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Full Paper:

Multi-Functional Glyconanosheets to Eradicate Drug-Resistant Bacteria on Wounds

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Abstract: Bacterial infection is becoming increasingly lethal with the emergence of antimicrobial resistance, and wounds plagued by such infection are notoriously difficult to heal. Here, we report the first use of galactose-black phosphorus nanosheets, (Gal-BP NSs) as a delivery platform for synergistic antibiotic (kanamycin, Kana) and photothermal treatments against the Gram-negative microbial strain, *P. aeruginosa* PAO1 (PAO1). Gal-BP NSs@Kana could actively target PAO1 and release kanamycin into the bacterial cytoplasm upon NIR laser irradiation. This strategy killed most of the PAO1 through a simultaneous burst of intracellular kanamycin release and photothermal treatment. Comparable antibacterial activities of Gal-BP NSs@Kana were observed within *in vivo* mouse models at their wound sites. In addition, this platform accelerated wound healing from PAO1 infection *via* promotion of neoangiogenesis and collagen production at the wound sites. This work

demonstrates the material properties of Gal-BP NS in fighting bacterial infections and in the treatment of wound healing.

1. Introduction

Bacterial infections are a major cause of high morbidity and mortality in human populations.^[1] The most commonly adopted treatments of antibiotics suffer from drawbacks such as low bioavailability, side effects, high costs, and environmental hazardousness.^[2] Antibiotics, which were hailed as miraculous drugs at their debut, have fulfilled the urgent medical need of treating bacterial infections for a long time.^[3] Unfortunately, bacteria are becoming increasingly resistant to antibiotics at an alarming rate and bacterial resistance has been spreading throughout the world among all species of bacteria.^[3b] This increasing antibiotic resistance of bacteria poses a serious threat to public health.^[4] Particularly, ESKAPE, namely the *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species,^[5] have been identified as the most deadly pathogens by The Infectious Diseases Society of America (IDSA). Amongst the ESKAPE family, the Gram-negative constituents are more difficult to treat,^[5a] due to the presence of an additional barrier in the form of lipopolysaccharide layer or efficient efflux pumps to clear drugs from the bacterial cytoplasm.^[6] To improve the clinical applicability of antibiotics, a great number of novel and efficient antibiotics delivery systems have been developed to meet diverse clinical needs.^[7] The synergistic combination of antibiotics and nanomaterials has proven to be especially helpful in delivering antibiotics efficiently, and preventing antibiotic resistance.^[8] 2D nanomaterials, such as graphene,^[9] MoS₂,^[4] and black phosphorus (BP)^[10] have been extensively studied for this purpose due to their extraordinary physicochemical properties.

Among the large family of 2D nanomaterials, BP has recently drawn great attention for biomedical applications due to its special physicochemical properties.^[11] Its puckered lattice configuration results in a surface area-to-volume ratio higher than most known 2D materials,

which brings increased drug loading capacity.^[12] Additionally, BP's attractiveness also lies in its inherent biocompatibility, as phosphorus occurs naturally in human bodies.^[13] Non-toxic phosphates and phosphonates are the final degradation products of BP NSs in aqueous media,^[14] and they are well tolerated. Furthermore, BP NSs have a broad absorption spectrum, covering the entire wavelength of visible light and even extending to near-infrared (NIR) regions, which allowed for photothermal treatment (PTT).^[12, 15] However, despite the few pioneering studies, the potential in BP NS-based nanocarriers for antibacterial application, such as antibiotic delivery, or active targeting of bacteria has not been demonstrated.

Figure 1. Schematic illustration of black phosphorus-based nanosheets as a platform to deliver antibiotics for antibacterial applications and to promote the regeneration activities of skin cells to accelerate wound healing from bacterial infection. The Gal-BP NSs@Kana could

actively target *P. aeruginosa* PAO1 (PAO1), and effectively kill the PAO1 via NIR-promoted kanamycin release, photothermal therapy, with intercellular ROS production. In addition, the platform promoted neoangiogenesis and collagen production to accelerate wound healing.

It was reported that the interactions between bacterial surface proteins and carbohydrates on the host cell surface was a vital step during infection. Hence, carbohydrates have been widely used to target bacteria.^[16] For example, monosaccharides galactose, mannose, fucose, and their derivatives could bind to bacterial lectins selectively.^[16a, 16b] Herein, we report an active targeting antibiotic delivery platform based on carbohydrates as targeting molecules and BP NSs as carriers (**Figure 1**) for synergistic photothermal and antibiotic treatment and wound healing applications. The BP-based NSs, as a robust synergistic bacteria-targeting platform with PTT capability, can promote antibacterial effect with low concentration of antibiotics, thus reducing antibiotic abuse and the resultant resistance development. The antibiotic release in cytoplasm is controlled by NIR laser irradiation, which elicit a high local concentration to effectively kill bacteria of *P. aeruginosa* PAO1 (PAO1) in combination with PTT of BP NSs. The results suggested that Gal-BP NSs could actively target PAO1, and kill the bacteria. In *in vivo* bacteria-induced wound model, Gal-BP NSs@Kana + NIR could eradicate PAO1 effectively at wound site. In addition, Gal-BP NSs@Kana + NIR could significantly decrease inflammatory cells at wound site, and promote neoangiogenesis and collagen production to accelerate wound healing from PAO1 infection. All these results indicated the potential of Gal-BP NS in fighting bacterial infections and wound healing application.

2. Results and Discussion

2.1. Characterization of the Gal-BP NSs

BP NSs were prepared through a modified mechanical exfoliation method,^[11b] where bulk BP was converted into BP NSs with probe sonication. After obtaining BP NSs, we proceeded to modification with (Gal)₃-NH₂ (nuclear magnetic resonance (NMR) was shown in Figure S1 and S2, Supporting Information),^[17] to actively target gram-negative *Pseudomonas*

aeruginosa. The chemical composition of BP NSs and crystal structure of Gal-BP NSs were determined by X-ray diffractometry (XRD) and X-ray photoelectron spectroscopy (XPS). As shown in Figure S3 (Supporting Information), the chemical composition of BP NSs was consistent with JCPDS No. 731358.^[11b, 18] The elements C, O, N were identified besides P, indicating that (Gal)₃-NH₂ was absorbed onto the surface of BP NSs (Figure S4a, Supporting Information). The BP characteristic peaks 2p^{3/2} and 2p^{1/2} were shown in Figure S4b (Supporting Information), and the weak peak is the signal of oxidized phosphorus, which agreed well with previous report.^[11a]

Figure 2. a) Schematic representation for liquid exfoliation of bulk BP with sonication into BP NSs and preparation of active targeting molecular and antibiotic loaded BP NSs via strong electrostatic interaction to form the Gal-BP NSs@Kana. TEM images of b) BP NSs with inserted distribution of diameter size and c) Gal-BP NSs with inserted distribution of diameter size (scale bar = 100 nm). AFM images of d) BP NSs and e) Gal-BP NSs (scale bar = 300 nm). AFM measured thickness of f) BP NSs (4.5 nm) and g) Gal-BP NSs (5.75 nm). h) Raman spectra of BP NSs and Gal-BP NSs. i) Kanamycin release from Gal-BP NSs with or

without 808 nm NIR laser irradiation (1.0 W/cm^2). Blue arrows marked the time point of NIR ON.

