Conceptually Novel Black Phosphorus/Cellulose Hydrogels as Promising Photothermal Agents for Effective Cancer Therapy

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Black phosphorus (BP) has recently emerged as an intriguing photothermal agent in photothermal therapy (PTT) against cancer by virtue of its high photothermal efficiency, biocompatibility, and biodegradability. However, naked BP is intrinsically characterized by easy oxidation (or natural degradation) and sedimentation inside the tumor microenvironment, leading to a short-term therapeutic and inhomogeneous photothermal effect. Development of BP-based nanocomposites for PTT against cancer therefore remains challenging. The present work demonstrates that green and injectable composite hydrogels based on cellulose and BP nanosheets (BPNSs) are of great efficiency for PTT against cancer. The resultant cellulose/BPNS-based hydrogel possesses 3D networks with irregular micrometer-sized pores and thin, strong cellulose-formed walls and exhibits an excellent photothermal response, enhanced stability, and good flexibility. Importantly, this hydrogel nanoplatform is totally harmless and biocompatible both in vivo and in vitro. This work may facilitate the development of BP-polymer-based photothermal agents in the form of hydrogels for biomedical-related clinic applications.

1. Introduction

As a representative 2D material, graphene exhibited considerable academic and industrial success during the past decade.[1] This has in turn prompted intensive explorations of other 2D materials.[2] Black phosphorus (BP), each layer of which is also well known as phosphorene, has emerged as a new 2D material in recent years.[3] Compared with graphene, BP has several distinct characteristics[4] including high anisotropy due to its wrinkled structure along the zigzag and armchair directions, both a direct and layer-dependent band gap ($E_p$) from 0.2 eV (bulk state) to 3.2 eV (monolayer) in contrast with semimetallic graphene, and, in particular, strong sensitivity to oxygen and water in association with visible light, resulting in poor stability under ambient conditions.[5] The largest drawback of BP is its poor environmental stability. Its degradation is the main cause of the deterioration of its intriguing physical properties under ambient condition. Many efforts have thus been made to protect BP from degradation in air.[6] However, recent investigations have demonstrated that this property of easy degradation is a valuable advantage when BP is used as a photothermal agent for photothermal therapy (PTT) against cancer.[7] First, as a direct band-gap semiconductor, BP shows excellent photothermal conversion efficiency and high photothermal cycle stability in water, either in the form of BP nanosheets (BPNSs) or BP
quantum dots.[7a,b] BP-based aerogels even exhibit an enhanced high-performance photothermal response.[8] Second, BP exhibits low toxicity,[9] is biocompatible (given that phosphorus is a vital element consisting of ~1% of the total weight of the human body), and is degradable in the living body, whose metabolites is believed to be phosphate with limited risks.[9] Considering their excellent photothermal and biodegradability properties, BP-based nanostructures may be superior to graphene in PTT-related biomedical applications. Another important point regarding BP is its appropriate modification because naked BP readily subsides in solvent such as water[10] or the microenvironment of the living body, which inevitably results in a short-term therapeutic and inhomogeneous photothermal effect. As a result, some typical strategies have been developed such as polymeric modification by simple physical blending[7a,c] or chemical treatment.[7b] In spite of these promising findings concerning PTT against cancer, previous studies of BP-based materials for PTT have been limited to BP/polymer-based nanoparticles and are still far from meeting the diverse requirements of PTT in practice.

Cellulose is a biodegradable polymer of the largest amount in nature. As an authentic green polymer, cellulose has many unique properties such as excellent biocompatibility, biodegradability, no toxicity, transparency, and large polarity. It has recently gained significant attention in the fields of food, energy, electronics, and especially biomedical applications.[13] The most concerning problem with cellulose is its dissolution since it is not soluble in common solvents. The unique polymer chain structures arranged with huge numbers of hydrogen bonding networks in both intrachains and interchains might be responsible for the difficulty of cellulose dissolution. Several well-established strategies to dissolve cellulose have been reported.[12] One promising green, low-cost, and highly efficient strategy is based on an aqueous NaOH–urea–H$_2$O system (w/w: 7%–12%–83%) at low temperature (−12 °C) within an ultrashort time (2 min); this strategy was first demonstrated by Zhang and co-workers.[13] With the help of this universal cellulose solution system, cellulose-based nanocomposites including film,[14] hydrogel (or aerogel),[15] electrospray nanofibers,[16] and cellulose/conductive blended polymers[17] that are not readily realized in tradition polymer modification have been successfully developed. More importantly, because of their biodegradability and biocompatibility, the use of cellulose or nanoparticle-integrated cellulose nanocomposites in tissue templates and photoluminescence in biomedicine fields can also be investigated.[18]

