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A Theranostic Nanocomposite with Integrated Black Phosphorus Nanosheet, Fe₃O₄@MnO₂-doped Upconversion Nanoparticles and Chlorin for Simultaneous Multimodal Imaging, Highly Efficient Photodynamic and Photothermal Therapy

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Abstract

As a novel photosensitizer for photodynamic therapy (PDT), black phosphorus (BP) has attracted increasing attention recently. However, some of its inherent drawbacks hinder its further biomedical applications. As with most conventional photosensitizers, the PDT efficiency of BP is severely limited by the hypoxia tumor microenvironment and low tissue penetration of ultraviolet/visible light. Moreover, BP also faces additional problems, such as mismatch wavelength between photothermal therapy (PTT) and PDT, as well as no imaging ability for cancer diagnosis. Herein, we addressed these challenges through the combination of Fe₃O₄@MnO₂-doped upconversion nanoparticles (UCNPs) and black phosphorus nanosheets (BPNs). By applying polyacrylic acid (PAA) as a chemical crosslinker, polylysine (PL) modified black phosphorus nanosheets were integrated with magnetic Fe₃O₄@MnO₂-doped NaYF₄:Yb/Er/Nd upconversion nanoparticles (MUCNPs) which could convert near-infrared (NIR) light into visible light. Then, a biocompatible photosensitizer, chlorin e6 (Ce6) was attached

onto the obtained MUCNPs@BPNs to prepare MUCNPs@BPNs-Ce6 nanocomposites. BPNs and Ce6 were exploited to activate PTT and PDT under a single irradiation light of 808 nm with strong tissue penetration ability. Meanwhile, the doped Fe₃O₄ and MnO₂ nanoparticles could provide T1 and T2 magnetic resonance (MR) imaging, which could form a multimodal imaging system with fluorescence imaging of Ce6. In addition, MnO₂ could decompose overexpressed H₂O₂ in tumor microenvironment to generate O₂ to enhance PDT. The magnetic BPNs-based nanocomposites provided multimodal imaging (including MR, fluorescence, optoacoustic, ultrasonic imaging) and displayed dramatically enhanced ability for tumor cell death with visualized synergistic phototherapy, which greatly facilitated the application of BPNs in biomedicine.

Keywords: photodynamic therapy, black phosphorus, photothermal therapy, multimodal imaging, synergistic phototherapy

1. Introduction

Photodynamic therapy (PDT) based on the photochemical reaction of a photosensitizer is an emerging clinical strategy for cancer treatment with minimal invasiveness [1-3]. The surface of photosensitizer could generate electrons under suitable light and reduce molecular oxygen into cytotoxic reactive oxygen species (ROS), like singlet oxygen (¹O₂) [4,5]. By interacting with a variety of biological macromolecules in cells, ROS can cause damage to the cell membrane system and destroy the structure and function of organelles at the target site by oxidative damage, eventually causing apoptosis and necrosis of target cells [6,7].

Recently, black phosphorus (BP) has emerged as a 2D layered photosensitizer [8,9]. As a potential PDT agent, BP possesses ultrahigh surface areas, fascinating photoelectronic properties, high quantum

yields (up to 0.91) and negligible cytotoxicity [10-13]. The ultra-thin BP nanosheets (BPNs) obtained with the liquid-phase exfoliation by breaking down BP have shown a strong ability for $^1\text{O}_2$ production for PDT irradiated at around 660 nm, which could be attributed to their low electron-hole recombination rate and plentiful supply of active sites on the surface [14,15]. Meanwhile, BPNs exhibit high photothermal conversion efficiency irradiated at 808 nm, which means that BPNs can also serve as photothermal therapy (PTT) agents [16,17]. Especially, PTT can produce heat in localized tissue to increase blood flow for enhanced O_2 transport and, ultimately achieving a synergetic effect with PDT [18]. However, a number of disadvantages of BPNs have hindered their practical application in the biomedical area. For instance, there is a mismatch of wavelengths for making optimal PTT (808 nm) and PDT (660 nm) work simultaneously. During the actual treatment process, it is difficult and uneconomic to use two light sources for phototherapy, and laser irradiation at 606 nm also suffers from strong photon intensity attenuation, causing poor tissue penetration depth [19,20]. In addition, like with most conventional photosensitizers, the PDT efficiency of BP is severely limited by the hypoxia tumor microenvironment [18,21]. Besides, BPNs usually lack imaging features for cancer diagnosis, which hampers its clinical application in theranostics [22].