Transmission electron microscopy (TEM) and atomic force microscopy (AFM) were used to characterize the morphology of BP NSs or Gal-BP NSs (**Figure 2**). TEM images shown that the average diameter was about 200 nm for both (Figure 2b, c) and the average thickness of BP NSs or Gal-BP NSs was determined by AFM to be 4.5 nm or 5.75 nm, respectively (Figure 2d, e, f, g). Dynamic light scattering revealed that the average hydrodynamic diameter of BP NSs and Gal-BP NSs was $156.2 \pm 1.7 \text{ nm}$ with PDI at 0.19 and $217.1 \pm 2.7 \text{ nm}$ with PDI at 0.29, respectively (Figure S5, Supporting Information). The slight increase in thickness and hydrodynamic size of Gal-BP NSs could be attributed to the layer of $(\text{Gal})_3\text{-NH}_2$ molecules coated on the BP NSs surface. The zeta potential of BP NSs was about -36 mV, and that of Gal-BP NSs was about -15.6 mV after electrostatically interaction with positively charged $(\text{Gal})_3\text{-NH}_2$ as shown in Figure S6 (Supporting Information). To further confirm that $(\text{Gal})_3\text{-NH}_2$ molecules were coated on the surface of BP NSs, we resorted to UV-vis-NIR spectroscopy and calculated the coating weight ratio according to the standard curve of $(\text{Gal})_3\text{-NH}_2$ molecules (Figure S7, Supporting Information). The results suggested that $(\text{Gal})_3\text{-NH}_2$ molecules were successfully coated on the surface of BP NSs, and the weight ratio is 10/1. In addition, we analyzed the Raman spectra for information on the structural transformation of BP NSs to Gal-BP NSs. As shown in Figure 2h, the typical peaks of BP NSs and Gal-BP NSs was located at about 361.5 , 436.5 and 463.6 cm^{-1} , which can be assigned to A_g^1 , B_{2g} , and A_g^2 modes, respectively. The results indicated that BP NSs and Gal-BP NSs maintained their structure from their corresponding bulk counterpart.^[11a] Compared with BP NSs, the Raman spectrum of Gal-BP NSs showed a slight shift toward a lower wavenumber, which could be caused by the slight thickness change upon coating with $(\text{Gal})_3\text{-NH}_2$ molecules.^[18-19]

According to previous reports,^[12, 14b] BP NSs has an absorbance across the whole NIR region, and exhibits a photothermal effect. In accordance, absorbance and photothermal property of BP NSs were explored in this study. As shown in Figure S8a (Supporting Information), (Gal)₃-NH₂ molecule had little influence on the UV-vis-NIR absorbance of BP NSs. From Figure S8b (Supporting Information), a broad absorption band was shown in the UV-vis-NIR absorbance spectra of Gal-BP NSs at different BP NSs concentrations (25, 50, and 100 µg/mL), which were in agreement with previous reports.^[11b] The photothermal properties of BP NSs and Gal-BP NSs were then quantified by monitoring temperature changes under the irradiation of 808 nm NIR laser. We found that the temperature of BP NSs solutions was increased from 25 °C to 57.2 °C, 45 °C, and 38.7 °C, at concentration of 100 µg/mL, 50 µg/mL, and 25 µg/mL, respectively (Figure S9a, Supporting Information). Meanwhile, the temperature of Gal-BP NSs solutions was increased from 25 °C to 57.9 °C, 45.9 °C, and 38.5 °C, at concentration of 100 µg/mL, 50 µg/mL, and 25 µg/mL, respectively (Figure S9b, Supporting Information). At a relatively low concentration of 100 µg/mL, the BP NSs without or with (Gal)₃-NH₂ showed considerable photothermal properties with a temperature increment of 32.2 °C or 32.9 °C, respectively. In contrast, the temperature was only increased by 3.3 °C for BP NSs-free solution under the same conditions. The results suggested that BP NSs were able to convert NIR light into thermal energy for photothermal therapy in a rapid and efficient manner. In addition, the photothermal stability of Gal-BP NSs was further investigated. From Figure S10 (Supporting Information), there were no significant changes of heating curves of Gal-BP NSs before and after one week of storage, and Gal-BP NSs could maintain a stable photothermal effect after being heated for five laser on/off cycles with an 808 nm laser irradiation power density of 1.0 W/cm², which confirmed the high photothermal stability of BP NSs.

Recently, 2D nanomaterials with high surface area to volume ratio, such as graphene, MoS₂, and their derivatives, have been reported as antibiotic carrier to interact with different types of antibiotic molecules.^[3a, 20] We thus had similar expectations on the potential antibiotic loading capability of BP NSs. Kanamycin, a member of aminoglycoside antibiotics, is produced by the bacterium *Streptomyces kanamyceticus* to combat severe *staphylococcal* and *mycobacterial* infections, but it is ineffective against *Pseudomonas aeruginosa*.^[21] So we chose the compound as the model antibiotic and *P. aeruginosa* as the model strain to be targeted. Herein, the loading and release behavior of BP NSs@Kana nanocomposites were studied. A solution of Gal-BP NSs was mixed with kanamycin solutions at different weight ratios of 0.25, 0.5, 1 and 2, and stirred for 24 h at room temperature. After collecting the unloaded kanamycin in excess, the kanamycin loading ratio on Gal-BP NSs was calculated by UV-vis-NIR spectra. As shown in Figure S11 (Supporting Information), no excess kanamycin was detected in the supernatants of those weight ratios of no more than 0.5/1, meanwhile, the excess kanamycin concentration in weight ratio of 1 and 2 were 15.5 µg/mL and 123.3 µg/mL, respectively. The results suggested that the loading capacity of kanamycin was increased with increasing of Gal-BP NSs@Kana feeding weight ratio, and reached a saturation level. After calculation, the saturation of kanamycin loading was about 80.6% that was noticeably higher than many conventional nanoparticle delivery systems.^[11b] In addition, NIR promoted kanamycin release from Gal-BP NSs@Kana was further investigated in this study. As shown in Figure 2i, the kanamycin release kinetics was determined by collecting the released kanamycin at different time intervals. While 26.3% of kanamycin was released from Gal-BP NSs@Kana over a span of 24 h, about 78.3% of kanamycin was released from the Gal-BP NSs@Kana irradiated five times under 808 nm NIR laser at a power density 1.0 W/cm² over a period of 10 min. Significant improvement in release of kanamycin was observed in a pulsed fashion after each irradiation. This result indicated that kanamycin release behavior from Gal-

BP NSs@Kana could be promoted under NIR laser irradiation which can induce local hyperthermia stimulus to control the on/off of kanamycin release.

