Research article

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Black phosphorus nanosheets and paclitaxel encapsulated hydrogel for synergistic photothermal-chemotherapy

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Abstract: Gastric cancer treatment remains a major challenge because of its aggressiveness and spread. In this study, we developed a hydrogel system for the treatment of gastric cancer, which can kill tumor cells through photothermal action and drug treatment. Based on the formation of Schiff base linkage, the OSA/AHA/BP/PTX hydrogel was prepared by mixing oxidized sodium alginate (OSA), aminated hyaluronic acid (AHA), black

phosphorus (BP), and paclitaxel (PTX) under physiological conditions, which exhibited excellent photothermal effect and slow release ability PTX. Moreover, CCK-8 and live/dead fluorescent confirmed that OSA/AHA/BP/PTX hydrogel could obvious inhibition the proliferation of gastric cancer cells (SGC7901). More importantly, *in vivo* experiments further show that the prepared hydrogel can significantly improve the tumor treatment effect of tumor-bearing mice by inducing tumor cell apoptosis and inhibiting the proliferation of new tumor cells. Compared with chemotherapy alone, photothermal combined chemotherapy had a better antitumor effect. The results of this study indicate that the composite hydrogel with controlled release of paclitaxel can be used as a candidate material for cancer treatment.

Keywords: black phosphorus nanosheets; chemotherapy; paclitaxel; photothermal therapy; thermosensitive hydrogel.

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1 Introduction

Gastric cancer is currently one of the most common malignant tumors in the world. The incidence rate in my country is very high, and it accounts for a large proportion in the world, almost reaching more than 40% [1]. Current treatment methods have great limitations. Only surgical treatment has a low cure rate. Radiotherapy and chemotherapy are auxiliary treatment methods before and after operation, which have no significant impact on the survival rate of patients with gastric cancer and have toxic and side effects [2, 3]. Therefore, the exploration of safe and effective treatment is an urgent problem to improve the cure rate of gastric cancer.

As an emerging cancer treatment method [4, 5], photothermal therapy (PTT) is based on the principle of photothermal conversion [6, 7]. Under the irradiation of near-infrared light, nanoparticles (NPs) with high photothermal conversion efficiency can produce heat to

kill tumor cells for treat cancer [8, 9]. Some PTT reagents including gold nanoparticles [10, 11] and some 2D nanomaterials such as graphene [12–14], and transition-metal dichalcogenides (TMDs), such as molybdenum disulfide (MoS_2) [15–17] and covalent–organic frameworks (COFs) [18,19], due to their superior near-infrared light efficiency, it is mainly used as a photothermal treatment reagent. However, most of them have certain limitations, such as lack of biodegradability, leading to their accumulation in the human body, which is a big challenge for their application [20, 21]. Therefore, it is urgent to explore new biodegradable PTT preparations with low cytotoxicity and high biological safety.

Black phosphorus (BP) nanosheet is a newly discovered two-dimensional nanomaterial with some excellent properties [22, 23], such as excellent biocompatibility, high extinction coefficient and photothermal conversion efficiency, which makes it widely used in the photothermal treatment of cancer [24, 25]. However, the biggest disadvantage of BP is its poor environmental stability. Its degradation is the main reason for its decline in physical properties under environmental conditions.

Therefore, some typical strategies have been developed, such as simple physical blending polymer modification or chemical treatment [26]. Hydrogels have been widely used in the fields of biomedicine due to their excellent swelling, permeability, biocompatibility, and nondeactivation of drug loading. In some studies, injectable thermosensitive hydrogels have been used to deliver PTT reagents and drugs. Li et al. [27] proposed that the thermosensitive hydrogel embedding BP for photothermal treatment, the local temperature of tumor tissue increased, and the therapeutic effect of hydrogel was better than that of blank hydrogel. Liu et al. prepared a photothermal therapy hydrogel containing BP, which has low cytotoxicity and high biodegradability. The photothermal controlled release drug accurately treats cancer [28]. Shi et al. prepared a new type of magnetic hydrogel. The hydrogel has selfhealing, sheer thinning, and smooth injection properties. The hydrogel is effective in tissue regeneration and anticancer treatment [29].

