, *27*, 1700371.

25. Wang, S.; Riedinger, A.; Li, H.; Fu, C.; Liu, H.; Li, L.; Liu, T.; Tan, L.; Barthel, M. J.; Pugliese, G.; De Donato, F.; D’Abbusco, M. S.; Meng, X.; Manna, L.; Meng, H.; Pellegrino, T. Plasmonic Copper Sulfide Nanocrystals Exhibiting Near-Infrared Photothermal and Photodynamic Therapeutic Effects. *ACS Nano* **2015**, *9*, 1788–1800.

26. Liu, Z.; Liu, X.; Du, Y.; Ren, J.; Qu, X. Using Plasmonic Copper Sulfide Nanocrystals as Smart Light-Driven Sterilants. *ACS Nano* **2015**, *9*, 10335–10346.

27. Liang, G.; Jin, X.; Qin, H.; Xing, D. Glutathione-Capped, Renal-Clearable CuS Nanodots for Photoacoustic Imaging and Photothermal Therapy. *J. Mater. Chem. B* **2017**, *5*, 6366–6375.

28. Zhou, M.; Song, S.; Zhao, J.; Tian, M.; Li, C. Theranostic CuS Nanoparticles Targeting Folate Receptors for PET Image-Guided Photothermal Therapy. *J. Mater. Chem. B* **2015**, *3*, 8939–8948.

29. Choi, H. S.; Liu, W.; Misra, P.; Tanaka, E.; Zimmer, J. P.; Itty, B. I.; Bawendi, M. G.; Frangioni, J. V. Renal Clearance of Quantum Dots. *Nat. Biotechnol.* **2007**, *25*, 1165–1170.

30. Zwicke, G. L.; Mansoori, A. G.; Jeffery, C. J. Utilizing the Folate Receptor for Active Targeting of Cancer Nanotherapeutics. *Nano Rev.* **2012**, *3*, 18496.

31. Sun, C.; Wen, L.; Zeng, J.; Wang, Y.; Sun, Q.; Deng, L.; Zhao, C.; Li, Z. One-Pot Solventless Preparation of PEGylated Black Phosphorus Nanoparticles for Photoacoustic Imaging and Photothermal Therapy of Cancer. *Biomaterials* **2016**, *91*, 81–89.

32. Jiang, Y.; Cui, D.; Fang, Y.; Zhen, X.; Upputuri, P. K.; Pramanik, M.; Ding, D.; Pu, K. Amphiphilic Semiconducting Polymer as Multifunctional Nanocarrier for Fluorescence/Photoacoustic Imaging Guided Chemo-Photothermal Therapy. *Biomaterials* **2017**, *145*, 168–177.
33. Qi, J.; Fang, Y.; Kwok, R. T. K.; Zhang, X.; Hu, X.; Lam, J. W. Y.; Ding, D.; Tang, B. Z. Highly Stable Organic Small Molecular Nanoparticles as an Advanced and Biocompatible Phototheranostic Agent of Tumor in Living Mice. *ACS Nano* **2017**, *11*, 7177–7188.
34. Wang, L. V.; Hu, S. Photoacoustic Tomography: *In Vivo* Imaging from Organelles to Organs. *Science* **2012**, *335*, 1458–1462.
35. Xu, M.; Wang, L. V. Photoacoustic Imaging in Biomedicine. *Rev. Sci. Instrum.* **2006**, *77*, 041101.
36. Yang, G.; Phua, S. Z. F.; Bindra, A. K.; Zhao, Y. Degradability and Clearance of Inorganic Nanoparticles for Biomedical Applications. *Adv. Mater.* **2019**, *31*, 1805730.
37. Zhao, L.; Yuan, W.; Tham, H. P.; Chen, H.; Xing, P.; Xiang, H.; Yao, X.; Qiu, X.; Dai, Y.; Zhu, L.; Li, F.; Zhao, Y. Fast-Clearable Nanocarriers Conducting Chemo/Photothermal Combination Therapy to Inhibit Recurrence of Malignant Tumors. *Small* **2017**, *13*, 1700963.
38. Wang, Y.; Liu, Y.; Zhang, J.; Wu, J.; Xu, H.; Wen, X.; Zhang, X.; Tiwary, C. S.; Yang, W.; Vajtai, R.; Zhang, Y.; Chopra, N.; Odeh, I. N.; Wu, Y.; Ajayan, P. M. Cryo-Mediated Exfoliation and Fracturing of Layered Materials into 2D Quantum Dots. *Sci. Adv.* **2017**, *3*, e1701500.
39. Rubio-Pereda, P.; Cocolletzi, G. H. Density Functional Theory Calculations of Biomolecules Adsorption on Phosphorene for Biomedical Applications. *Appl. Surf. Sci.* **2018**, *427*, 1227–1234.
40. Yasaei, P.; Kumar, B.; Foroozan, T.; Wang, C.; Asadi, M.; Tuschel, D.; Indacochea, J. E.; Klie, R. F.; Salehi-Khojin, A. High-Quality Black Phosphorus Atomic Layers by Liquid-Phase

Exfoliation. *Adv. Mater.* **2015**, *27*, 1887–1892.