Among the cellulose-based composites, cellulose hydrogels integrated with functional inorganic nanoparticles have shown substantial potential in biomedical applications.[15] First, cellulose hydrogels filled with substantial amounts of water are totally biocompatible within tissues, cells, and other components of the living body and are real green materials capable of gradual degradation within the living body into water, carbon dioxide, and other substances. Second, the addition of nanoparticles into cellulose hydrogel matrices can not only improve their mechanical property but also provides them with functionality originating from the nanoparticles themselves, such as conductivity, photoluminescence, and catalytic and magnetic properties.[15]

Clinically, materials used in PTT should be effective, stable, and nontoxic. Thus, based on the considerations above, it can be reasonably anticipated that BP-integrated cellulose hydrogels can realize biosafety, enhanced stability, and controlled degradability of BP-based nanoparticles. In the present study, we demonstrate that both the cellulose hydrogel itself and that loaded with a high concentration of BPNSs show no cytotoxicity in vitro or tissue toxicity in vivo, indicating that BPNSs entrapped in hydrogels do not contact the outside environment. Moreover, the BPNSs in the cellulose hydrogel composites retained their high photothermal efficiency as illustrated by the rapid temperature increase after near-infrared (NIR) irradiation, resulting in efficient tumor elimination. The integration of the biocompatibility, biodegradation, and photothermal property of cellulose/BPNSs makes them a promising PTT agent against cancer.

2. Results and Discussion

2.1. Synthesis of BPNS-Integrated Cellulose Hydrogels

Figures 1 and 2 depict a typical synthesis route of cellulose/BPNS composite hydrogels and their corresponding characterizations, respectively. As shown in Figure 1, cellulose can be fully resolved in an aqueous NaOH (7 wt%)/urea (12 wt%)/H$_2$O (81 wt%) system at a low temperature of ~12 °C (Step I). As reported previously,[13b,2] with the help of three hydrates [Na$_x$-based hydrate (Na$_x$/H$_2$O), OH$^-$-based hydrate (OH$^-$/H$_2$O), and urea-based hydrate (urea/H$_2$O)], cellulose polymer chains can be disentangled and then resolved in water. Old huge hydrogen bonding networks of cellulose in both intrachains and interchains have been shown to be destroyed by these hydrates, and new ones between cellulose chains and Na$_x$/H$_2$O as well as OH$^-$/H$_2$O, urea/H$_2$O, and Na$_x$/H$_2$O as well as OH$^-$/H$_2$O are formed at low temperatures (Step I, left). This favors rapid dissolution of cellulose chains in water. The final cellulose chains in water exist in a channel-like form called inclusion complex supramolecules (Step I, right). To obtain BPNSs, a liquid-phase exfoliation method was adopted (Step II), and the exfoliation process was conducted in N-methyl-2-pyrrolidone (NMP), a solvent that effectively exfoliates BPNSs.[9a] The as-prepared BPNSs have a lateral dimension range from 50 to 430 nm shown in Figure 2a. Their high-resolution transmission electron microscopy (TEM) image in Figure 2b displayed a crystal-like lattice finger of 0.323 and 0.223 nm assigned to the (012) and (014) planes, respectively. The clear fast Fourier transform photograph (Figure 2c) and the corresponding selected electron diffraction pattern (Figure 2d) of the BPNSs suggested reservation of the crystalline features of the BPNSs during the exfoliation procedure. The thickness of the as-prepared BPNSs was evaluated using atomic force microscopy (AFM), as shown in Figure 2e. Results showed that these BPNSs have a broad thickness range of 5.1–10.8 nm (Figure 2f). The synthesis of BPNS-integrated cellulose (cellulose/BPNSs) hydrogels was performed by gelating cellulose chains with the aid of a crosslinker of epichlorohydrin (ECH) in the presence of BPNSs in water (Step III). The homogeneous cellulose/solution with ECH (Figure 2g) was heated to 65 °C for 1.5 h to form raw cellulose/BPNS hydrogels (Figure 2h). In this process, we observed both
chemical cross-linking (i.e., the substantial amount of −OH groups from the cellulose chains reacted with epoxy groups from ECH) (Figure S1, Supporting Information) and physical cross-linking (i.e., self-aggregation of cellulose chains).[19] The regeneration of cellulose/BPNS hydrogels was completed by adding excess dilute H2SO4 solution as shown in Figure 2i. The inorganic salts (such as NaOH, urea, Na2SO4, and H2SO4) and excess ECH molecules were completely removed by a dialysis process in water for 4 d (Figure 2j,k). Notably, degradation of the BPNSs in these hydrogels can be largely suppressed under low-temperature, dark conditions during the long dialysis process. The resultant cellulose-based hydrogels (Figure 2l) possessed a special macroscopic shape that was dependent on the shape of the vessels used and exhibited excellent flexibility and elasticity (described in the following text). Sedimentation of the BPNSs in the cellulose hydrogels can be totally avoided because of the high viscosity of the hydrogel matrices, distinct from those in water.[7a,10]