The main purpose of the project is to prepare a hydrogel system for the treatment of tumors, which can combine photothermal therapy with chemotherapy to effectively inhibit the proliferation of tumor cells. The OSA/AHA/BPNS/PTX hydrogel was successfully prepared by dynamic Schiff base bonding. Among them, oxidized sodium alginate (OSA) and aminated hyaluronic acid (AHA) have good biocompatibility, biodegradability, and easy modification, so they are selected as the preparation materials of the hydrogel matrix. Paclitaxel (PTX), a type of

microtubule depolymerization inhibitor, is one of the main components in gastric cancer chemotherapy. The hydrogel is prepared by the Schiff base formation between the aldehyde group of OSA ($-\text{CHO}$) and the amino group of AHA. The controlled release of paclitaxel in the hydrogel can be achieved by the effect of black phosphorescence and heat, which effectively inhibits tumor cell proliferation. The purpose of this study is to determine the therapeutic effect of OSA/AHA/BPNS/PTX hydrogel on tumor-bearing mice, and to provide a feasible plan for the treatment of cancer.

2 Experimental section

2.1 Material

Sodium alginate, oxalic acid dihydrazide (ADH), 1-hydroxybenzotriazole hydrate (HOBt), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and dimethyl sulfoxide were bought from Macleans Biochemical Technology Co., Ltd. Sodium hyaluronate was obtained from Huaxi Biological Technology Co., Ltd. Sodium periodate was purchased from Tianjin Damao Chemical Reagent Factory. The bulk BP was ordered from Xianfeng Nano Material Technology Co., Ltd.

2.2 Preparation of BP nanosheets

The BP nanosheets were prepared by a simple liquid exfoliation technique. In short, 30 mg of the purchased BP was added to 60 mL of ultrapure water, which bubbled with argon to eliminate dissolved oxygen molecules, thereby reducing oxidation during the peeling process. Then the mixture solution was sonicated in ice bath for 12 h (amplifier: 25%, on/off cycle: 15 s/5 s). The preparing brown dispersion was centrifuged at 2000 rpm for 5 min to remove the residual clumps of BP. The supernatant containing BP NSs was collected for further use and stored under 4 °C.

2.3 Preparation of oxidized sodium alginate (OSA)

An amount of 5 g of sodium alginate was dissolved in 200 mL of deionized water under stirring at 400 rpm, and then 50 mL of ethanol was added to disperse and dissolve. A total of 10 mL 0.5 g mL⁻¹ sodium periodate aqueous solution was slowly added dropwise to the above solution in

the dark. After reaction for 24 h at 25 °C, 5 mL of ethylene glycol was added to the mixture to terminate the reaction. After the solution stranded for at least 2 h, 500 mL of ethanol was added to precipitate the OSA. After that, the solid OSA was collected and re-dissolved in 100 mL deionized water and dialyzed (dialysis bag, Mw cut-off value was 3000 Da) for 3 days. Finally, the OSA was freeze-dried for use.

2.4 Preparation of aminated hyaluronic acid (AHA)

AHA was prepared using aqueous conditions following previously described procedures with slight modifications [30]. 0.5 g of Sodium hyaluronate (HA) was dissolved with 100 mL of deionized water. An amount of 10 g of ADH was added to the HA solution. An amount of 0.8 g of EDC and 0.7 g of HOBt were dissolved in DMSO/H₂O (v/v = 1:1, 5 mL each), which added to the above solution. The diluted hydrochloric acid was added to the solution for adjusting the pH to 5.0. After reacting at room temperature for 24 h, 5 g of NaCl was added to the reaction solution and AHA was precipitated with ethanol. The precipitate was washed three times with water and dialyzed for three days to remove the salt. The purified product was freeze dried at -80 °C and kept at 4 °C.

2.5 Preparation of hybrid hydrogel

For the preparation of OSA/AHA hydrogels, 2.0 wt% AHA with different concentrations of OSA (10, 15 and 20 wt%) were used to synthesize hydrogel AHA/10% OSA, AHA/15% OSA and AHA/20% OSA. Briefly, AHA and OSA were dissolved in PBS (0.1 M, pH = 7.2) at room temperature. Then, the OSA solution and AHA solution was mixed thoroughly in equal proportions, and then reacted at room temperature for gelation.