The biocompatibility and the cytotoxicity of nanomaterials are an important concern for their biomedical application. In this study, we investigated the cytotoxicity of BP NSs *via* cell counting kit-8 (CCK-8) assays before further antibacterial application. As shown in Figure S12 (Supporting Information), there was no obvious cytotoxicity in NIH 3T3 cell line for BP NSs and Gal-BP NSs at different concentrations (6.25, 12.5, 25, 50, 100 $\mu\text{g/mL}$) for 24 h incubation.

2.2. Evaluation of Antibacterial Activity *In Vitro*

We then advanced to test the selectivity of BP NSs as an antibiotic carrier, with (Gal)₃-NH₂ targeting molecules, against *P. aeruginosa*. For confocal laser scanning microscopy (CLSM) imaging studies, a fluorophore (Fluo) was first synthesized according to the method in experimental section, and the physicochemical properties were characterized by NMR (Figure S13 and S14, Supporting Information) and fluorescence spectroscopy (Figure S15, Supporting Information). Loading of Fluo onto the surface of BP NSs and Gal-BP NSs was performed using the same method for kanamycin. After loading, we incubated *P. aeruginosa* PAO1 with those NSs, and the respective uptake was determined by using CLSM. As shown in **Figure 3a**, the green color marked uptake of BP NSs@Fluo into PAO1. Significantly higher cellular fluorescence intensity was observed in PAO1 incubated with Gal-BP NSs@Fluo, compared with samples incubated with BP NSs@Fluo. This suggests that the targeting effect of (Gal)₃-NH₂ molecules induce more NSs taken up into PAO1. Moreover, the cellular fluorescence intensity in PAO1 dropped remarkably with pretreatment of the bacteria sample by free (Gal)₃-NH₂ molecule. This result suggests that (Gal)₃-NH₂ molecules drove the internalization of Gal-BP NSs *via* interactions with acceptors on the surface of PAO1, which could be blocked by saturating free (Gal)₃-NH₂ molecules. On the other hand, a lower fluorescence intensity in *E. coli* ATCC 8739 was observed with the same protocols, which demonstrated

the high targeting selectivity of (Gal)₃-NH₂ molecules for PAO1. In addition, the Z-stack analysis (Figure S16, Supporting Information) confirmed that BP NSs were taken up into bacteria instead of merely localizing to the bacterial surface exterior.

Figure 3. a) Uptake of BP-based NSs into PAO1 and ATCC 8739 (green color fluorophore, excitation wavelength = 488 nm, BF: bright field, scale bar = 1 μm). b) CFU of PAO1 after different treatment with or without NIR laser. c) Visible colonies of PAO1 after different treatment with (down row) or without (top row) NIR laser. Data are shown as mean ± SD; statistical significance was determined using a two-tail, Student's *t*-test versus the PBS group; * $p \leq 0.05$, ** $p \leq 0.01$.

To evaluate the antibacterial efficacy of BP NSs, colony forming units (CFU) of PAO1 was counted after different treatment conditions, with or without NIR laser. From Figure S17 (Supporting Information), it was found that the CFU of PAO1 after incubation with different concentration of BP NSs or Gal-BP NSs without NIR laser had no significant decrease compared with PBS incubation. However, the CFU of PAO1 treated with NIR laser had a significant concentration-dependent decrease. It was shown that 256 μg/mL of BP NSs or 128 μg/mL of Gal-BP NSs could eradicate PAO1. In addition, the CFU of PAO1 after treatment with kanamycin alone with or without NIR laser was measured as shown in Figure S18

(Supporting Information). The CFU of PAO1 was found to be only concentration-dependent, with no influence from NIR laser treatment. For comparison, 64 $\mu\text{g/mL}$ BP NSs or Gal-BP NSs were loaded with varying concentrations of kanamycin, and the CFU of PAO1 after treatment, with or without NIR laser, was recorded as shown in Figure S19 (Supporting Information). The results indicated that BP NSs@Kana or Gal-BP NSs@Kana with NIR laser could kill PAO1 with a significantly higher efficiency compared with the same concentration BP NSs, Gal-BP NSs, or kanamycin alone, with 8 $\mu\text{g/mL}$ of Gal-BP NSs@Kana being the most effective. Based on these results, 8 $\mu\text{g/mL}$ of kanamycin loaded into 64 $\mu\text{g/mL}$ of Gal-BP NSs was chosen for the subsequent antibacterial application studies *in vitro* and *in vivo*. Therefore, the CFU of PAO1 treated with same concentration of BP NSs or Gal-BP NSs and kanamycin was counted, as shown in Figure 3b, followed with visible colony imaging of PAO1 diluted 1000-fold after treatment, as shown in Figure 3c. There was no difference in CFU or visible colony under different treatment conditions without NIR laser. However, Gal-BP NSs@Kana could effectively kill PAO1 after laser irradiation. The results demonstrated the high efficiency of Gal-BP NSs as carriers of kanamycin against PAO1. With the aid of NIR irradiation, the delivered kanamycin could be released into the bacterial cytoplasm in a controlled burst to gain higher local concentrations, while the photothermal effect of Gal-BP NSs could simultaneously bring up local temperatures to kill PAO1 effectively.

The results were further confirmed by Live/Dead staining assay, spread plate method and scanning electron microscope (SEM) examination. As shown in **Figure 4a**, the majority of bacteria were dead after optimal treatment with Gal-BP NSs@Kana and NIR laser irradiation. In contrast, other BP NSs led to less dead bacteria, and PBS or kanamycin alone was ineffective. We also analyzed the morphology of bacterial cells with SEM examination, and the results are shown in Figure 4b. The typical morphologies of PAO1 *i.e.* smooth surface and rod shape, were observed for the PBS and kanamycin groups. Distorted and wrinkled membranes were observed on the surfaces of PAO1 after BP NSs treatment with NIR

irradiation. The most apparent damage was identified for the bacteria cells treated with Gal-BP NSs@Kana with a NIR irradiation for 10 min, which was consistent with the results of the Live/Dead staining assay and the spread plate assay. All the results above suggested the effective targeting of PAO1 by Gal-BP NSs@Kana, with the carried kanamycin undergoing controlled release into cytoplasm upon irradiation by NIR laser. The synergistic combination achieved both high bacteriostatic and bactericidal efficacy.