2.2. Morphology and Physical Properties

The typical morphology and physical properties of cellulose hydrogels with and without BPNSs are shown in Figure 3 and Figure S2 (Supporting Information). Neat cellulose hydrogel without BPNSs displays typical 3D networks with irregular porous structures (pore diameter of ≈50–300 µm) (Figure 3a1). These large pores provide sufficient space to both absorb and preserve a large amount of water. The formation of pores in cellulose hydrogels can be ascribed to the phase separation between rich- and poor-cellulose regions during the cross-linking process. The pore walls formed by cellulose have thin dimensions and exhibit strong supports, leading to a stable 3D network (Figure 3a2,a3). Similar 3D networks can be observed for the BP-integrated cellulose hydrogels in Figure 3b1–b3, suggesting that the addition of BPNSs does not have a significant influence on the above-mentioned phase separation behaviors and thus the 3D networks of cellulose hydrogels. In addition, the BPNSs are totally embedded within the 3D networks’ walls, as shown in Figure 1. Such a unique embedment structure of BP components in cellulose/BPNS composite hydrogels can keep BP themselves from directly contacting with living cells, thus almost avoiding their potential toxicity and endowing cellulose/BPNS composite hydrogels with outstanding biocompatibility features (described in the following text).

We also evaluated the physical properties that are of great importance in tissue engineering, including photothermal performance, viscoelasticity, and mechanical properties (Figure 4). As a promising photothermal agent, BP has shown excellent photothermal properties against tumor cells in recent investigations.[7a–c] More importantly, its total biodegradability and good biocompatibility in the living body may push it ahead in future biomedical applications. In the present study,
the obtained cellulose/BPNS composite hydrogels were based on BPNSs and cellulose, which are expected to exhibit unique photothermal properties. Figure 4a depicts the photothermal performance of BP-integrated cellulose composite aerogels and neat cellulose aerogel, respectively. The results showed that BP-integrated cellulose composites in the form of aerogels had quite high final light-induced temperatures with a short incubation time (Figure 4a). For instance, with a power laser density of 1 W cm$^{-2}$, the composite aerogel with a BP concentration of 380 ppm can reach a temperature as high as 149 °C with illumination by only an 808 nm light for as short as 18 s (Figure 4c). In sharp contrast, the cellulose aerogel without additional BP (i.e., neat cellulose aerogel) only reached a temperature of 25 °C for 120 s of illumination (Figure 4d); this is similar to room temperature, indicating that cellulose has a negligible photothermal contribution. Notably, the 380 ppm BP-integrated cellulose aerogel reached temperatures that were higher than our thermal imager's measurement range (150 °C), leading to failure of presenting a temperature plateau (Figure 4a). The remaining BP-integrated cellulose aerogels exhibited enhanced final temperatures with increasing BP contents. However, their hydrogels exhibited different characteristics as shown in Figure 4b. Because of their large amount of water, the cellulose/BPNS composite hydrogels displayed a significant decrease in final temperature with the same power density of 1 W cm$^{-2}$ compared with their aerogel counterparts. For example, the cellulose composite hydrogel with a 380 ppm BP content reached a temperature of 50 °C during 600 s of light illumination in the first cycle (Figure 4b), about 26 °C higher than room temperature but far lower than its aerogel counterpart (i.e., 149 °C at 18 s). Similarly, other cellulose hydrogels with the addition of BP exhibited close loading dependence on the light-induced final temperatures. Good photothermal cycle stabilities were also observed in all of the cellulose/BPNS hydrogels (Figure 4b). Moreover, after the photothermal measurements, the cellulose/BPNS hydrogels were still in the form of hydrogels (Figure 4e), indicating their good thermodynamic stability in practical applications. We can therefore conclude that both cellulose/BPNS composite aerogels and hydrogels exhibit excellent photothermal properties, which have been shown to be determined by the BP content and their forms of existence. As shown in Figure 4f, all the hydrogel samples showed little frequency dependence of the dynamic storage moduli ($G'$) in the whole measured frequency regime, demonstrating their highly elastic behaviors. Moreover, the $G'$ values of cellulose hydrogels could be gradually improved as the BP concentration in the cellulose/BPNS hydrogels increased. In Figure 4g, in the case of the neat cellulose hydrogel, a typical strain–stress
A “J” shape, and the corresponding compressive strength slowly increased first with increasing strain (0–30%) and then abruptly increased (30–50%) until the sample collapsed. Such a phenomenon suggests that the neat cellulose hydrogel has great flexibility under external compressive treatment. Compared with cellulose hydrogels without BPNSs, cellulose/BPNS hydrogels with 380 ppm BP content depict enhanced mechanical properties. For instance, the elastic modulus, strength at break, and strain at break of the sample with 380 ppm BP content were 30 ± 2.1 Pa, 13.9 ± 1.6 kPa, and 50.0 ± 4.9%, respectively, which were higher than those of the neat cellulose hydrogel (27.5 ± 1.3 Pa, 8.3 ± 2.2 kPa, and 46.5 ± 1.7%, respectively). These results indicate that the addition of BP into cellulose hydrogel can improve the mechanical properties of cellulose hydrogels, which is in good agreement with the viscoelasticity results shown in Figure 4f. Notably, collapse of the cellulose hydrogels was triggered by tiny cracks on their surfaces during the compressive procedure, and the final hydrogels could hold their initial water without any squeezing of the water (Figure S3, Supporting Information), demonstrating their highly stable 3D networks. Such mechanical properties of cellulose hydrogels are of great significance for their injection into the living body via syringes.