One promising solution to overcome these disadvantages is to combine BPNs with multifunctional upconversion nanoparticles (UCNPs). This kind of material can absorb long wavelength photons, which have strong tissue penetration and emit high-energy light with shorter wavelengths to activate photosensitizers [23-25]. Moreover, some metal elements with imaging capabilities (Gd, Mn^{2+} etc.) were doped into UCNPs for endowing UCNPs with the ability of imaging to build a UCNPs guided multimodal imaging therapeutic systems [26,27]. Lin et al. [28] have successfully conjugated $\text{NaGdF}_4\text{:Yb,Er@Yb@Nd@Yb}$ UCNPs with BPNs for 808 nm laser-mediated

PDT. However, they did not demonstrate the PTT effect, and the nanosystem had no imaging functionality.

Scheme 1. (A-C) Schematic illustration of the fabrication of BP-based nanoplatforms. (D) Schematic illustration of BP-based nanoplatform for enhanced PDT and PTT therapy upon single 808 nm laser irradiation.

There are some problems associated with BP serving as PDT agents, such as severe poor O₂ production in the tumor environment, low tissue penetration from activating light, mismatch of wavelength between simultaneous PTT and PDT, and lack of imaging functionality in real time. As a solution, we conjugated Fe₃O₄@MnO₂ doped UCNPs with BPNs and fabricated a novel BPNs-based theranostic platform that could simultaneously achieve: 1) PTT/PDT at single 808 laser irradiation; 2) self-support of O₂ in tumor microenvironment; 3) multimodal imaging. We innovatively integrated the ultrathin BPNs with the small Fe₃O₄@MnO₂-doped NaYF₄:Yb/Er/Nd UCNPs (MUCNPs) and the chlorin e6 (Ce6) in one nanosystem. As shown in **Scheme 1A**, the BPNs were firstly obtained by ultrasonic liquid phase stripping from BP powder. The MUCNPs obtained from oleic acid (OA) wrapped Fe₃O₄ nanoparticles (NPs) was modified with the strong coordination molecule poly (acrylic acid) (PAA) to acquire good hydrophilicity (**Scheme 1B**) [29,30]. Then, the polylysine (PL) covered

BPNs by electrostatic interaction to enhance the stability of BPNs [31] and the functional groups of the two materials were integrated by chemical bond crosslinking. Finally, the Ce6 was loaded onto the nanoplatfrom (**Scheme 1C**). Ce6 has high sensitizing efficacy and high fluorescent emission between the wavelengths of 660-670 nm, which can be utilized for both optical imaging and PDT *in vivo* [32-34]. The PTT of BPNs was firstly activated when the nanocomposites were irradiated with 808 nm laser. Then, the high wavelength laser was adjusted to between 660-670 nm by the MUCNPs, which was the specific excitation wavelength of PDT for both BPNs and Ce6 [35-37]. The ultraviolet (UV) and visible light emitted from the MUCNPs core upon NIR light irradiation activated the electrons which reacted with the electron acceptors (dissolved O₂ from H₂O₂) to produce ¹O₂. At the same time, the MnO₂ transformed from the doped Mn²⁺ under the high temperature and pressure during the hydrothermal process could efficiently catalyze excessive intracellular H₂O₂ in tumor microenvironment to generate O₂ [38]. This process was essential to sustain BPNs and Ce6-mediated PDT therapy (**Scheme 1D**). Additionally, as a result of the reduced pH of the tumor microenvironment, the obtained MnO₂ decomposed to Mn²⁺ and provided the T1 and T2 signal with Fe₃O₄ for *in vivo* magnetic resonance (MR) imaging applications, which we could combine with fluorescence imaging of Ce6 to form a multimodal imaging system. The ROS generation efficiency, photothermal conversion efficiency, tumor therapeutic effect and imaging capabilities were performed well in both *in vitro* and *in vivo* experiments and it was found that the prepared MUCNPs@BPNs-Ce6 possessed excellent tumor inhibition efficiency [39,40].