Figure 4. a) LIVE/DEAD staining of PAO1 after treated with PBS, BP NSs, Gal-BP NSs, Kana, BP NSs@Kana, and Gal-BP NSs@Kana without (top row) or with (bottom row) NIR laser irradiation (the green and red color showed live and dead cells, respectively, scale bar = 20 μm). b) SEM images of PAO1 after different treatments without (top row) or with (bottom row) NIR laser irradiation. Magnification: 10,000 ×, scale bar = 1 μm.

According to previous reports, nanomaterials, including V_2O_5 ,^[22] Fe_3O_4 ,^[23] MoS_2 ^[4] and graphene^[24] have a peroxidase-mimicry ability to elicit high level of intracellular reactive oxygen species (ROS). ROS exert high antibacterial activity with no possibility for bacterial resistance, because there is no detoxifying cell enzyme.^[25] Moreover, ROS can rapidly cause oxidative damage to bacterial cell walls and membranes, which increases membrane-permeability and heat sensitivity.^[26] BP is also a 2D material with photothermal and photodynamic properties like MoS_2 and graphene, so we investigated the intracellular ROS production induced by BP NSs, using CM- H_2DCFDA as the probe. As shown in Figure S20

(Supporting Information), there were no significant differences across all treatments without NIR laser irradiation. However, upon irradiating with NIR laser, the intracellular ROS production from BP NSs treatment was much higher than from PBS or kanamycin. The results were highly consistent with SEM examination (Figure 4b), demonstrating the existence of an extra powerful weapon, intracellular ROS, against PAO1 in the combined arsenal of Gal-BP NSs@Kana and NIR radiation.

2.3. Application for Treatment of *P. aeruginosa* Infection on Wounds *In Vivo*

In our above studies, systematic cellular compatibility tests and *in vitro* antibacterial experiments demonstrated excellent applicability of Gal-BP NSs@Kana in the treatment of PAO1 infection. To examine the antibacterial efficiency of the Gal-BP NSs@Kana in wound sterilization, we choose four representative groups and designed a PAO1 infection model on mouse skin *in vivo*. First, we investigated the photothermal properties of BP NSs *in vivo* to account for the photothermal treatment effectiveness of BP NSs. As shown in **Figure 5a**, the BP NSs showed extremely robust PTT efficiency during irradiation, and the temperature of BP NSs and Gal-BP NSs@Kana was increased to 47.8 °C and 48.9 °C, respectively after NIR irradiation for 10 min. In contrast, in the PBS and kanamycin groups, the skin underwent a lower increase in temperature under the same conditions. Subsequently, the residual PAO1 around the wound was quantified after treatment with PBS, BP NSs, kanamycin and Gal-BP NSs@Kana for 2 days in this study. As seen in Figure 5b, viable bacterial colonies were observed in almost all groups except the one treated by Gal-BP NSs@Kana with NIR laser irradiation, where colonies could be barely seen, and the corresponding CFU per wound were calculated and showed in Figure 5c. The results suggested that the synergistic therapy was effective against PAO1 infection for wound healing *in vivo*.

Figure 5. a) Photothermal images of mice treated with PBS, BP NSs, Kana, and Gal-BP NSs@Kana during an 808 nm NIR laser irradiation for 10 min at power density of 1.0 W/cm². The images of 0 min, 5 min, and 10 min were shown in this figure. b) Formed visible colonies and c) Log CFU per wound of PAO1 harvested from every wound after different treatments. $n = 5$. Data are shown as mean $\pm$ SD; statistical significance was determined using a two-tail Student's t -test; * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Lastly, the size of wounds on skin of female BALB/c mice was calculated from photos taken on the 1, 3, 5, 7 and 9 days, with the aid of Image J software. From **Figure 6a, c**, the shrinkage in wound size was far more significant with BP NSs or Gal-BP NSs@Kana treatment with NIR irradiation, especially the Gal-BP NSs@Kana group, compared with all other treatments. Based on statistical analysis of wound sizes, the wound was almost healed after treatment with Gal-BP NSs@Kana + NIR after 7 days, and was fully healed after 9 days, while the wound sizes of other groups were only partially decreased (Figure 6b, d). The results indicated that Gal-BP NSs@Kana + NIR could effectively eradicate the bacteria PAO1 and decrease inflammatory cells at wound site, and promoting wound healing from PAO1 infection.

Figure 6. The digital images for the PAO1 infected mice skin wounds after treated with PBS, BP NSs, Kana, and Gal-BP NSs@Kana a) without or c) with NIR laser irradiation at 1, 3, 5, 7, and 9 days. The change of mice skin wounds size after different treatments b) without or d) with NIR laser irradiation. $n = 5$, scale bar: 6 mm. Data are shown as mean $\pm$ SD; statistical significance was determined using a one-way analysis of variance (ANOVA); * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Furthermore, hematoxylin and eosin (H&E) staining of mice skin wounds and major organs were performed. From Figure S21 (Supporting Information), histological analysis of major organs (heart, liver, spleen, lung, and kidney) of all groups showed no abnormalities and damage after a 9-day treatment, which indicated that BP NSs were a biocompatible delivery platform for treating bacterial infections during wound healing. **Figure 7** showed the H&E staining, Masson's trichrome staining and immunohistochemistry (IHC) staining of F4/80, CD31 and α -SMA of mice skin wounds at 9 days post treatment with PBS, BP NSs, kanamycin, and Gal-BP NSs@Kana without or with NIR laser irradiation. From the H&E staining, the whole wound was displayed, and the scar and local tissue necrosis could be observed in PBS, BP NSs, Kana, Kana + NIR, and PBS + NIR groups. Meanwhile, the scar nearly disappeared in Gal-BP NSs@Kana, and BP NSs + NIR. Finally, the wound in Gal-BP NSs@Kana + NIR group was healed, and skin layers were formed completely. F4/80 was a

cellular marker of macrophage which was used for inflammatory cell infiltration. From IHC staining of F4/80, the inflammatory cells were labeled with red color arrow. There were a large number of inflammatory cells infiltrated into wounds in PBS, BP NSs, Kana, Kana + NIR, and PBS + NIR groups, meanwhile only a small number of inflammatory cells were observed in Gal-BP NSs@Kana, and BP NSs+NIR treatments, and then nearly no inflammatory cells were observed in Gal-BP NSs@Kana + NIR groups. In addition, it was noticed that neoangiogenesis and collagen production were gradually formed, which was supported by Masson's trichrome staining and IHC staining of CD31 and α -SMA. CD31 was a cellular marker of endothelial cells, which was used for new blood vessel. From IHC staining of CD31, positive cells were endothelial cells labeled with purple color arrow, most of them were distributed in endothelium of new blood vessel. Significantly more positive cells were found in mice treated with BP NSs or Gal-BP NSs@Kana than other groups, with Gal-BP NSs@Kana + NIR being the best. α -SMA was a cellular marker of myofibroblasts. Myofibroblasts were contractile cells that brought the edges of a wound together and underwent apoptosis in the late stage of wound repair. From IHC staining of α -SMA, it was found that there were lots of α -SMA positive cells labeled below yellow color line in PBS, Kana, Gal-BP NSs@Kana, BP NSs + NIR, and Kana + NIR groups. However, the α -SMA positive cells almost disappeared after Gal-BP NSs@Kana + NIR treatment, with collagen in place from Masson's trichrome staining. In Masson's trichrome staining, the blue color marked collagen, and the red color marked keratin or muscle fibers. It was noticed that collagen was produced and moved from the edge of wound to wound site in Kana, Gal-BP NSs@Kana, BP NSs + NIR, and Kana + NIR groups. In addition, the majority of collagen was produced and distributed in dermis after treated with Gal-BP NSs@Kana + NIR. These results suggested that Gal-BP NSs@Kana + NIR could promote neoangiogenesis and collagen production to accelerate wound healing from PAO1 infection after eradicated PAO1 at wound site.