2.3. Toxicity Assays

The potential toxicity of cellulose and cellulose/BPNS hydrogels was thoroughly investigated both in vitro and in vivo. To measure the cytotoxicity toward both normal cells and cancer cells, the viability of B16 (mouse melanoma), SMMC-7721 (human hepatocellular carcinoma), and J774A.1 (mouse macrophage) cells incubated with cellulose/BPNS hydrogels with a consecutive concentration was determined by Cell Counting Kit-8 (CCK8) assays (Beyotime Biotechnology). J774A.1 cells were chosen because macrophages comprise the major cell population in the tumor microenvironment. As shown in Figure 5a, the neat cellulose hydrogel was absolutely nontoxic for the three kinds of measured cells. In the case of BPNS-integrated cellulose hydrogels, no detectable toxicity was observed, even at a BP concentration of 380 ppm. This phenomenon indicates that both cellulose and cellulose/BPNS hydrogels have rather high biocompatibility and biosafety. In contrast, despite its promising applications in PTT in view of its high light-to-heat conversion efficiency, biocompatibility, and biodegradability, BP is readily and quickly oxidized in environments containing oxygen, such as under typical biological circumstances. Additionally, BP without proper chemical or physical modifications by polymers can readily subside in water for a long time, resulting in an unstable concentration of BP near tumor sites and thus inhomogeneous photothermal effects. Fortunately, in the present study, the BPNSs were totally embedded within 3D cellulose networks’ walls, not only diminishing the chances of BP contacting oxygen but also totally stopping the BPNSs from undergoing sedimentation. Additionally, it can be speculated that the embedment structure of BP components in cellulose/BPNS composite hydrogels can effectively cut off the direct contact of BP from surrounding cells, thus suppressing the potential toxicity of BP when it is used at a high loading level. The in vivo toxicity of the cellulose/BPNS hydrogels was also assessed, as shown in Figure 5b. Six week old female BALB/c nude mice were randomly divided into three groups of five mice each and subjected to different treatments. Because of their high flexibility and toughness, making them
unsuitable for intravenous injection, the cellulose-based hydrogels after grinding were subcutaneously injected into the mice to determine their toxicity in vivo. The mice were injected with saline (negative control), cellulose hydrogel, or cellulose/BPNS hydrogel at a 380 ppm BP concentration, respectively. As depicted in Figure 5b, the body weights of the mice in the three groups showed no significant difference after 2 weeks, suggesting that the cellulose-based hydrogels with or without additional BP had no significant influence on the growth of the mice, similar to the saline injection. Moreover, the corresponding histological changes in the major organs, including the heart, liver, spleen, lung, and kidney, were evaluated by hematoxylin and eosin (H&E) staining. Again, no noticeable organelle damage was observed in the cellulose hydrogel or cellulose/BPNS groups, as in the saline group (Figure 5c). We also evaluated immunotoxicity of the cellulose/BPNS hydrogel at a 380 ppm BP concentration, by using immune-competent C57BL/6 mice, and monitored their inflammatory cytokines in the serum, including TNFα, IL-1β, and IL-6, respectively. Our results showed that the protein level of these cytokines had no significant change after hydrogel subcutaneous injection, demonstrating that the hydrogels used did not induce inflammation in vivo (Figure 5d). These results indicate that both cellulose hydrogel and cellulose/BPNS hydrogels have little influence on the cell viability of both normal cells and cancer cells and that they do not damage the living bodies, suggesting that cellulose-based hydrogels with or without the addition of BP are of high biocompatibility and biosafety.
2.4. PTT against Cancer