2. Results and discussion

The novel theranostic nanoplatfrom was rationally designed and fabricated by coating MUCNPs

on amino modified BPNs, and loading Ce6 through coupling the carboxyl groups of the Ce6 molecules and PAA onto MUCNPs with the exposed amino groups on the surface of BPNs. To prepare MUCNPs, OA capped Fe_3O_4 with a mean diameter of 15 nm (**Figure 1A**) were initially synthesized through a coprecipitation method [41]. Afterwards, the as-synthesized OA- Fe_3O_4 NPs were performed as seeds, on which the red upconversion luminescent shell (MnO_2 -doped $\text{NaYF}_4:\text{Yb}/\text{Er}/\text{Nd}$) was formed by hydrothermal synthesis [23,42]. The high-resolution transmission electron microscope (HRTEM) image in **Figure 1B** shows that the lattice fringes of the Fe_3O_4 cores and MUCNPs were 0.26 nm (inset **Figure 1A,B**) and 0.31 nm (**Figure 1B**) respectively, which ascribed to the (331) lattice planes of the magnetite face-centered cubic structure and the (111) lattice planes of cubic NaYF_4 . Therefore, this result demonstrated that MnO_2 -doped $\text{NaYF}_4:\text{Yb}/\text{Er}/\text{Nd}$ shells were successfully wrapped onto magnetic seed nanocrystals. The X-ray diffraction (XRD) pattern (**Figure S1**) further supports the successful preparation of Fe_3O_4 (JCPDS Card No.76-1849) and MUCNPs core-shell structures (JCPDS Card No. 16-0342).

The BPNs were prepared by a simple liquid exfoliation technique [43]. The transmission electron microscope (TEM) image shows that the as prepared BPNs were free standing with a 2D nanostructure. It could be observed that the BPNs had a lattice distance of ca. 0.28 nm, and the size ranged from 50 to 100 nm (**Figure 1C**). The Raman spectrum from the BP powder and BPNs dispersion showed the three representative BP Raman modes at 362 cm^{-1} , 439 cm^{-1} , and 466 cm^{-1} (**Figure S2**). After that, PL was grafted on BPNs by electrostatic interaction to provide numerous amino groups for working as reactive functional groups. After PL conjugation, the zeta potential of BPNs increased from -19.5 mV to 3.8 mV (**Figure S3**). The positive charge indicated that PL was successfully attached onto BPNs. Afterwards, MUCNPs@BPNs were obtained by assembling BPNs- NH_2 and MUCNPs-PAA onto a theranostic nanoplatform. The morphology and size distribution of the sample were investigated with

TEM. As shown in **Figure 1D**, a polymer layer and the MUCNPs were successfully coated onto the BPNs. The quantitative energy dispersive X-ray spectroscopy (EDS) (**Figure 1E**) confirms that the distribution of the Fe, Mn, Nd, Yb, and P atoms were homogeneously over the surfaces of the MUCNPs@BPNs. The corresponding EDX spectrum (**Figure S4**) shows the characteristic energy lines of Fe, Mn, Er, Yb, Nd, Y, C, N, and P arising from the MUCNPs and BPNs, and verifies the successful integration of BPNs and UCNPs, which X-ray photoelectron spectroscopy (XPS) also confirmed (**Figure 1F**). Finally, Ce6 was loaded onto the MUCNPs@BPNs *via* the formation of BPNs-Ce6 complexes with covalent interactions. After Ce6 loading, the MUCNPs@BPNs showed red fluorescent and good dispersion in phosphate buffered solution (PBS), Roswell Park Memorial Institute (RPMI) medium, and Dulbecco's modified eagle medium (DMEM) (**Figure 1G**). In addition, MUCNPs@BPNs-Ce6 showed high photoluminescence intensity for fluorescence, although the signal was significantly lower compared to free Ce6 at the same concentration (**Figure 1H**).

Figure 1. (A) TEM image of OA-Fe₃O₄. (B) TEM (a) and HRTEM (b) image of MUCNPs, (C) TEM image of BPNs. Inset is the HRTEM image of BPNs. (D) TEM image of MUCNPs@BPNs. (E) The corresponding elemental mapping images of MUCNPs@BPNs. (F) XPS spectra of MUCNPs@BPNs. (G) Photograph of MUCNPs@BPNs-Ce6 in water, PBS, DMEM and RPMI under bright or UV light. (H) The photoluminescence spectra of Ce6, MUCNPs@BPNs and MUCNPs@BPNs-Ce6. (I) Upconversion mechanism of MUCNPs@BPNs excited using an 808 nm laser. (J) Upconversion luminescence emission spectra of the MUCNPs and MUCNPs@BPNs under 808 nm irradiation. (K) Magnetic hysteresis loop for MUCNPs and MUCNPs@BPNs-Ce6. Inset is the photographic image of MUCNPs@BPNs-Ce6 solution under a magnet.