2.6 Preparation of OSA/AHA/BPNS/PTX hydrogel

A quantity of 1 mL of the 200 µg mL⁻¹ black nanosheets (BPNS) was thoroughly mixed with 4 mL of AHA solutions as well as 2 mL of OSA aqueous and 1 mL of the 1 mg mL⁻¹ paclitaxel (PTX) for 30 min. The above mixed solution formed an OSA/AHA/BPNS/PTX hydrogel matrix.

2.7 Characterization

Chemical structure of AHA and OSA was characterized by ¹H NMR spectroscopy (300 MHz, Varian, USA) using deuterium oxide (D₂O) as the solvent. The shape and morphology of the synthesized BPNS were studied with transmission electron microscopy (JEOL TEM-1210) at 120 kV. The morphology of the synthesized hydrogels was tested by scanning electron microscopy (SEM, LEO1530 VP, Germany). Fourier transform infrared spectrum of all samples was collected in a PerkinElmer Spectrum 100 FT-IR spectrometer (PerkinElmer Inc., USA) under the transmittance mode with KBr plates. And the optical property of BP was evaluated by UV-Vis absorption spectra (UV-3100PC, Mapada Instruments, Shanghai, China). The gelation time was measured using the inverted tube test.

2.8 Rheological characterization

Rheological characterization of hydrogels was performed using a rheometer (Kinexus, Ma Erwen instruments, Britain). The storage modulus (*G'*) and loss modulus (*G''*) of the hydrogels were measured in the oscillation mode using a parallel plate configuration (diameter 25 mm). In the oscillation time sweep experiment, *G'* and *G''* were recorded at 10% strain, at frequency of 1 Hz and 0.5 mm gap (CD mode) for 300 s. In order to characterize the linear viscoelastic range, a dynamic strain sweep test with a frequency of 0.1–100 Hz was carried out.

2.9 In vitro degradation

The degradation profile of the hydrogels was tracked *in vitro* by measuring weight loss in PBS containing either 0 or 100 U mL⁻¹ of hyaluronidase solution in a horizontal shaker at 37 °C over time. At predetermined time intervals of 3, 7, 14, and 21 days, the hydrogels were taken out, washed with distilled water and lyophilized. The degradation rate was calculated using the formula, in which *W*₀ represented the initial weight of the freeze-dried hydrogel and *W*_{*t*} is the weight of the freeze-dried hydrogel at time *t*. All tests were done on five samples (*n* = 5).

2.10 Compression test

The compression test of the hydrogels was performed on a universal testing machine (Model 5543; Instron, Norwood, MA). Hydrogel samples with a diameter of 8 mm and a thickness of 4 mm were prepared in advance and

equilibrated in PBS. The compressive strain rate was set to 1 mm min^{-1} with a 5 N load cell under 40% constrain. The measurements were performed three times ($n = 3$).

2.11 Photothermal effect of the OSA/AHA/BPNS

The photothermal performance and photothermal stability of the system were studied. The BPNS, OSA/AHA, OSA/AHA/BPNS, and OSA/AHA/BPNS/PTX were irradiated with 808 nm laser at 1.0 W cm^{-2} . And the OSA/AHA/BPNS was exposed to 808 nm laser with different power. A thermal imaging camera was used to measure the temperature of these samples at different points in time. In order to obtain photothermal stability, the hydrogel was irradiated with 808 nm laser for four on/off cycles. Measure the temperature change before and after irradiation with infrared thermal imaging camera.

2.12 *In vitro* drug release

To investigate the release profile of paclitaxel from the hydrogel, 1 mL of OSA/AHA/BPNS/PTX was put into a glass tube and continuously stirred (50 rpm) on a constant temperature shaker. A quantity of 1 mL of 37°C PBS (pH 7.4) was added to the top of the sample. At different time periods, the hydrogel was exposed to 808 nm laser irradiation (1.0 W cm^{-2} , 10 min), and all the culture medium was taken out at pre-determined intervals and replaced with an equal volume of fresh culture medium. And use UV-Vis to detect the light and heat of OSA/AHA/BPNS/PTX to control drug release.

