pH-Responsive Pluronic F127–Lenvatinib-Encapsulated Halogenated Boron-Dipyrromethene Nanoparticles for Combined Photodynamic Therapy and Chemotherapy of Liver Cancer

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ABSTRACT: Combination therapy such as photodynamic therapy (PDT)-enhanced chemotherapy is regarded as a promising strategy for cancer treatment. Boron-dipyrromethene (BODIPY), as close relatives of porphyrins, was widely used in PDT. However, poor water solubility, rapid metabolism by the body and lack of targeting limits its clinical application. Lenvatinib, as the first-line drug for molecular-targeted therapy of liver cancer, restricted its clinical application for its side effects. Herein, to achieve the synergy between PDT and chemotherapy, we synthesized two halogenated BODIPY, BDPBr₂ and BDPCl₂, which were prepared into self-assembly nanoparticles with lenvatinib, and were encapsulated with Pluronic F127 through the nanoprecipitation method, namely, LBPNPs (LBBr₂ NPs and LBCl₂ NPs). The fluorescence quantum yields of LBPNPs were 0.73 and 0.71, respectively. The calculated loading rates of lenvatinib for LBBr₂ NPs and LBCl₂ NPs were 11.8 and 10.2%, respectively. LBPNPs can be hydrolyzed under weakly acidic conditions (pH 5.0) to generate reactive oxygen species (ROS), and the release rate of lenvatinib reached 88.5 and 82.4%. Additionally, LBPNPs can be effectively taken up by Hep3B and Huh7 liver cancer cells, releasing halogenated BODIPY and lenvatinib in the acidic environment of tumor cells to enhance the targeting performance of chemotherapeutics. Compared with free lenvatinib and separate halogenated BODIPY, LBPNPs can inhibit tumor growth more effectively through pH-responsive chemo/photodynamic synergistic therapy and significantly promote the cascade of caspase apoptotic protease. This study shows that LBPNPs can be a promising nanotheranostic agent for synergetic chemo/photodynamic liver cancer therapy.

1. INTRODUCTION

Liver cancer is the second leading cause of cancer death worldwide,¹ and hepatocellular carcinoma (HCC) is the most common pathological type in liver cancer.²⁻³ The pathogenesis of HCC is complex, and the proliferation, invasion, and metastasis of cancer cells are closely related to the various cascade signal transduction pathways.⁴⁻⁵ Lenvatinib is a new multitarget tyrosine kinase inhibitor (TKI) that has recently been demonstrated to be noninferior to sorafenib in phase 3 randomized controlled trial.⁶⁻⁸ In a real-world study, lenvatinib was demonstrated to be more efficacious than sorafenib as a salvage therapy for transarterial treatments in unresectable HCC.⁹ However, the poor solubility in aqueous environments and low oral bioavailability limited its therapeutic efficacy.¹⁰ Therefore, new approaches are urgently needed to improve the therapeutic effectiveness of lenvatinib and to reduce its side effect.

However, in most cases, tumor resistance to chemotherapeutic agents almost universally occurs during the chemotherapy process. To address this problem, chemo/photodynamic synergistic cancer therapy has been extensively studied for its multiple advantages in overcoming drug resistance and enhancing therapeutic efficacy.¹¹⁻¹² Phototherapy, including photodynamic therapy (PDT) is a new method for the treatment of cancer.¹³⁻¹⁴ It can trigger a series of photochemical and photobiological reactions under specific wavelength laser irradiation by a photosensitizer,¹⁵ the most important of which is the production of reactive oxygen species (ROS, such as singlet oxygen $^1\text{O}_2$, superoxide anion radical $\text{O}_2^-\cdot$, and hydroxyl radical $\cdot\text{HO}$),¹⁶⁻¹⁸ thereby killing tumor cells. Boron-dipyrromethene (BODIPY) as a fluorescent dye is widely used in PDT due to its efficient singlet oxygen yield and gentle synthesis process.¹⁹⁻²¹ However, due to its poor biocompatibility, its application is limited. Thus, BODIPY-
containing nano-photosensitizers have recently been designed and widely used.\textsuperscript{22} Linking BODIPY with amphiphilic molecules can result in the formation of NPs via self-assembly.\textsuperscript{23} In addition, BODIPY nano-photosensitizers can be prepared via direct small molecule self-assembly and supramolecular host–guest interactions.\textsuperscript{24}