In MUCNPs, Nd³⁺ ions acted as sensitizers to absorb the 808 nm NIR excitation energy, while Yb³⁺ ions behaved as bridging ions to receive energy from Nd³⁺ ions and then transferred it to the Er³⁺ acceptor ions in a so-called “cascade sensitized UC process” (**Figure 1I**) [44]. To explore the efficiency of upconversion emission for more efficient PDT, the upconversion luminescence spectra of MUCNPs under 808 nm NIR light were measured. In **Figure 1J**, the emissions at 520-560 nm and 645-685 nm were ascribed to ⁴S_{3/2} → ⁴I_{15/2} and ⁴F_{9/2} → ⁴I_{15/2} transitions of Er³⁺ [45], demonstrating that the efficient energy transferred from UCNPs to BPNs and Ce6. This was caused by the fluorescence resonance energy transfer process. As predicted, there was an obvious decrease in the whole emission region after MUCNPs were modified onto BPNs, due to the nonspecific absorbency [42,46]. XPS was applied to investigate the chemical state of manganese element in the as-made MUCNPs. The two characteristic peaks at 652.8 and 641.3 eV, correspond to the Mn (IV) 2p_{2/3} and Mn (IV) 2p_{1/2} spin-orbit peaks of MnO₂, respectively [47], proving that Mn²⁺ was oxidized into MnO₂ under the high temperature and pressure (**Figure S5**). **Figure 1K** shows the magnetic hysteresis measurements of the superparamagnetic MUCNPs with saturation magnetization value of approximately 53.0 emu/g, and that of MUCNPs@BPNs-Ce6 was approximately 5.2 emu/g at room temperature. The decrease was caused by the thick shell coating on the magnetic NPs. Inset in **Figure 1K** indicates that the MUCNPs@BPNs-Ce6 exhibited a sufficiently strong magnetic separation for biomedical applications.

As illustrated in **Figure 2A**, the MUCNPs@BPNs-Ce6 exhibited high stability in physiological media during 7 days, including in water, PBS, RPMI, and DMEM, due to the modification of PL [48]. The absorbance spectra of the MUCNPs@BPNs-Ce6 in physiological media also demonstrates the fluorescent properties of MUCNPs@BPNs-Ce6 (**Figure 2B**). The absorbance peak matched well with the emission peak of Ce6 at around 370 nm and 640 nm. Under continuous laser irradiation at 808 nm (2.0 W/cm^2), the temperature elevation of the various aqueous suspensions containing MUCNPs@BPNs-Ce6 was measured and MUCNPs@BPNs-Ce6 solutions showed an obvious concentration-dependent heating effect (**Figure 2C**). The control experiment demonstrated that the temperature of the MUCNPs@BPNs-Ce6 solution increased by $30 \text{ }^\circ\text{C}$ from room temperature with a concentration of only $200 \text{ }\mu\text{g/mL}$ (**Figure 2D**). Afterwards, we measured the photothermal conversion efficiency (η) of the MUCNPs@BPNs-Ce6. Based on the obtained data (**Figure 2E,F**), the η value of MUCNPs@BPNs-Ce6 was determined to be ca. 33.4%, enabling the use of such nanocomposites as a photothermal agent.

As we know, the Mn^{2+} can be oxidized to MnO_2 under high temperature and pressure. The obtained product could further react with H_2O_2 and efficiently induce the catalytic decomposition of H_2O_2 to generate O_2 [48-50]. To investigate the O_2 generation capability of MUCNPs@BPNs-Ce6, the dissolved O_2 in MUCNPs@BPNs-Ce6 solution was measured with an O_2 probe. As shown in **Figure 2G**, O_2 was generated only when the MUCNPs@BPNs-Ce6 and H_2O_2 coexisted, and no O_2 was detected in pure H_2O_2 , MUCNPs@BPNs-Ce6/water or MUCNPs@BPNs-Ce6/PBS. Since the MUCNPs@BPNs-Ce6 could effectively generate O_2 from H_2O_2 , the electron spin resonance (ESR) technique was performed to measure the $^1\text{O}_2$ -generation capability of the MUCNPs@BPNs-Ce6 that was dispersed in acidic H_2O_2 with 2,2,6,6-tetramethylpiperidine-N-oxyl (TEMPO) as an $^1\text{O}_2$ trapper. As shown in **Figure 2H**, a characteristic spin adduct with three resolved peaks was found, which revealed that $^1\text{O}_2$ were generated from MUCNPs@BPNs-Ce6 upon NIR light irradiation. Besides, 1,3-diphenylisobenzofuran (DPBF) could react with H_2O_2 and cause an irreversible decrease in the