Figure 7. The H&E staining, Masson's trichrome staining, and IHC staining of F4/80, CD31 and α -SMA of mice skin wounds at 9 day after different treatment with or without NIR laser (scale bar = 50 μ m). Inflammatory cells were labeled with red color arrow in IHC staining of F4/80. In Masson's trichrome staining, the blue color was the collagen, red color was keratin and muscle fibers. Positive cells were endothelial cells labeled with purple color arrow in CD31 IHC staining, and myofibroblast cells labeled below yellow color line in α -SMA IHC staining. There were obvious epidermis and dermis only after treatment with Gal-BP NSs@Kana + NIR.

3. Conclusion

In summary, we have demonstrated the antibacterial application of BP NSs, in the form of a galactose functionalized antibiotic delivery platform. Moreover, the Gal-BP NSs@Kana effectively killed the bacteria PAO1 at wound sites and promoted neoangiogenesis, and collagen production, thus facilitating wound healing from the bacteria infection. The biocompatibility of BP NSs was further demonstrated both *in vitro* and *in vivo*, paving the way for diverse biomedical application in real life scenarios. With this work, we hope to

promote more studies on the antibacterial application of 2D materials, and conduct further investigations into various ways of applying black phosphorus in biomedicine.

4. Experimental Section

Materials: The bulk black phosphorous (BP) was purchased from Smart-Elements and stored in a dark Ar glovebox. LIVE/DEAD BacLight Bacterial Viability Kit and CM-H₂DCFDA were purchased from ThermoFisher Scientific (MA, USA). Any other AR agents were purchased from Sigma-Aldrich. HyPure Molecular Biology Grade Water (Hyclone TM) was used to prepare all the solutions.

Preparation of black phosphorous nanosheets (BP NSs): The BP NSs were prepared using a modified liquid exfoliation method. In brief, 50 mg of bulk BP was dispersed in 50 mL of HyPure Molecular Biology Grade Water eliminated dissolved oxygen molecules with argon for reducing oxidation during preparing procedure. Then, the mixture solution was sonicated in ice water keeping a relatively low temperature for 12 h (Amplifier: 25%, 9 s on/3 s off). Afterward, the suspension was centrifuged at 2,000 rpm for 10 min to remove the residual unexfoliated bulk BP. The supernatant was then centrifuged at 10,000 rpm for 10 min to get rid of water. The precipitate was carefully collected and re-suspended in PBS or HyPure Water for further use and stored in 4 °C.

Preparation of Gal-BP NSs: In order to prepare Gal-BP NSs, 500 μ L of (Gal)₃-NH₂ (20 μ g/mL) was added into BP NSs solution (200 μ g/mL, 500 μ L), then, the mixture was stirred for 2 h at room temperature. The resulting mixture was centrifuged at 10,000 rpm (4 °C) for 10 min to remove the excess (Gal)₃-NH₂, and washed 3 times with same method. The Gal-BP NSs were suspended in PBS or HyPure Water for further use stored in 4 °C. In order to calculate the weight ratio of BP and (Gal)₃-NH₂ of Gal-BP NSs, 1% (w/v) phenol was added into Gal-BP NSs solutions, then, 62.5% (v/v) H₂SO₄ was added into the mixtures and stirred for 15 min at room temperature. The final mixtures were orange-red color, which have

absorption peaks at 485 nm and were determined by using UV-vis-NIR to calculate concentration of (Gal)₃-NH₂ coating on BP NSs.

Preparation of Gal-BP NSs@Kana: For kanamycin loading, the kanamycin solutions with different concentrations (0, 25, 50, 100, 200 µg/mL) were added into Gal-BP NSs (100 µg/mL), and then the mixtures were stirred for 24 h at room temperature. The mixtures were centrifuged at 10,000 rpm for 10 min to collect the excess unloading kanamycin, and the supernatants were collected to quantitate kanamycin loading on Gal-BP NSs using UV-vis-NIR spectrophotometer. The loading capacity was calculated according to unloading kanamycin, (total kanamycin - unloading kanamycin)/total kanamycin. Then, the Gal-BP NSs@Kana was suspended in PBS or HyPure Water for further use and stored in 4 °C.

NIR promoted kanamycin release from Gal-BP NSs@Kana: Kanamycin released from Gal-BP NSs@Kana with or without 808 nm irradiation was measured in this release study. Briefly, 0.5 mL Gal-BP NSs@Kana (200 µg/mL) was added into 0.5 mL PBS each group. Then, the solution was irradiated with or without 808 nm NIR laser at a power density 1.0 W/cm² over a period of 10 min. At the desired time points, 175 µL supernatant was collected and the same volume of PBS was added into the solution after centrifugation at 10,000 rpm for 5 min. And then the absorption of kanamycin in the supernatant was measured by using UV-vis-NIR absorbance spectrometer. The concentration of kanamycin was calculated according to the standard curve.

Characterization of based-BP NSs: The morphology and thickness of based-BP NSs were observed by transmission electron microscope (TEM, JEOL JEM 1400) and atomic force microscope (AFM, Bruker system in contact mode). UV-vis-NIR absorption spectra were recorded by a Shimadzu UV-vis-NIR spectrophotometer. Raman spectra of based-BP NSs were performed on a high-resolution confocal Raman microscope (Olympus BX41) with laser excitation wavelength of 633 nm at room temperature. X-ray photoelectron spectroscopy (XPS) spectra were collected on an X-ray photoelectron spectroscopy (SPECS, PHOIBOS

100). X-ray diffraction (XRD) was performed on a Bruker D8 Advance XRD. Hydrodynamic diameter, polydispersity index and zeta-potential of based-BP NSs were determined by the dynamic light scattering (DLS) techniques using a Malvern Zetasizer Nano-ZS instrument. The data were obtained with the average of three times.