2.13 *In vitro* cytotoxicity assays

SGC7901 and L929 cells were seeded in 96-well plates at a density of 1×10^4 cells per well and incubated at 37°C and 5% CO_2 for 24 h. The OSA/AHA, OSA/AHA/BPNS, OSA/AHA/PTX or OSA/AHA/BPNS/PTX + NIR (0.1 g) were soaked in 1 mL of complete medium and incubated at 37°C with 5% CO_2 for 24 h to get leach liquor. After adherence, the cells were untreated or treated with leach liquor 24 h. Five multiple wells were used for each sample. According to the manufacturer's instructions, the cells were subjected to CCK8 analysis or calcian acetoxymethyl/propidium iodide staining (Beyotime Biotechnology) to observe the difference between live and dead cells under a fluorescence microscope.

2.14 *In vivo* photothermal performance

To explore the *in vivo* photothermal efficacy of AHA/OSA/BPNS, tumor-bearing nude mice were divided into two groups and injected intratumor with PBS and AHA/OSA/BPNS respectively. One hour after the injection, the mice were irradiated with an 808 nm laser at a power density of 1.0 W cm^{-2} for 5 min. During the irradiation, an infrared thermal imaging camera (Fotric 226) was used to record the temperature change of the tumor.

2.15 *In vivo* antitumor efficacy

Female BALB/c nude mice bearing SGC7901 tumors were adopted to carry out the *in vivo* antitumor study. When the tumor volume reached approximately 100 mm^3 , the mice were randomly divided into six groups (three mice per group): (1) PBS, (2) NIR, (3) PTX, (4) OSA/AHA/BPNS plus 808 nm irradiation, (5) OSA/AHA/BPNS/PTX, (6) OSA/AHA/BPNS/PTX plus 808 nm irradiation. For group 1, the mice were intratumorally injected 100 μL of PBS. For group 2, the mice were received an intratumoral injection of PBS. For group 3, the mice were received an intratumoral injection of PTX (20 mg kg^{-1} of PTX). For group 4, the mice were given intratumoral injection of OSA/AHA/BPNS (1.5 mg kg^{-1} of BPNS). For group 5 and group 6, the mice were given intra tumoral injection of OSA/AHA/BPNS/PTX. For group 2, group 4, and group 6, the mice were exposed to 808 nm laser at the power density of 1.5 W cm^{-2} for 5 min. For *in vivo* antitumor study, the tumor volume and body weight variation were measured every other day. The day of treatment was recorded as day 0. And the tumor volume was calculated by the following formula: $\text{volume} = [\text{length} \times (\text{width})^2]/2$.

3 Results and discussion

3.1 Synthesis and characterization of OSA/AHA and OSA/AHA/BPNS

The BP nanosheets were prepared the liquid stripping method was used to prepare a modified liquid exfoliation technique reported by previous studies [31]. The morphology of BP was characterized by transmission electron microscopy (TEM). As shown in Figure 1A, the TEM image shows that the BP nanosheets have a uniform 2D sheet-like morphology with an average lateral size of 200 nm. The particle size distribution of BP was further

Figure 1: (A) TEM of BP. (B) DLS of BP. (C) Absorbance spectra of BPNSs dispersed in IPA at different concentrations. (D) Schematic diagram of gelation of hydrogel over time. (E) Statistic diagram of gel forming time of hydrogels with different ratios. (F) ^1H NMR spectra for AHA and OSA. (G) FT-IR spectra of OSA, AHA, and AHA/OSA.

tested by dynamic light scattering (DLS), and the average particle size was 216.9 ± 1.2 nm (PDI 0.24), as shown in Figure 1B. The optical properties of BP nanosheets dispersed in aqueous solution were measured. As shown in Figure 1C, BP nanosheets have a typical broad absorption band spanning the ultraviolet and near-infrared region, which are similar to other two-dimensional layered materials.