Having confirmed the biological safety of cellulose/BPNS hydrogels, we explored their possibilities for PTT against cancer. Both in vitro cell killing efficiency and in vivo tumor regression efficiency of the cellulose/BPNS hydrogels after NIR light irradiation were determined. Using the B16, SMMC-7721, and J774A.1 cells as model cell lines, we determined the relative cell viability after incubation with the cellulose/BPNS hydrogels (at consecutive concentrations of 0, 95, 190, 285, and 380 ppm) followed by NIR irradiation (1 W cm\(^{-2}\), 10 min). As shown in Figure 6a, the cellulose hydrogels with BP contents of 95 and 190 ppm exhibited insufficient photothermal properties to kill tumor cells, as did those without the addition of BP. With increasing BP loading levels, the cell killing efficiency reached 40–60% at 285 ppm and almost 100% at 380 ppm.

Figure 5. Toxicity assays. a) In vitro toxicity assays. Relative cell viability of B16, SMMC-7721, and J774A.1 cells after incubation with Mock (no treatment) and hydrogels without BPNSs or with BPNS concentrations of 95, 190, 285, and 380 ppm for 24 h. b,c) In vivo toxicity assays after subcutaneous injection of 100 µL of saline, hydrogel, or cellulose/BPNSs. (b) Body weight was monitored on days 1, 3, 5, 7, 9, 11, 13, and 15. (c) Hematoxylin and eosin (H&E) staining of the heart, liver, spleen, lung, and kidney on day 15. d) Immunotoxicity assays in vivo for protein levels of TNF\(\alpha\), IL-1\(\beta\), and IL-6 in the serum of C57BL/6 mice after indicated treatments. Data were shown as mean ± SD, Student’s t test, \(n=5\), n.s. means \(p > 0.05\), nonsignificant.
The difference between the cell lines may be caused due to different survival abilities under heat stress. Similar results were obtained from the fluorescence images, as shown in Figure 6b. The live SMMC-7721 cells were green (stained by calcein acetoxymethyl) and the dead ones were red (stained by propidium iodide). The SMMC-7721 cells incubated with cellulose-based hydrogels and irradiated by NIR light began to die and were fully dead as the concentration of BP increased from 285 to 380 ppm. These fluorescence results are in good agreement with the above-mentioned results of the cell killing efficiency, demonstrating that cellulose/BPNS composite hydrogels possess effective light-to-heat conversion efficiency and can be reasonably utilized for PTT against cancer in vivo.

To investigate the potential application of cellulose/BPNS composite hydrogels for cancer PTT in vivo, human hepatocellular carcinoma models were established by subcutaneous injection of SMMC-7721 cells (4 × 10⁶ cells for each mouse) into 6 week old female BALB/c nude mice. When the tumor volumes reached 100–200 mm³, the mice were randomly divided into four groups of five mice each and subjected to the following therapies: Group 1 (G1) underwent cellulose hydrogel treatment, Group 2 (G2) underwent cellulose/BPNS (380 ppm) treatment, Group 3 (G3) underwent cellulose hydrogel plus NIR irradiation treatment, and Group 4 (G4) underwent cellulose/BPNS hydrogel (380 ppm) plus NIR irradiation treatment. The temperature of the NIR-irradiated spot was continuously measured with an infrared thermal detector. As shown in Figure 6c,d, when the tumor-bearing mice were treated with cellulose hydrogel (i.e., G3), the temperature of the tumor site was only slightly augmented by NIR irradiation (41.2 °C) with a slow speed. In contrast, when treated with cellulose/BPNS hydrogel with a 380 ppm BP concentration (i.e., G4), the temperature reached 64.1 °C with a sharp increase in speed in the initial minute. After the treatments, the tumor volume of