Photothermal properties of based-BP NSs: 1 mL of BP NSs (100 µg/mL), Gal-BP NSs (100 µg/mL) aqueous solution was added into a transparent glass vial. The temperature change of different solutions was monitored by using a thermal infrared imaging camera (Fluke, USA) under the irradiation of a fiber-coupled continuous semiconductor diode laser. According to absorbance of BP NSs, the first NIR window laser with 808 nm (Beijing Viasho Technology Co Ltd) with power density of 1.0 W/cm² was used in this study.

In vitro cytotoxicity assay: NIH/3T3 cell line (from ATCC) was used for *in vitro* cytotoxicity assay of based-BP NSs, which was cultured in DMEM with 10% FBS and 1% penicillin/streptomycin at 37 °C with 5% CO₂. The cytotoxicity of based BP NSs to cell was determined by cell counting kit-8 (CCK-8, Dojindo, Japan) assay. In brief, cells were seeded into the 96-well plates at density of 1×10⁴ cells/well and cultured overnight. Then the old medium was replaced with 100 µL fresh medium containing different concentration of based-BP NSs (0, 6.25, 12.5, 25, 50, 100 µg/mL) for 24 h. Then, the old medium was replaced with CCK-8 solution. After incubation for another 2 h, the OD_{450 nm} of the media was measured by using a microplate reader (Bio-Rad Benchmark Plus, US). The cell viability was calculated using the formula: % cell viability = (AbsTest - AbsBlank) / (AbsControl - AbsBlank) ×100%.

In vitro bacterial targeting assay: To observe the bacterial targeting assay, a fluorescent molecule was firstly synthesized. In general, 4-bromo-1,8-naphthalic anhydride (277 mg, 1.0 mmol) and *N*-Boc-ethylenediamine (481 mg, 3.0 mmol) were refluxed in 20 mL 2-methoxyethanol for 24 hours. Then the solvent was removed *in vacuo* and the residue purified by flash column chromatography to give Boc-Fluo (285 mg, 59%) as a yellow solid. The product was stirred in a solution of 15 mL 1 M HCl in MeOH overnight, and then dried to

give Fluo (200 mg, 96%) as a yellow solid, the product was used without further purification. Then, the *in vitro* bacterial targeting effect of Gal-BP NSs was investigated by using a confocal laser scanning microscopy. The targeting assay was divided into five groups: a) BPS; b) treated with BP NSs@Fluo; c) treated with Gal-BP NSs@Fluo; d) pre-treated with (Gal)₃-NH₂ and then treated with Gal-BP NSs@Fluo; e) *E. coli* ATCC 8739 (ATCC 8739) was treated with Gal-BP NSs@Fluo. For group (a, b, c), 100 μ L *Pseudomonas aeruginosa* PAO1 (PAO1) (5×10^8 CFU/mL) was incubated with PBS, BP NSs@Fluo, or Gal-BP NSs@Fluo for 2 h at 37 °C, respectively. For group d, 100 μ L PAO1 (5×10^8 CFU/mL) was pre-treated with (Gal)₃-NH₂ for 1 h, and incubated with Gal-BP NSs@Fluo for 2 h at 37 °C. For group e, 100 μ L ATCC 8739 (5×10^8 CFU/mL) were incubated with Gal-BP NSs@Fluo for 2 h at 37 °C. After treatment, all groups were collected with centrifugation at 5,000 rpm for 5 min, and then washed with PBS three times. Finally, 10 μ L bacterial was drop onto a slide for observation using CLSM.

In vitro antibacterial activity: The *in vitro* antibacterial abilities of samples against bacteria were evaluated *via* CFU, spread plate method, Live/Dead staining and SEM examination. PAO1 was grown overnight at 37°C in MHB medium and diluted to 10^5 CFU/mL in MHB. And then, 100 μ L the PAO1 suspension was added into a 96-well plate, followed by the addition of a two-fold dilution series of 100 μ L samples in MHB. For the CFU, after incubated at 37 °C for 2 h, the samples were irradiated with or without 808 nm NIR laser at a power density 1.0 W/cm^2 over a period of 10 min. The suspensions were serially diluted with MHB medium, and $4 \times 5 \mu\text{L}$ of diluted samples were dropped onto the agar plates. After incubation at 37 °C for 18-24 h, the colonies formed were counted to calculate CFU of PAO1. For spread plate method, after incubated at 37 °C for 2 h, the samples were irradiated with or without 808 nm NIR laser at a power density 1.0 W/cm^2 over a period of 10 min. 50 μ L bacterial suspensions (diluted 1/1000 with MHB) were spread on a standard agar culture plate and incubated at 37 °C for 18-24 h. These plates contained bacterial colonies were observed

by using camera. For Live/Dead staining, the bacterial were harvested by centrifugation at $5,000 \times g$ for 5 min after treated with or without NIR laser, and washed 3 times with 0.85% NaCl. Then the bacterial were suspended with 1 mL 0.85% NaCl and mixed with 3 μ L Live/Dead dye mixture, then incubated at room temperature in the dark for 30 min. The images of bacterial live/dead staining were captured with CLSM. For SEM examination, 10 μ L bacterial suspensions after treated with or without NIR laser were dropped onto a wafer, and followed with a dry cycle at room temperature overnight. The dry samples were dehydrated with gradient diluted ethanol 25%, 50%, 75%, and 100%. After the ethanol completely volatilized, the samples were sprayed with gold and observed with SEM.

Production of intracellular ROS: Bacterial intracellular ROS production was analyzed by using a microplate reader with a ROS indicator CM-H₂DCFDA. Briefly, PAO1 was grown overnight at 37 °C in MHB medium and diluted to 10^5 CFU/mL in pre-warmed PBS buffer containing the CM-H₂DCFDA probe with a concentration of 10 μ M. After incubation for 30 min, the PAO1 was washed 3 times with PBS. Then, 100 μ L the PAO1 suspension was added into a 96-well plate, followed by the addition of 100 μ L samples in MHB. After incubation at 37 °C for 1 h, the PAO1 was irradiated with 808 nm NIR laser at a power density 1.0 W/cm² over a period of 10 min. ROS signal was measured by using a microplate reader, after incubation for 2 h.