absorbance of DPBF at 416 nm, it was applied as the trapping agent to assess the $^1\text{O}_2$ quantum yield of the MUCNPs@BPNs-Ce6 (**Figure 2I**). Under 808 nm laser (0.1 W/cm^2) irradiation, the absorbance of DPBF decreased gradually during the irradiation with the presence of the MUCNPs@BPNs-Ce6, indicating that MUCNPs@BPNs-Ce6 could generate enough ROS and be potentially utilized in PDT.

Figure 2. (A) The dispersed stability of the MUCNPs@BPNs-Ce6 in physiological mediums. (B) Absorbance spectra of the MUCNPs@BPNs-Ce6 in physiological mediums. (C) The photothermal heating curves of PBS and MUCNPs@BPNs-Ce6 solutions with different concentrations under the 808 nm laser irradiation (2.0 W/cm^2). (D) Temperature infrared images of $200 \mu\text{g/mL}$ MUCNPs@BPNs-Ce6 aqueous solution under 808 nm laser irradiation (2.0 W/cm^2) irradiation. (E) Photothermal response of MUCNPs@BPNs-Ce6 aqueous solution under irradiation for 5 min under 808 nm laser irradiation (2.0 W/cm^2) and then the laser was shut off. (F) Linear time data versus $-\text{Ln}\theta$ obtained from the heating up time of (E). (G) The O_2 concentration in H_2O_2 solutions and different solutions after incubation with MUCNPs@BPNs-Ce6. (H) ESR spectra of the MUCNPs@BPNs-Ce6 in different reaction systems. (I) Absorbance spectra of DPBF mixed with the MUCNPs@BPNs-Ce6.

The biocompatibility of MUCNPs@BPNs-Ce6 was also investigated through erythrocyte shapes, hemolysis assay, and methyl thiazolyl tetrazolium (MTT) assay. Morphologically aberrant forms of

erythrocytes could be applied to the diagnosis of various medical conditions, including hemolytic anemia. The photomicrographs from **Figure 3A** indicates that the number and shape of erythrocytes in the test blood did not change after the treatment of MUCNPs@BPNs-Ce6. As shown in **Figure 3B**, no significant hemolysis was observed when red blood cells (RBCs) were co-cultured with MUCNPs@BPNs-Ce6. It was found that MUCNPs@BPNs-Ce6 alone had no toxic effect on HeLa cells, even though their concentrations were up to 500 $\mu\text{g/mL}$ (**Figure 3C**). Besides, morphology change and cell apoptosis of HeLa cells were hardly observed under optical microscope from morphology imaging of cells (**Figure 3D**). These results suggested that MUCNPs@BPNs-Ce6 had insignificant cytotoxicity and possessed good biocompatibility *in vitro* [28,51].

Figure 3. (A) Erythrocyte shapes of the rat after injection of water, PBS and MUCNPs@BPNs-Ce6. Scale bar: 50 μm . (B) Hemolysis percentage of RBCs at various concentrations of MUCNPs@BPNs-Ce6. Inset is the photograph of the RBCs solution cultured with MUCNPs@BPNs-Ce6. (C) Cell viability of HeLa cells with various concentrations of MUCNPs@BPNs-Ce6. (D) Morphology of HeLa cells incubated with various concentrations of MUCNPs@BPNs-Ce6. Scale bar: 100 μm . (E) Confocal fluorescence images of HeLa cells

incubated with (a) PBS or (b) MUCNPs@BPNs-Ce6 for 4 h. Scale bar: 30 μm . (F) Fluorescence images of SOSG stained HeLa cells in a N_2 atmosphere. Scale bar: 50 μm . Cell viability of HeLa cells with various concentrations of MUCNPs@BPNs-Ce6 under different conditions: (G) 0.1 W/cm^2 + acid H_2O_2 solution; (H) 2.0 W/cm^2 ; (I) 2.0 W/cm^2 + acid H_2O_2 solution for 5 min. Inset is the confocal fluorescence images of calcein AM/PI-stained HeLa cells incubated with various treatments. Scale bar: 100 μm .