Figure 6. Photothermal cancer therapy. a,b) In vitro photothermal assays. (a) Relative cell viability of B16, SMMC-7721, and J774A.1 cells after treatment with Mock or hydrogels and followed by additional NIR irradiation (808 nm laser, 1 W cm⁻², 5 min), as measured by Cell Counting Kit-8 assays. (b) SMMC-7721 cells were treated as in (a) and subjected to calcein acetoxymethyl/propidium iodide staining for fluorescence visualization. Scale bar, 50 µm. c–g) In vivo photothermal cancer therapy. Human hepatocellular carcinoma mice models were established as described in the Experimental Section. The tumor-bearing mice were intratumorally injected with blank hydrogel or cellulose/BPNSs (380 ppm), with or without NIR irradiation as shown in (c). The temperature changes after NIR irradiation of the hydrogel and cellulose/BPNS groups are graphically shown. (d) Time-dependent temperature changes during irradiation. (e) Time-dependent tumor volume changes after treatment with hydrogel, cellulose/BPNSs, hydrogel+NIR irradiation, and cellulose/BPNSs+NIR. The tumor volumes of Group 1 (G1), Group 2 (G2) and Group 3 (G3) were compared with the Group 4 (G4). Data were shown as mean ± SD, **p < 0.001, n = 5, Dunnett’s multiple comparison test. (f) Representative tumors isolated from the four groups. (g) Time-dependent body weight changes of the mice in the four groups.
each mouse was measured every other day, and the day of treatment was marked day 1. The tumor volume \( (V) \) was calculated by the equation: \( V = \text{length} \times \text{width}^2 \div 2 \). As shown in Figure 6e, compared with the cellulose hydrogel (G1), the addition of BPNSs into the cellulose hydrogel (G2) had no effect on tumor growth even at a high concentration of 380 ppm; however, upon NIR irradiation (G4), the tumor exhibited significant regression (was almost totally killed). G3 (cellulose hydrogel with NIR irradiation) showed a slightly decreased tumor volume, which may have resulted from the inhibition of tumor cell growth by a nonfatal temperature increase. Figure 6f shows representative tumors in each group of mice on day 15. These results demonstrate that the use of cellulose/BPNS hydrogels for cancer PTT is a quite efficient strategy to both kill the cancer cells and inhibit the development of cancer. We also measured the body weight of the mice during the treatment period, as depicted in Figure 6g. Compared with the control groups (G1, G2, and G3), mice treated with cellulose/BPNS hydrogel together with NIR irradiation (G4) experienced a reduction in body weight in the early phase (1–5 d), then showed a normally increased trend. We speculate that this phenomenon may have been ascribed to the high-temperature-induced damage to the skin at the tumor site, which may have influenced the mice’s food intake, wound recovery, and normal daily activity. Furthermore, the time-dependent in vivo biodistribution of the hydrogels using a composite cellulose/BPNS/cy7 hydrogel was also investigated. As shown in Figure S4 (Supporting Information), the hydrogel maintains for a relatively long time, which could prevent BPNSs from rapid sedimentation. Overall, our results demonstrate that the cellulose/BPNS hydrogels acted as efficient photothermal agents that can be used to kill both cancer cells and normal cells in the tumor microenvironment, and this photothermal efficiency could be preserved in vivo. The photothermal property makes cellulose/BPNSs an ideal material for photothermal cancer therapy.

3. Conclusion

We have developed green, smart cellulose/BPNS nanocomposite hydrogels via a facile, green chemical cross-linking reaction in alkaline solutions. BPNS-integrated cellulose hydrogels have excellent photothermal properties due to the presence of BP and good mechanical properties owing to their 3D networks with multipores and substantial water. Comprising cellulose and water together with homogeneous BPNS dispersion, these composite hydrogels have demonstrated biosafety and biocompatibility for both cells and organs in the living body. Moreover, by intratumoral injection, the cellulose/BPNS composite hydrogels can act as effective photothermal agents against cancer in mice. Our findings highlight the significance of gelation of BP-based nanomaterials with biodegradable polymers and will greatly impact PTT against cancer in the clinical setting.

However, to finally realize clinical and translational applications of cellulose/BPNS hydrogels, future explorations of the biosafety, cost effectiveness, and controlled synthesis of high-quality few-layer BP nanomaterials with much higher efficiency, controlled size/thickness, and reduced adverse effects are compulsory.

However, the present study has some limitations. First, cellulose-based hydrogels are excellent drug delivery systems (Figure S5, Supporting Information), and it would thus be meaningful to study the combined action of PTT and photodynamics therapy applied with cellulose/BPNS hydrogels in the future. Second, subcutaneous xenografts are superficial to the skin, whereas many tumors, including liver cancer, are much deeper. It could be more close to the clinical practice through the use of an orthotopic liver cancer model. In the future, cellulose/BPNS hydrogels might also be developed in the treatment of superficial tumors such as melanoma and breast cancer.