In vivo mice wound model and healing process: Female BALB/c mice (8 weeks old) were purchased from HFK Bioscience Co., Ltd. (Beijing, China). The animal experiments were approved by the Laboratory Animal Ethics Committee of Jinan University and conformed to the legal mandates and national guidelines for the care and maintenance of laboratory animals. Mice were randomly divided into four groups (n = 5 per group): PBS, BP NSs, Kana, and Gal-BP NSs@Kana with or without NIR laser. Two wounds were created with a disposable 6 mm skin biopsy punch one time on the back (left and right) of each mouse after anesthesia. The wounds were infected by a 10 μ L bacterial suspension of PAO1 with 1.0×10^7

CFU/wound and covered with Tegaderm (3M™) to prevent contamination. After 24 h, Tegaderm film was removed and treatment was applied by using 10 μ L PBS, BP NSs, Kana, and Gal-BP NSs@Kana, respectively. After treated for 30 min, the left wound on the back of each mouse was irradiated with 808 nm NIR laser at a power density 1.0 W/cm² over a period of 10 min, the right wound was only treated with samples without NIR laser. All mice were housed in cages under standardized temperature and humidity on a 12 h light/dark cycle. For checking the antibacterial activity *in vivo*, the wounds were harvested using scalpel blade. And then the wounds were homogenized and plated on agar plates to determine CFU. For wound healing process, the wounds were observed and photographed every two days for 9 days, and then the skin tissue sample were excised and fixed with 4% Paraformaldehyde for pathological slides. For IHC, the slides were blocked with 3% BSA, and followed with incubation of antibodies, F4/80 antibody (Servicebio, GB11027, dilution, 1:1000), CD31 antibody (Servicebio, GB12063, dilution, 1:300), α -SMA antibody (Servicebio, GB13044, dilution, 1:1000), and then incubated with HRP-IgG (Servicebio, dilution, 1:200). Finally, the slides were staining with diaminobenzidine (DAB) solution and hematoxylin for cell nucleus. The histological images were observed by using a microscope after staining with H&E, immunofluorescence, Masson's trichrome, and IHC. Finally, the major organs (heart, liver, spleen, lung, kidney) were harvested and stained with H&E after treatment.

Statistical analysis: All data were evaluated as means values $\pm$ standard deviation of three times at least and analyzed by an unpaired two-tail Student's *t*-test or a one-way analysis of variance (ANOVA) followed by LSD multiple comparisons using GraphPad Prism, with values of $p < 0.05$ considered significant.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

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References

- [1] A. P. Anisimov, K. K. Amoako, *J. Med. Microbiol.* **2006**, 55, 1461.
- [2] a) T. P. Cushnie, A. J. Lamb, *Int. J. Antimicrob. Agents* **2005**, 26, 343; b) M. H. Xiong, Y. Bao, X. Z. Yang, Y. H. Zhu, J. Wang, *Adv. Drug Deliv. Rev.* **2014**, 78, 63.
- [3] a) X. Zhang, W. Zhang, L. Liu, M. Yang, L. Huang, K. Chen, R. Wang, B. Yang, D. Zhang, J. Wang, *Nanotechnology* **2017**, 28, 225101.
- [4] W. Yin, J. Yu, F. Lv, L. Yan, L. R. Zheng, Z. Gu, Y. Zhao, *ACS Nano* **2016**, 10, 11000.
- [5] a) H. W. Boucher, G. H. Talbot, J. S. Bradley, J. E. Edwards, D. Gilbert, L. B. Rice, M. Scheld, B. Spellberg, J. Bartlett, *Clin. Infect. Dis.* **2009**, 48, 1; b) L. B. Rice, *J. Infect. Dis.* **2008**, 197, 1079.
- [6] H. Nikaido, *J. Bacteriol.* **1996**, 178, 5853.
- [7] R. Langer, D. A. Tirrell, *Nature* **2004**, 428, 487.
- [8] a) X. Li, S. M. Robinson, A. Gupta, K. Saha, Z. Jiang, D. F. Moyano, A. Sahar, M. A. Riley, V. M. Rotello, *ACS Nano* **2014**, 8, 10682; b) W. Hu, C. Peng, W. Luo, M. Lv, X. Li, D. Li, Q. Huang, C. Fan, *ACS Nano* **2010**, 4, 4317.
- [9] S. Panda, T. K. Rout, A. D. Prusty, P. M. Ajayan, S. Nayak, *Adv. Mater.* **2018**, 30, 1702149.