Figure 1F showed the ^1H NMR spectrum of AHA. The 1.9 ppm peak was assigned to the acetylamino part of the *N*-acetyl-D-glucosamine residue of HA, and the peak between 2.2 and 2.4 ppm referred to the integral of the methylene proton of ADH [32]. In the ^1H NMR spectrum of OSA, all new peaks appeared at 5.12–5.72 ppm, which were produced by the combination of aldehyde groups and hydroxyl groups in OSA to form hemiacetals. Compared with the infrared spectrum analysis, oxidized sodium alginate was successfully prepared.

Under mild conditions, the AHA and OSA solutions were mixed thoroughly to form a hydrogel within a few minutes. This is mainly due to the Schiff base reaction between the amino group of AHA and the aldehyde group of OSA. Figure 1D showed that OSA/AHA was formed after mixing with OSA and gelatin, allowing for *in situ* generation of hydrogels in tumor tissues. The gel time of OSA/AHA with different concentrations of OSA was

measured, the gelation time of AHA/10% OSA was about 130 s, and AHA/15% OSA and AHA/20% OSA could form gels within 30 s, as shown in Figure 1E. As the amount of OSA increases, the gel time was gradually shortened, because the higher the amino content, the greater the possibility of contact with aldehyde groups. As the results of the FT-IR spectrum shown in Figure 1G, the C=O stretching vibration of AHA/OSA was significantly increased at 1660 cm^{-1} , indicating that the aldehyde group was cleaved and a tertiary amide bond was formed. There were many hydroxyl groups in the molecular structure of sodium alginate (SA), which makes it exhibit a broad peak at 3424.9 cm^{-1} . After oxidation, this peak narrows significantly, which can indirectly indicate the decrease in the number of –OH.

3.2 Physical and mechanical properties of hydrogels

In this study, we designed a kind of hydrogel using a dynamic Schiff base, which has good biological description and good compressibility. According to the synthetic route shown in Scheme 1, OSA and AHA macromolecules were successfully obtained.

The gelation behavior of hydrogels was measured by rheological analysis. As presented in Figure 2A, the G' surpassed G'' immediately when OSA was added to the AHA solution because of the quick formation of the hydrogel. When the polymer solution was injected into the body, the polymer solution diffuses into the surrounding tissues, thereby forming a hydrogel. The final G' of OSA/AHA hydrogel can reach a plateau of 10^4 Pa, which could preserve a structurally robust 3D network shape. At the same time, the storage modulus G' of OSA/AHA hydrogels increased due to the increase in the concentration of the

hydrogel and the dynamic Schiff base bond. As demonstrated in Figure 2B, the G' exceeded G'' under the shear frequency from 0.1 to 100 rad s^{-1} due to the Schiff's base. It is indicated that the hydrogel was stable.

The ideal hydrogel should have good mechanical properties to maintain its integrity during use. The compression moduli of the hydrogels are presented in Figure 2C. The results show that AHA/20% OSA hydrogel had a higher modulus (~ 3 kPa) than AHA/10% OSA hydrogel and AHA/15% OSA. The mechanical properties are related to the structure of the hydrogel, As shown

Figure 2: (A) The rheological properties. (B) The gel viscosity with frequency ranging from 0.1 to 100 Hz. (C) The compression modulus test. (D) SEM images of the cross-section of hydrogels. (E) Weight loss rate of the hydrogels in pure PBS solution and PBS solution containing 100 U mL^{-1} hyaluronidase. (F) SEM images of hydrogel at different time points of degradation.

Figure 2D, the pore size of the AHA/15% OSA hydrogel with higher crosslinking density was smaller than that of the AHA/10% OSA hydrogel, resulting in a more compact structure. Our results indicated that the compressive modulus of the hydrogel could be improved by increasing the amount of OSA.

In PBS (with or without 100 U mL⁻¹), the weight loss curve of the prepared hydrogel (Hyaluronidase) gradually decreased with increasing incubation time (Figure 2E). All hydrogels degrade 10–15% in 3 days, 30–40% in 7 days, 45–50% in 14 days, and 60–70% in 21 days. When the hydrogel was placed in PBS containing hyaluronidase, the weight loss from degradation was significantly higher than that in PBS solution. The hydrogel degrades 81–91% after 21 days, and the rapid degradation rate is related to the β elimination mechanism caused by hyaluronidase. In addition, we observed the degradation of the *in vitro* simulated degradation experiment with and without enzymes through the SEM (Figure 2F). We could clearly see that as the degradation time increases, the hydrogel's network structure collapses more seriously.