4. Experimental Section

Material Synthesis—Preparation of Few-layer BPNSs: Few-layer BPNSs were fabricated by liquid-phase exfoliation. Briefly, bulk BP (50 mg) was first ground in agate mortar with the aid of NMP solvent for 30 min and then transferred into a plastic tube with NMP (total volume of 50 mL). The BP/NMP mixture was first treated by tip sonication for 6 h (power of 300 W) and then subjected to bath ultrasonication for 72 h (power of 600 W). The resultant BP/NMP mixture was then centrifuged at a speed of 7000 rpm to obtain a BPNS/NMP solution. Few-layer BPNSs were then obtained by centrifugation at a speed of 18 000 rpm. The residual NMP solvent for the BPNSs was totally removed by centrifuging the BPNS/ethanol solution at least three times. Finally, the few-layer BPNSs were dispersed in water for the following procedure.

Material Synthesis—Preparation of Cellulose Solution: The cellulose solution was prepared as described in previous reports.\(^{[13a,b,d,e]}\) In brief, NaOH–urea–H\(_2\)O solution (w/w: 7%–12%–81%) was first precooled to –12 °C, and cellulose powder was then added into this aqueous solution with vigorous stirring by a high-speed mixer machine (7000 rpm) for 2 min. The solid mass weight of cellulose relative to the NaOH–urea–H\(_2\)O solution was 4 wt%. The obtained transparent cellulose solution was subjected to bath ultrasonication for 1 min to be degassed and stored at room temperature.

Material Synthesis—Preparation of Cellulose/BPNS Hydrogels and Aerogels: The cellulose/BPNS hydrogels were synthesized as follows: 5 mL of cellulose solution was thoroughly mixed with a certain volume of BP/H\(_2\)O solution (0.25, 0.5, 0.75, and 1 mL) as well as corresponding deionized water (0.75, 0.5, 0.25, and 0 mL) and 0.5 mL of ECH at room temperature for 30 min. The vial containing the mixture was then placed into an oil bath at 65 °C for 1.5 h. Then, 0.5 mL of ECH was added to the mixture, with the aid of NMP solvent for the BPNSs was totally removed by centrifuging the few-layer BPNS/NMP mixture for 600 W. The resultant BP/NMP mixture was then subjected to bath ultrasonication for 72 h (power of 600 W). The resultant BP/NMP mixture was then centrifuged at a speed of 7000 rpm to obtain a BPNS/NMP solution. Few-layer BPNSs were then obtained by centrifugation at a speed of 18 000 rpm. The residual NMP solvent for the BPNSs was totally removed by centrifuging the BPNS/ethanol solution at least three times. Finally, the few-layer BPNSs were dispersed in water for the following procedure.

Figure S4 (Supporting Information), the hydrogel maintains for a relatively long time, which could prevent BPNSs from rapid sedimentation. Overall, our results demonstrate that the cellulose/BPNS hydrogels acted as efficient photothermal agents that can be used to kill both cancer cells and normal cells in the tumor microenvironment, and this photothermal efficiency could be preserved in vivo. The photothermal property makes cellulose/BPNSs an ideal material for photothermal cancer therapy.
AFM (Bruker). The morphology of the cellulose/BPNS hydrogels was characterized using field emission scanning electron microscopy (SEM) (Hitachi SU8010). To maintain their pore structures, these hydrogels were subjected to freeze-drying treatment, and the as-prepared aerogels were used for SEM measurement. The samples were coated with a thin layer of Pt before characterization. An acceleration voltage of 5 kV was used for the samples. The working distance ranged from 8.0 to 9.4 mm. Fourier-transform infrared spectroscopy (PerkinElmer) was performed using an attenuated total reflectance model with 64 scans. The rheological behaviors of the samples were characterized using an Anton Paar MCR 52 instrument. Dynamic frequency sweep experiments were measured from 1 to 100 rad s⁻¹ at a fixed oscillatory strain of 0.2% at room temperature. The compressive measurements were conducted using universal material testing system (model: Instron 5966). All the measurements were performed at room temperature at a fixed crosshead speed of 3 mm min⁻¹ and a gauge length of 24 mm. Five samples were tested and averaged for each formulation. The photothermal properties of the samples were characterized using an 808 nm (near-infrared light) NIR laser instrument equipped with an infrared imaging camera (FLIR-160) to monitor the temperature change of the samples.

**Cell Lines:** The human hepatocellular carcinoma cell line SMMC-7721 was cultured in DMEM/F12 1:1 medium (Hyclone), and the mouse melanoma B16 cells and mouse macrophage J774A.1 cells were cultured in DMEM/high glucose (Hyclone). The medium contains 10% FBS (Gibco) and 1% Pen/Strep (Gibco), and the cells were obtained from the American Type Culture Collection and cultured in an incubator (Thermo Fisher Scientific) at 37 °C and 5% CO₂.