The cellular uptake behaviors of MUCNPs@BPNs-Ce6 inside HeLa cells could be guided by the Ce6 fluorescence. Red fluorescence came from Ce6 and blue fluorescence in cell nucleus came from 4',6-diamidino-2-phenylindole (DAPI). As shown in **Figure 3E**, the cytoplasm of HeLa cells emitted strong red fluorescence, and no red fluorescence signal was observed in cell nucleus, demonstrating that MUCNPs@BPNs-Ce6 had entered into cells but had been hampered by the numerous small nuclear pore complexes of the nuclear membrane [52-54]. In addition, a singlet oxygen sensor green (SOSG) probe was used to measure the intracellular $^1\text{O}_2$ generation of the MUCNPs@BPNs-Ce6 assembly, which could react with $^1\text{O}_2$ from PDT and generate an endoperoxide product with green fluorescence. To protect the cellular morphology, 5% paraformaldehyde was used to immobilize HeLa cells and were incubated with the MUCNPs@BPNs-Ce6. Then, the fixed cells were washed with PBS and further incubated with SOSG for 5 min. When the fixed cells were irradiated with an 808 nm laser for 5 min, only weak green fluorescence of SOSG was observed (**Figure 3F**) in the HeLa cells. In contrast, the cells showed enhanced fluorescence once the cells were mixed with H_2O_2 and subsequently irradiated by 808 nm laser, indicating that the MUCNPs@BPNs-Ce6 could efficiently generate intracellular $^1\text{O}_2$ for PDT.

To evaluate the therapeutic effect of our nanocarriers, a live/dead assay and MTT assay were carried out by incubating cancer cells with MUCNPs@BPNs-Ce6, then the cells were illuminated with different NIR laser. After proper staining, a control experiment, where cancer cells were incubated without nanocarriers but exposed to the laser irradiation, showed high cell viability, indicating the irradiation alone had no adverse impact on cell survival [55,56]. However, it was observed that MUCNPs@BPNs-Ce6 treated cells under NIR light showed obvious viabilities of reductions (**Figure 3G**), indicating that MUCNPs@BPNs-Ce6 had a good PDT ability. In addition, a lower cell viability

was found when MUCNPs@BPNs-Ce6 was used under increasing laser power intensities (**Figure 3H**). Interestingly, the cell viability further decreased when MUCNPs@BPNs-Ce6 with H₂O₂ solution was applied along with laser irradiation (2.0 W/cm²), indicating that the cell death was not only PTT-dependent, but also due to a synergetic effect coming from the MUCNPs@BPNs-Ce6 (**Figure 3I**). As expectant, this synergetic effect could be clearly testified by 250 µg/mL of MUCNPs@BPNs-Ce6 under laser irradiation, resulting in almost 100 % cell death. Confocal fluorescence images of calcein AM and propidium iodide (PI) contained cells further confirmed the efficiency of photothermal and photodynamic ablation induced by MUCNPs@BPNs-Ce6 (inset of **Figure 3I**).

As mentioned above, the obtained MnO₂ could decompose into Mn²⁺ at a reduced pH in tumor microenvironment. It has been known that the Mn²⁺ and Fe₃O₄ have been widely applied as contrast agents for MR imaging. Therefore, the obtained MUCNPs@BPNs-Ce6 showed an obvious concentration-dependent brightening effect under T1-weighted MR imaging, as well as darkening effect under T2-weighted MR imaging. **Figure 4A,B** displays different relaxation time (T1 and T2) of MUCNPs@BPNs-Ce6 with different concentrations. The T1 and T2 longitudinal relaxivity of MUCNPs@BPNs-Ce6 (r1 and r2) was measured to be 9.79 mM⁻¹s⁻¹ and 5.71 mM⁻¹s⁻¹, which was linearly enhanced along with the increase of equivalent Mn²⁺ and Fe concentrations. The blood circulation and biodistribution profiles are important to study the *in vivo* behavior of nanocomposites. Uptake of MUCNPs@BPNs-Ce6 in blood was investigated by detecting Fe concentration, and it decreased to 4% ID/g at 24 h in MUCNPs@BPNs-Ce6 injected mice, and remained at a relatively high level for a long time (**Figure 4C**). As shown in **Figure 4D**, a clear magnetic resonance response from *in vivo* T1 MR imaging was observed at the tumor site at 24 h post injection, owing to the gradual generation of Mn²⁺ from the MnO₂ of MUCNPs. In addition, the quantitative MR imaging of the quantification of the region-of-interest (ROI) also verified higher T1-weighted MR intensity, further proving the effective tumor accumulation of nanocomposites (**Figure 4E**). Meanwhile, reduced T2-weighted MR signals in the tumor showed up, and the signals stayed at an almost constant level after