3.3 Photothermal performance

Strong absorption in the near-infrared region is a prerequisite for photothermal conversion [33]. As shown in

Figure S2, BP exhibits broad and strong absorption in the wavelength range from ultraviolet to near-infrared. According to Beer–Lambert's law [34], the extinction coefficient at 808 nm is estimated to be 54.2 L·g⁻¹·cm⁻¹, which may be applied to in-depth clinical treatment.

BP had strong absorption ranging from UV to NIR wavelengths, and had efficient light-to-heat conversion ability. In order to evaluate the near-infrared photothermal performance, Hydrogels with different BP concentrations (0, 50, 100, and 200 $\mu\text{g mL}^{-1}$) were dispersed in an aqueous solution and exposed to a near-infrared laser at 808 nm and 1.0 W cm⁻². The solution temperature was monitored as a function of the irradiation time (Figure 3A). At low concentration (200 $\mu\text{g mL}^{-1}$), the solution temperature increased by 22.5 °C after 10 min of irradiation, while the temperature of water only increased by 3.9 °C. It indicated that BP nanosheets can quickly and effectively convert near-infrared light into heat. In addition, we explored the temperature evolution of BPNS, OSA/AHA/BPNS/PTX, OSA/AHA, and OSA/AHA/BPNS (BPNS:50 $\mu\text{g mL}^{-1}$) with 1 W cm⁻² for 10 min. As shown in Figure S3, the BPNS solution temperature increased by 17.5 °C after 10 min of irradiation. There was no significant difference between the temperature rise of OSA/AHA/BPNS/PTX, OSA/AHA/BPNS and BPNS, while the temperature of the water only increased by 3.5 °C. This

Figure 3: (A) Photothermal heating curves of the OSA/AHA/BPNS with different concentrations of BP nanosheets (0, 50, 100 and 200 $\mu\text{g mL}^{-1}$) irradiated with an NIR laser (808 nm, 1.0 W cm⁻²) for 10 min. (B) Temperature change versus time under 808 nm laser with power of 0.5 W cm⁻² during 0–60 min, 1 W cm⁻² during 60–120 min, and 1.5 W cm⁻² during 120–180 min. (C) The photothermal stability test formula of OSA/AHA/BPNS hydrogel. The power density of the near-infrared light was 1 W cm⁻². (D) PTX standard curve. (E) Absorbance spectra of released PTX. (F) Release rate of PTX with and without laser exposure.

indicated that the hydrogel does not affect the thermal performance of BP.

Photothermal stability was one of the most important indicators for evaluating PTT agents, and it helped the long-term treatment of hydrogels. Therefore, the photothermal stability of AHA/OSA/BPNS was studied. Figure 3C showed the heating profile of a hydrogel with 1 W cm^{-2} different power densities. The results showed that even after four on–off cycles, the temperature change was small, which proved that the BP nanosheets had satisfactory photothermal stability.

3.4 NIR-light-controlled AHA/OSA/BPNS degradation

The near-infrared degradability of AHA/OSA/BPNS could increase its clinical application potential. In order to further evaluate the biodegradation of AHA/OSA/BPNS, the temperature of the gel was monitored over time under near-infrared laser irradiation. As shown Figure 3B, compared with the ambient temperature under 0.5 or 1 W cm^{-2} irradiation, AHA/OSA/BPNS could work well when the laser power was lower and the temperature rises above 10 or 20 °C. These cycles were repeated six times, and the hydrogel underwent reversible softening. When the laser power was increased to 1.5 W, the temperature increased sharply, the hydrogel gradually melts, and the increase in temperature relative to the ambient temperature gradually decreased.