Therefore, the combination of PDT with chemotherapy can reduce the side effects of chemotherapy drugs and improve the biocompatibility of photosensitizers to overcome the drawbacks of monotherapy. Here, two halogenated BODIPY were designed, and bromine and chlorine substituents were introduced to the BODIPY skeleton to increase the generation of $^{1}\text{O}_2$, quantum yield. In our study, the multifunctions of the self-assembly nanodrug with lenvatinib and halogenated BODIPY (LBPNPs) were studied. With the $\pi-\pi$ stacking effect between lenvatinib and halogenated BODIPY, the one-step nanoprecipitation method was employed to obtain LBPNPs (LBBr$_2$ NPs and LBCl$_2$ NPs). Pluronic F127 was used to encapsulate to further improve the stability of nanoparticles in an aqueous environment (Scheme 1).

**Scheme 1. Multifunctions of Our Self-Assembly Nanoparticles with Lenvatinib and Halogenated BODIPY**

![Scheme 1. Multifunctions of Our Self-Assembly Nanoparticles with Lenvatinib and Halogenated BODIPY](image)

"The nanodrug was developed using a novel, one-step nanoprecipitation method. In this system, lenvatinib was employed as a chemotherapy drug, while halogenated BODIPY was used for PDT. Under 660 nm laser irradiation, halogenated BODIPY could have synergistic antitumor effects with lenvatinib which were mediated by ROS induction. LBPNPs can sense and respond to environmental changes via different chemical and physical mechanisms. Halogenated BODIPY could have synergistic antitumor effects with lenvatinib, which are mediated by ROS induction under 660 nm laser irradiation. The stability, characterization, singlet oxygen generation, ROS production, cytotoxicity, and apoptosis ability of the LBPNPs were evaluated in vitro by using HCC cell lines Hep3B and Huh7. We found that our designed LBPNPs had excellent biocompatibility and stability in vitro. Pluronic F127 was hydrophilic, while the inner part was hydrophobic for loading halogenated BODIPY. The core–shell structured micelles were degraded to release halogenated BODIPY and lenvatinib at low pH conditions in tumor cells. Meanwhile, LBPNPs could rapidly enter Hep3B/Huh7 cells and produce a great amount of ROS under laser irradiation to achieve potent cytotoxicity. LBPNPs exhibited low toxicity in the dark, while they were strongly phototoxic to tumor cells via the generation of $^{1}\text{O}_2$, which were activated by physiologically acidic pH under light irradiation. Meanwhile, LBPNPs could induce apoptosis of HCC cells by upregulating apoptosis-related proteins. Our studies in vitro demonstrated that LBPNPs can effectively accumulate in HCC cells and produce ROS to inhibit the growth of tumor cells through the chemo/photodynamic synergistic therapy. In general, BODIPY-containing nano-photosensitizers could be responsive to the tumor microenvironment and it can improve the PDT specificity and reduce toxic side effects.