intravenous injection from the *in vivo* T2 MR imaging (**Figure 4D**, and **Figure S7**). These results proved that MUCNPs@BPNs-Ce6 were a good application prospect in magnetic resonance imaging *in vivo* and could serve as a T1 and T2 contrast agent simultaneously.

Figure 4. (A) Linear correlation between longitudinal relaxivities (r_1) and equivalent Mn concentration of MUCNPs@BPNs-Ce6 at pH 6. The inset shows T1-weighted MR images of MUCNPs@BPNs-Ce6 solution with various Mn concentrations at pH 6. (B) Linear correlation between longitudinal relaxivities (r_2) and equivalent Fe concentration of MUCNPs@BPNs-Ce6. The inset shows T2-weighted MR images of MUCNPs@BPNs-Ce6 solution with various Fe concentrations. (C) Blood time-activity curve for MUCNPs@BPNs-Ce6 by measuring Fe concentration. (D) *In vivo* MR images of the tumor of mice before and after injection with MUCNPs@BPNs-Ce6 for 24 h. (E) T1-weighted MR signals in the tumor before and after injection with MUCNPs@BPNs-Ce6 for 24 h. (F) Biodistribution results of HeLa tumor-bearing mice before and after injection with MUCNPs@BPNs-Ce6 in MR imaging for 24 h. (G) *In vivo* fluorescence images of HeLa tumor-bearing mice before and after injection with MUCNPs@BPNs-Ce6 for 24 h. (H) *Ex vivo* fluorescence images of organs and tumor after injection with MUCNPs@BPNs-Ce6 for 24 h. (I) *In vivo* US imaging of HeLa tumor-bearing mice after injection with MUCNPs@BPNs-Ce6 for different time.

Analysis of radioactivity in isolated organs and tissues injected with MUCNPs@BPNs-Ce6 for 24 h could be observed in **Figure 4F**. A considerable signal of Fe ($> 10\%$ ID/g) in the liver organs was measured after the injection of MUCNPs@BPNs-Ce6, and the signal was lowest in the heart which

was around 1.5% ID/g. Significant differences in blood retention and tumor uptake became evident in the image analysis depending on the nanocomposites surface modification. **Figure 4G** shows the *in vivo* fluorescence imaging of HeLa tumor-bearing mice injected with MUCNPs@BPNs-Ce6. Compared with the image prior to injection, a strong signal could be observed in the tumor region of HeLa tumor-bearing mice injected with MUCNPs@BPNs-Ce6 for 24 h, indicating the efficient accumulation of those nanocomposites in the tumor. *In vivo* fluorescence imaging of isolated organs further confirmed that nanocomposites accumulated mainly in the liver and almost no fluorescence signal was found in the heart (**Figure 4H** and **Figure S8**). Furthermore, it was obviously through observation that the ultrasound signals appeared at the tumor site after intravenous injection, which was attributed to O₂ bubbles coming from the decomposition of H₂O₂ (**Figure 4I**).