41. Li, L.; Rashidi, L. H.; Yao, M.; Ma, L.; Chen, L.; Zhang, J.; Zhang, Y.; Chen, W. CuS Nanoagents for Photodynamic and Photothermal Therapies: Phenomena and Possible Mechanisms. *Photodiagn. Photodyn. Ther.* **2017**, *19*, 5–14.

42. Li, N.; Sun, Q.; Yu, Z.; Gao, X.; Pan, W.; Wan, X.; Tang, B. Nuclear-Targeted Photothermal Therapy Prevents Cancer Recurrence with Near-Infrared Triggered Copper Sulfide Nanoparticles. *ACS Nano* **2018**, *12*, 5197–5206.

43. Cai, Y.; Zhang, G.; Zhang, Y.-W. Layer-Dependent Band Alignment and Work Function of Few-Layer Phosphorene. *Sci. Rep.* **2014**, *4*, 6677.

44. Tirado, J.; Roldan-Carmona, C.; Muñoz-Guerrero, F. A.; Bonilla-Arboleda, G.; Ralaifarisoa, M.; Grancini, G.; Queloz, V. I. E.; Koch, N.; Nazeeruddin, M. K.; Jaramillo, F. Copper Sulfide Nanoparticles as Hole-Transporting-Material in a Fully-Inorganic Blocking Layers n-i-p Perovskite Solar Cells: Application and Working Insights. *Appl. Surf. Sci.* **2019**, *478*, 607–614.

45. Yang, J.; Xu, R.; Pei, J.; Myint, Y. W.; Wang, F.; Wang, Z.; Zhang, S.; Yu, Z.; Lu, Y. Optical Tuning of Exciton and Trion Emissions in Monolayer Phosphorene. *Light Sci. Appl.* **2015**, *4*, No. e312.

46. Wang, H.; Jiang, S.; Shao, W.; Zhang, X.; Chen, S.; Sun, X.; Qun Zhang, Q.; Luo, Y.; Xie, Y. Optically Switchable Photocatalysis in Ultrathin Black Phosphorus Nanosheets. *J. Am. Chem. Soc.* **2018**, *140*, 3474–3480.

47. Wang, H.; Zhang, X.; Xie, Y. Photocatalysis in Two Dimensional Black Phosphorus: The Roles of Many-Body Effects. *ACS Nano* **2018**, *12*, 9648–9653.

48. Qu, G.; Xia, T.; Zhou, W.; Zhang, X.; Zhang, H.; Hu, L.; Shi, J.; Yu, X.-F.; Jiang, G. Property–Activity Relationship of Black Phosphorus at the Nano–Bio Interface: From Molecules

to Organisms. *Chem. Rev.* **2020**, *120*, 2288-2346.

49. Lee, S. J.; Shim, Y.-H.; Oh, J.-S.; Jeong, Y.-I.; Park, I.-K.; Lee, H. C. Folic-Acid-Conjugated Pullulan/Poly(*DL*-Lactide-co-Glycolide) Graft Copolymer Nanoparticles for Folate-Receptor-Mediated Drug Delivery. *Nanoscale Res. Lett.* **2015**, *10*, 43.

50. Cheng, H.; Zhu, J.-L.; Zeng, X.; Jing, Y.; Zhang, X.-Z.; Zhuo, R.-X. Targeted Gene Delivery Mediated by Folate-Polyethylenimine-Block-Poly(Ethylene Glycol) with Receptor Selectivity. *Bioconjugate Chem.* **2009**, *20*, 481–487.

51. Goldin, A.; Mantel, N. The Employment of Combinations of Drugs in the Chemotherapy of Neoplasia: A Review. *Cancer Res.* **2010**, *17*, 635–654.

52. Chou, T.-C. Theoretical Basis, Experimental Design, and Computerized Simulation of Synergism and Antagonism in Drug Combination Studies. *Pharmacol. Rev.* **2006**, *58*, 621–681.

53. Tham, H. P.; Xu, K.; Lim, W. Q.; Chen, H.; Zheng, M.; Thng, T. G. S.; Venkatraman, S. S.; Xu, C.; Zhao, Y. Microneedle-Assisted Topical Delivery of Photodynamically Active Mesoporous Formulation for Combination Therapy of Deep-Seated Melanoma. *ACS Nano* **2018**, *12*, 11936–11948.

54. Plutnar, J.; Sofer, Z.; Pumera, M. Products of Degradation of Black Phosphorus in Protic Solvents. *ACS Nano* **2018**, *12*, 8390–8396.

Table of Contents Graphic