- [10] a) C. Mao, Y. Xiang, X. Liu, Z. Cui, X. Yang, Z. Li, S. Zhu, Y. Zheng, K. W. K. Yeung, S. Wu, *ACS Nano* **2018**, 12, 1747; b) T. Guo, Y. Wu, Y. Lin, X. Xu, H. Lian, G. Huang, J.-Z. Liu, X. Wu, H.-H. Yang, *Small* **2018**, 14, 1702815; c) X.-W. Huang, J.-J. Wei, M.-Y. Zhang, X.-L. Zhang, X.-F. Yin, C.-H. Lu, J.-B. Song, S.-M. Bai, H.-H. Yang, *ACS Appl. Mater. Interfaces* **2018**, 10, 35495.
- [11] a) H. Wang, X. Yang, W. Shao, S. Chen, J. Xie, X. Zhang, J. Wang, Y. Xie, *J. Am. Chem. Soc.* **2015**, 137, 11376; b) W. Tao, X. Zhu, X. Yu, X. Zeng, Q. Xiao, X. Zhang, X. Ji, X. Wang, J. Shi, H. Zhang, L. Mei, *Adv. Mater.* **2017**, 29, 1603276; c) L. Tan, J. Li, X. Liu, Z. Cui, X. Yang, K. W. K. Yeung, H. Pan, Y. Zheng, X. Wang, S. Wu, *Small* **2018**, 14, 1703197; d) Z. Xiong, X. Zhang, S. Zhang, L. Lei, W. Ma, D. Li, W. Wang, Q. Zhao, B. Xing, *Ecotoxicol. Environ. Saf.* **2018**, 161, 507; e) Q. Wu, M. Liang, S. Zhang, X. Liu, F. Wang, *Nanoscale* **2018**, 10, 10428.
- [12] W. Chen, J. Ouyang, H. Liu, M. Chen, K. Zeng, J. Sheng, Z. Liu, Y. Han, L. Wang, J. Li, L. Deng, Y. N. Liu, S. Guo, *Adv. Mater.* **2017**, 29, 1603864.
- [13] D. L. Childers, J. Corman, M. Edwards, J. J. Elser, *Bioscience* **2011**, 61, 117.
- [14] a) X. Ling, H. Wang, S. Huang, F. Xia, M. S. Dresselhaus, *Proc. Natl. Acad. Sci. U. S. A.* **2015**, 112, 4523; b) J. Shao, H. Xie, H. Huang, Z. Li, Z. Sun, Y. Xu, Q. Xiao, X. F. Yu, Y. Zhao, H. Zhang, H. Wang, P. K. Chu, *Nat. commun.* **2016**, 7, 12967.
- [15] Z. Sun, H. Xie, S. Tang, X. F. Yu, Z. Guo, J. Shao, H. Zhang, H. Huang, H. Wang, P. K. Chu, *Angew. Chem. Int. Ed. Engl.* **2015**, 54, 11526.
- [16] a) M. D. Disney, P. H. Seeberger, *Chem. Biol.* **2004**, 11, 1701; b) C. Sabin, E. P. Mitchell, M. Pokorna, C. Gautier, J. P. Utille, M. Wimmerova, A. Imberty, *FEBS Lett.* **2006**, 580, 982; c) G. Cioci, E. P. Mitchell, C. Gautier, M. Wimmerova, D. Sudakevitz, S. Perez, N. Gilboa-Garber, A. Imberty, *FEBS Lett.* **2003**, 555, 297.
- [17] S. H. Mahadevegowda, S. Hou, J. Ma, D. Keogh, J. Zhang, A. Mallick, X. W. Liu, H. Duan, M. B. Chan-Park, *Biomater. Sci.* **2018**, 6, 1339.
- [18] X. Zhang, H. Xie, Z. Liu, C. Tan, Z. Luo, H. Li, J. Lin, L. Sun, W. Chen, Z. Xu, L. Xie, W. Huang, H. Zhang, *Angew. Chem. Int. Ed. Engl.* **2015**, 54, 3653.
- [19] A. Favron, E. Gaufres, F. Fossard, A. L. Phaneuf-L'Heureux, N. Y. Tang, P. L. Levesque, A. Loiseau, R. Leonelli, S. Francoeur, R. Martel, *Nat. Mater.* **2015**, 14, 826.
- [20] a) X. Fan, F. Yang, C. Nie, Y. Yang, H. Ji, C. He, C. Cheng, C. Zhao, *ACS Appl. Mater. Interfaces* **2018**, 10, 296; b) X. Xie, C. Mao, X. Liu, Y. Zhang, Z. Cui, X. Yang, K. W. K. Yeung, H. Pan, P. K. Chu, S. Wu, *ACS Appl. Mater. Interfaces* **2017**, 9, 26417.
- [21] a) K. Poole, *Antimicrob. Agents Chemother.* **2005**, 49, 479; b) Z. Pang, R. Raudonis, B. R. Glick, T.-J. Lin, Z. Cheng, *Biotechnol. Adv.* **2019**, 37, 177.
- [22] F. Natalio, R. Andre, A. F. Hartog, B. Stoll, K. P. Jochum, R. Wever, W. Tremel, *Nat. Nanotechnol.* **2012**, 7, 530.
- [23] L. Gao, J. Zhuang, L. Nie, J. Zhang, Y. Zhang, N. Gu, T. Wang, J. Feng, D. Yang, S. Perrett, X. Yan, *Nat. Nanotechnol.* **2007**, 2, 577.
- [24] H. Sun, N. Gao, K. Dong, J. Ren, X. Qu, *ACS Nano* **2014**, 8, 6202.
- [25] F. Vatansever, W. C. de Melo, P. Avci, D. Vecchio, M. Sadasivam, A. Gupta, R. Chandran, M. Karimi, N. A. Parizotto, R. Yin, G. P. Tegos, M. R. Hamblin, *FEMS Microbiol. Rev.* **2013**, 37, 955.
- [26] W. Y. Pan, C. C. Huang, T. T. Lin, H. Y. Hu, W. C. Lin, M. J. Li, H. W. Sung, *Nanomedicine* **2016**, 12, 431.

Table of Contents

A new black phosphorus nanosheets-based delivery platform, Gal-BP NSs with kanamycin loaded, is prepared for synergistic antibiotic and photothermal treatment of *Pseudomonas aeruginosa* infection. The formation shows an efficient active targeting to *P. aeruginosa* PAO1, and bacterial inhibition and killing against *P. aeruginosa* PAO1 infection *in vitro* and *in vivo*.

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Supporting Information

Multi-Functional Glyconanosheets to Eradicate Drug-Resistant Bacteria on Wounds

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and Xue-Wei Liu**

Figure S1. ¹H NMR of (Gal)₃-NH₂ molecule. Figure S2. ¹³C NMR of (Gal)₃-NH₂ molecule

Figure S3. XRD pattern of BP NSs.

Figure S4. XPS spectra of Gal-BP NSs and BP NSs: (a) Survey spectrum of Gal-BP NSs, (b) P2p spectrum of Gal-BP NSs, (c) Survey spectrum of BP NSs, and (d) P2p spectrum of BP NSs.

Figure S5. DLS size distribution of (a) BP NSs and (b) Gal-BP NSs.

Figure S6. Zeta potentials of BP NSs, (Gal)₃-NH₂, Gal-BP NSs.

Figure S7. The UV-vis-NIR spectra of $(\text{Gal})_3\text{-NH}_2$ molecule at different concentrations.

Figure S8. UV-vis-NIR spectra of (a) BP NSs, Gal-BP NSs. (b) UV-vis-NIR spectra of Gal-BP NSs at different concentrations.

Figure S9. Photothermal properties of water, BP NSs and Gal-BP NSs solution at different concentrations under irradiation with 808 nm NIR laser (1.0 W/cm^2) for 10 min.

Figure S10. Photothermal stability of Gal-BP NSs. (a) Photothermal heating curve of Gal-BP NSs before and after one week storage at 4 °C irradiated with 808 nm NIR laser for 10 min at power density of 1.0 W/cm². (b) Photothermal heating curve of Gal-BP NSs while heated for five on/off cycles with 808 nm NIR laser at power density of 1.0 W/cm².

Figure S11. (a) The UV-vis-NIR spectra of kanamycin at different concentrations. (b) The UV-vis-NIR spectra of collected supernatant unloading kanamycin at different weight ratios of kanamycin : Gal-BP NSs.

Figure S12. Cell viability of NIH 3T3 cells after incubation with BP NSs and Gal-BP NSs at different concentrations for 24 h.

Figure S13. ¹H NMR of fluorophore Figure S14. ¹³C NMR of fluorophore

Figure S15. (a) The fluorescent spectra of the fluorophore at different concentrations. (b) Purified Gal-BP NSs@Fluo after fluorophore interacted with Gal-BP NSs. The weight ratio was 1/8.

Figure S16. The Z-stack analysis of internalization of based-BP NSs in PAO1 and ATCC 8739.

Figure S17. CFU of PAO1 after treated with different concentration of BP NSs or Gal-BP NSs with or without NIR laser. * $p \leq 0.05$, ** $p \leq 0.01$.

Figure S18. CFU of PAO1 after treated with different concentration of Kana with or without NIR laser. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Figure S19. CFU of PAO1 after treated with different concentration of Kana loaded into 64 µg/mL BP NSs or Gal-BP NSs with or without NIR laser. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Figure S20. The PAO1 intracellular ROS production after different treatments with or without NIR laser irradiation (using CM-H₂DCFDA as probe). * $p \leq 0.05$, ** $p \leq 0.01$.

Figure S21. H&E staining of heart, liver, spleen, lung and kidney tissue slides (200 $\times$) for different treatments at day 9.