In vivo exploration was carried out in four groups of HeLa tumor-bearing nude mice (n=3) to test the therapeutic efficacy of MUCNPs@BPNs-Ce6 *via* the tail vein (**Figure 5A**). The tumor temperature under irradiation was monitored by the thermal camera. Five minutes after the irradiation, tumor temperature increased to higher than 50 °C (**Figure 5B**) in MUCNPs@BPNs-Ce6 with 808 nm laser (2.0 W/cm²) treated groups, which was sufficient to cause tumor cells damage *in vivo*. By comparison, for mice treated with PBS and low NIR light exposure, tumor temperature did not show any obvious increase. These results suggested that light energy can transfer into thermal energy efficiently in the body by MUCNPs@BPNs-Ce6. **Figure 5C** shows the therapeutic effects of different groups. The tumor-bearing nude mice were treated as follows: group 1, PBS (control); group 2, PBS irradiated with 808 nm laser (2.0 W/cm²); group 3, MUCNPs@BPNs-Ce6 irradiated with 808 nm laser (0.1 W/cm²); and group 4, MUCNPs@BPNs-Ce6 irradiated with 808 nm laser (2.0 W/cm²). The tumor volumes were calculated every 2 days. At the end of the experiment, all nude mice were put down and the tumors were collected. The tumor sizes of the MUCNPs@BPNs-Ce6 (**Figure 5D,E**) under 808 nm laser (2.0 W/cm²) irradiation group were notably smaller than those of the other three groups. Consequently, these results demonstrated that the laser irradiation-controlled MUCNPs@BPNs-Ce6

possessed an excellent tumor ablation effect *in vivo*. In mice treated with MUCNPs@BPNs-Ce6 followed by laser irradiation and applied magnetic field, the tumor tissues were necrotic, which exhibited karyolysis, pyknosis, and degradation (**Figure 5F**). By contrast, the tumor tissues of the PBS group exhibited a normal organized cellular structure.

Figure 5. (A) Thermal images of tumor-bearing mice exposed to the 808 nm laser irradiation for 5 min. (B) Temperature changes of tumors after 808 nm laser irradiation. (C) Photographs of excised tumors after different treatment. (D) *In vivo* tumor volume curves of tumor-bearing mice in different groups after various treatments indicated. (E) The weight of excised tumor. (F) Hematoxylin and eosin (H&E) staining photomicrographs of tumor tissue after different treatments. Size bar = 100 μ m. (G) Body weights after different treatments. (H) Percent survival after different treatment groups. (I) Histopathological analysis of major organs after different treatments: (a) PBS; (b) PBS+2.0 W/cm² laser; (c) sample+0.1 W/cm² laser; and (d) sample+2.0 W/cm² laser. Size bar = 100 μ m.

Furthermore, the body weight of mice was not significantly affected, demonstrating that there were no acute side effects along with our combined therapy (**Figure 5G**). Noticeably, the MUCNPs@BPNs-Ce6 with 2.0 W/cm² treated group showed a 100% survival rate after 45 days. In contrast, mice of other treated groups had lower survival or even died owing to the extensive tumor

burden (**Figure 5H**). The *in vivo* biosafety study showed that MUCNPs@BPNs-Ce6 caused no damage to the main organs (**Figure 5I**). It is well known that most of the intravenously injected nanocomposites are taken up and eliminated by mononuclear phagocyte system (MPS), including liver and kidney tissue [57]. Therefore, acute inflammation in the liver and kidney caused by MUCNPs@BPNs-Ce6 could be characterized by an increase in biochemical parameters, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN), and increased total bilirubin (TBIL). As shown in **Figure S9**, the biochemical examination of the serum shows that the ALT, AST, BUN, and TBIL levels in the test group had no obvious difference compared to that in the control group. These results indicate that the MUCNPs@BPNs-Ce6 had no noticeable acute toxicity in small experimental animals.

3. Conclusion

In summary, a BPNs nanocarrier was designed and prepared to load the MUCNPs and Ce6 through chemical crosslinking, for tumor phototherapy guided by multimodal imaging. The obtained MUCNPs@BPNs-Ce6 demonstrated excellent light conversion efficiency, biocompatibility, and imaging properties. Through enhanced permeability and retention (EPR) effect, the nanocarriers could efficiently aggregate at the site of the tumor, which effectively improved therapeutic effects and enhanced imaging. Upon irradiation at 808 nm NIR light, the MUCNPs@BPNs-Ce6 possessed efficient ROS generation and high photothermal conversion efficiency, benefiting from PTT/PDT synergistic therapy of BPNs and Ce6, as well as a higher O₂ supply for MnO₂. Meanwhile, the multimodality imaging of MUCNPs@BPNs-Ce6 could be applied for diagnosis. Both *in vitro* and *in vivo* experiments verified the safety and enhanced antitumor effect of this nanoplatform. Taken together, the current work demonstrated that MUCNPs@BPNs-Ce6 can be exploited as a good theranostic agent for cancer diagnosis and treatment.

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