**In Vitro Cytotoxicity Assays:** B16, SMMC-7721, and J774A.1 cells were seeded in 96-well plates. After adherence, the cells were untreated (Mock) or treated with hydrogel (without BPNSs) or cellulose/BPNSs (the concentration of BPNSs in the hydrogel was 95, 190, 285, or 380 ppm). The hydrogels were sliced into 8 mm³ cubes (2 mm in each dimension) before use so that they could be placed into the wells. At 24 h post-treatment, the hydrogels were removed and the cells were subjected to CCK8 assays according to the manufacturer's instructions.

**In Vivo Toxicity Assay:** All animals in this study were obtained from the Guangdong Medical Laboratory Animal Center (Guangzhou, China), and all of the in vivo experiments followed the protocols approved by the Animal Care and Use Committee of the Shenzhen University. Fifteen 6 week old female BALB/c nude mice were randomly divided into three groups, and their body weight was measured (day 1). They were then subcutaneously injected with 100 µL of saline, cellulose hydrogel (without BPNSs), or cellulose/BPNSs (the BPNS concentration was 380 ppm (1.5 mg kg⁻¹)) in the left flank. The hydrogels were first ground into slurry to facilitate injection. Body weights were recorded on days 1, 3, 5, 7, 9, 11, 13, and 15. On day 15, the mice were killed and the major organs including the heart, liver, spleen, kidney, and lung were collected for hematoxylin and eosin staining as described elsewhere.[20]

**ELISA Assay of Inflammatory Cytokines:** Saline, cellulose hydrogel, and cellulose/BPNS composite hydrogel (380 ppm BP concentration) were subcutaneously injected in the left flank of 6 week old female C57BL/6J mice, the group size was 5. After 15 d of postinjection, the serum of each mouse was collected and subjected to ELISA assays according to the manufactures' instructions (R&D systems).

**In Vitro Photothermal Assays:** The cells were seeded and treated with Mock or hydrogels as in vitro cytotoxicity assays and further treated with NIR irradiation (808 nm laser, 1 W cm⁻², 10 min; the distance between the laser exciter and culture plates was about 5 cm) after incubation with Mock or hydrogels for 2 h. At 24 h postirradiation, the cells were subjected to CCK8 assays or calcine acetoxymethyl/propidium iodide staining (Beyotime Biotechnology) to visualize the difference between live and dead cells under fluorescence microscopy according to the manufacturer's instructions.

**Cell Viability:** Cell viabilities after treatments of hydrogels with/without NIR irradiation were detected by light absorption at 450 nm (OD 450 nm). For each cell line, the mean OD of Mock group was set to be 100%, respectively, which stands for the cell viabilities of the negative control. The ODs of other groups of each cell line were normalized to the corresponding Mock groups, shown as percentage of the Mock group.

**In Vivo Photothermal Cancer Therapy:** SMMC-7721 cells were prepared in phosphate-buffered solution at a concentration of 4 × 10⁶. Each 6 week old female BALB/c nude mouse was subcutaneously injected with 4 × 10⁶ SMMC-7721 cells to establish a human hepatocellular carcinoma model in vivo. The tumor volumes were measured every 2 days and calculated using the equation length × width²/2. About 12 d postinjection, the tumor volume reached 100–200 mm³ and was thus ready for cancer therapy research. The mice bearing tumors were then randomly divided into four groups for further therapy: G1, cellulose hydrogel with neither BPNSs nor NIR irradiation; G2, cellulose hydrogel with 380 ppm BPNSs (cellulose/BPNSs) and no NIR irradiation; G3, cellulose hydrogel without BP, but with NIR irradiation (hydrogel+NIR); and G4: hydrogel with both BP (380 ppm BPNSs) and NIR irradiation (cellulose/BPNS+NIR). The homogenous grounded hydrogels were intratumorally injected at 100 µL (day 1), and the amount of BPNSs was adjusted to 1.5 mg kg⁻¹. The mice in all the four groups were then anesthetized with pentobarbital sodium (60 mg kg⁻¹), and the mice in G3 and G4 were further subjected to NIR irradiation. The settings for NIR irradiation were an 808 nm laser, 1 W cm⁻², 5 min. The temperatures at the tumor sites after irradiation were recorded with an infrared thermal detector. The tumor volume and body weight were monitored every 2 d until day 15, when all the mice were killed and the tumors of each group were isolated from the mice for further assays.

**Statistical Analysis:** Where indicated, data were analyzed for statistical significance and reported as p values. Statistical analysis was performed with GraphPad Prism software.

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

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