3.5 NIR-light controlled drug releasing

Near-infrared light with a wavelength of 808 nm was used to study the photothermal effect of the embedded BPNS on the drug release rate. UV/Vis spectrometer was used to monitor the drug concentration released in the PBS solution (pH 7.4) in real time. The light intensity was set to 1 W cm^{-2} with an exposure time of 5 min, followed by another 5 min under dark. The UV–Vis calibration curve was used to calculate the drug concentration from the UV–Vis absorbance. As shown in Figure 3E, the absorption spectrum of PTX at 234 nm gradually increased, indicating that the concentration of released PTX varies with the exposure time. The release rate is measured by the slope of the drug release curve with or without visible light irradiation.

The release rate was measured by the slope of the drug release curve with or without visible light irradiation. Figure 3F depicted the drug release rate during the first four

consecutive switching cycles. It could be seen that the release rate with light irradiation was much higher than release rate without visible light irradiation, indicating that AHA/OSA/BPNS/PTX could be used as an effective drug release light switch.

3.6 *In vitro* cell experiments

To evaluate the possible cytotoxicity, mouse fibroblasts (L929) and human liver cancer cells (SGC7901) were incubated with AHA/OSA, AHA/OSA/BPNS gel, and AHA/OSA/BPNS/PTX hydrogel at 37 °C for 24 h and studied using the cell counting kit-8 (CCK-8) assay. Figure 4A showed that the OSA/AHA hydrogel matrix had almost no effect on the growth of cells and could ensure the normal growth and reproduction of cells. When BPNS was added to the matrix, the cells could still carry out normal life activities without infrared light. But after adding PTX to the hydrogel, PTX was released from the gel and caused a lot of cell apoptosis.

After confirming the biocompatibility of AHA/OSA/BPNS, our next step was to study its *in vitro* therapeutic effect. In cell experiments, acridine orange/propidium iodide (live cells, green fluorescence; dead cells, red fluorescence) were used to distinguish live/dead cells by comatching cells. Under the NIR laser at different times, SGC7901 cells were gradually killed by the released drugs, as shown in Figure 4C. The CCK-8 assay was used to test cell viability (Figure 4B). The combination of photothermal and chemotherapy could better inhibit the proliferation of tumor cells.

3.7 *In vivo* photothermal imaging and antitumor effect

In this section, we first performed the photothermal effect of AHA/OSA/BPNS gel in SGC7901 tumor mice, and PBS was used as a control. Mice were injected intratumor with PBS and AHA/OSA/BPNS gel. One hour after the administration, the mice were exposed to 808 nm laser (1.0 W cm^{-2}) for 5 min. Figure S1A shows photothermal images of tumor tissue at different time intervals. It can be seen that the PBS-treated mice showed a slow and relatively low temperature increase after irradiation, while the mice treated AHA/OSA/BPNS exhibited a sharp temperature increase. The tumor tissue was irradiated with a laser with a wavelength of 808 nm, and the temperature reached 53.1 °C (Figure S1B). The results showed that AHA/OSA/BPNS gel has good photothermal conversion ability *in vivo*.

Figure 4: (A) The effect of different experimental groups on cell viability of SGC7901 and L929. (B) Relative cell viabilities of SGC7901 cells treated with different laser irradiation times. (C) Fluorescent images of acridine orange (green, live cells) and propidium iodide (red, dead cells) contained SGC7901 cells after addition of OSA/AHA/BPNS/PTX and exposure to laser irradiation for 0, 5, 10, and 20 min.

Favorable photothermal conversion efficiency and good cell compatibility of BP NS prompted us to further study the antitumor efficiency of AHA/OSA/BPNS/PTX *in vivo*. Tumor volume and weight are used as indicators to monitor the antitumor effect of different treatment methods. In 14 days, different treatment methods can observe obvious tumor growth curves in mice (Figure 5A and B). The tumor volume after irradiation (without any drug injection) was almost the same as that in the PBS group, which confirmed that irradiation had no significant effect on tumor growth. The chemotherapy using free PTX or AHA/OSA/BPNS/PTX gel chemotherapy without near-infrared radiation could partially inhibit tumor growth, but the tumor volume was still significantly elevated. The AHA/OSA/BPNS/PTX was slightly different from PTX, which may be because the hydrogel forms a “depot” at the tumor site and continuously released PTX to the tumor. For PTX solution, it could quickly spread to surrounding tissues. Compared with PTX solution, the efficacy of AHA/OSA/BPNS/PTX gel was enhanced, which may be mainly due to the longer residence time of the drug in the tumor area. Among them, compared with other control groups, PTT/chemotherapy with AHA/OSA/BPNS/PTX laser irradiation significantly delayed tumor growth. The powerful antitumor effect of AHA/OSA/BPNS/PTX may be due to the increase in drug concentration and temperature

increase after NIR irradiation in the tumor. The results showed that AHA/OSA/BPNS/PTX enhanced the efficacy of photothermal therapy and combined chemotherapy to fight cancer. The final weight of tumors in each group after 14 days treatment was measured (Figure 5C). The weight change of mice can be used to evaluate the systemic toxicity of AHA/OSA/BPNS/PTX gel. The body weight of the mice during treatment was shown in Figure 5D. Compared with other groups, the weight of mice treated with free PTX group decreased. We speculated that the side effects of free drugs may affect the feeding and normal daily activities of mice. It could be found that the weight fluctuations of the other groups after treatment were negligible; indicating that the effective load of the gel could lower the toxic and side effects of the free drug.

Fourteen days after PTT treatment, the tumor tissues of each group (three in each group) were collected for H&E, Ki67, and TUNEL staining. As shown in Figure 5E, tumor cell nuclei in the AHA/OSA/BPNS + NIR group, PTX group, and AHA/OSA/BPNS/PTX group all showed different degrees of shrinkage, and the tumor cell nucleus in the AHA/OSA/BPNS/PTX + NIR group was almost ruptured. In the TUNEL stained image, more green fluorescence appeared in the AHA/OSA/BPNS/PTX + NIR group, which was consistent with H&E staining. Ki67 immunohistochemical staining was performed on tumor sections of each

Figure 5: *In vivo* therapy evaluation. Tumor-bearing nude mice were divided to six groups randomly with treatment of PBS, PBS + NIR, PTX, OSA/AHA/BPNS + NIR, OSA/AHA/BPNS/PTX, and OSA/AHA/BPNS/PTX+NIR. (A) Images of tumor tissues in each group collected on day 14. (B) Tumor growth curve in terms of volume within 14 days under different treatments. (C) Average tumor weight in each group after 14 days treatment. (D) Body weight changes of BALB/c mice bearing SGC7901 tumors recorded every other day after different treatment. (E) H&E, TUNEL, and immunohistochemical Ki67 staining of tumor sections in each group.

group to monitor the changes in tumor tissue proliferation activity. Ki67 staining showed that after AHA/OSA/BPNS/PTX + NIR treatment, tumor cell proliferation activity was also significantly inhibited.

3.8 In vivo biocompatibility evaluation

Biocompatibility was a prerequisite for the safe application of materials in nanomedicine. Therefore, we studied the *in vivo* toxicity of AHA/OSA/BPNS, AHA/OSA/BPNS/PTX, and PTX to the main organs of mice. Figure 6 shows the H&E images of the main organs of mice after 14 days of different treatments. Compared with the PBS control, most of the formula-treated organs did not show any visible damage, indicating that most of the formulas have good biosafety.

4 Conclusion

In this study, an injectable hydrogel coated BP nanosheet and paclitaxel was designed for use in synergistic photothermal chemotherapy. The hydrogel exhibited good

photothermal performance and photothermal stability. Importantly, *in vitro* experiments proved that it had inherent biodegradability. Finally, BP nanosheets and paclitaxel coated hydrogels showed high photothermal therapeutic effects *in vivo*, and exhibited potential anti-tumor effects after intratumorally administration. This was due to the increase in temperature under near-infrared irradiation, the internal drug concentration increased. At the same time, AHA/OSA/BPNS/PTX gel showed negligible systemic toxicity. Therefore, we believe that the system has great potential in tumor treatment.

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Figure 6: *In vivo* biocompatibility evaluation of materials, H&E staining images of mice organs underwent different treatments for 14 days. Note that 1–6 represent mice groups with different treatment.

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