2. RESULTS AND DISCUSSION

2.1. Characterization of BDPBr$_2$, BDPCl$_2$, and LBPNPs (LBBr$_2$ NPs and LBCl$_2$ NPs). According to the methods reported in the literature studies,\textsuperscript{25,26} BDPBr$_2$ and BDPCl$_2$ were obtained successfully. The characterizations of BDPBr$_2$ and BDPCl$_2$ were further tested by UV−vis−NIR and fluorescence spectra. As demonstrated in Figure 1a, the UV−vis spectrum of BDPBr$_2$ and BDPCl$_2$ showed prominent absorption peaks at 524 and 521 nm, respectively, in tetrahydrofuran (THF). As shown in Figure 1b, according to the fluorescence spectra, intense emission peaks at 536.8 and 527.6 nm, respectively, are observed for BDPBr$_2$ and BDPCl$_2$ upon excitation at 488 nm in THF, respectively. Their absorption spectrum and fluorescence spectrum are typical mirror symmetry. They showed strong fluorescence ($\Phi_F = 0.82$ and 0.75). These observations indicated the photosensitizers, BDPBr$_2$ and BDPCl$_2$, that we synthesized have potential for bioimaging application. However, BDPBr$_2$ and BDPCl$_2$ cannot be dissolved in water, so they cannot be used for tumor treatment. Therefore, the nanoprecipitation method was used to self-assemble BDPBr$_2$/BDPCl$_2$ and lenvatinib and then they were encapsulated by the amphiphilic substance Pluronic F127 to prepare nanoparticles. The synthesized photosensitizers, halogenated BODIPY-BDPBr$_2$/BDPCl$_2$ confirmed the $\pi-\pi$ stacking effect with lenvatinib and were encapsulated within Pluronic F127 by the one-step nanoprecipitation method, obtaining hydrophilic LBPNPs (LBBr$_2$ NPs and LBCl$_2$ NPs). The successful loading of halogenated BODIPY and lenvatinib onto LBPNPs was further confirmed by UV−vis−NIR and fluorescence spectra. As presented in Figure 1c, LBBr$_2$ NPs and LBCl$_2$ NPs in ddH$_2$O exhibited clear and transparent purple-red solution. In Figure 1c, LBBr$_2$ NPs showed two absorption peaks at 303.5 and 560.5 nm, corresponding to the characteristic of lenvatinib (Figure 1f) and BDPBr$_2$ (Figure 1a), with a 3 nm blue shift compared with lenvatinib and a 36.5 nm red shift compared with BDPBr$_2$ (524 nm). As shown in Figure 1d, furthermore, the fluorescence intensity of LBBr$_2$ NPs was lower than that of free BDPBr$_2$ at the same concentration, resulting from the quenching effect of LBBr$_2$ NPs. Compared with the photosensitizer, BDPBr$_2$/BDPCl$_2$, the fluorescence quantum yields of nanoparticles were 0.73 and 0.71, respectively. At the same time, LBBr$_2$ NPs had two new fluorescence intensity peaks at 635 and 690 nm. The above results fully illustrated the successful self-assembly of LBPNPs, and these results can be ascribed to the increased interchain interactions induced by self-assembly, which was more suitable for both imaging and therapy in vivo.\textsuperscript{27} Meanwhile, LBCl$_2$ NPs also displayed two absorption peaks at 309.5 and 574.5 nm in ddH$_2$O (Figure 1c), corresponding to the characteristic of lenvatinib (Figure 1f) and BDPCl$_2$ (Figure 1a), with a 2 nm red shift compared with
lenvatinib and a 53.5 nm red shift compared with BDPCl2 (521 nm). LBBr2 NPs had two fluorescence intensity peaks at 601 and 682 nm (Figure 1d). The fluorescence decay of LBBr2 NPs and LBCl2 NPs were monoexponential, and the fluorescence lifetimes of them were relatively long (Figure 1e). These results indicated that the two nanoparticles—LBPNPs (LBBr2 NPs and LBCl2 NPs)—had similar photochemical and physical properties and can be used in the next step of research.

The morphological dimensions and ultrastructure of LBBr2 NPs and LBCl2 NPs were studied by scanning electron microscopy (SEM), transmission electron microscopy (TEM), and dynamic light scattering (DLS). As shown in Figure 2a,b, the SEM and TEM images indicated that LBBr2 NPs and LBCl2 NPs were uniformly distributed in aqueous solution and exhibited good spherical morphology with a diameter around 78 and 85 nm, respectively. The distribution of nanoparticles in organisms has great relationship with their particle size. Nanoparticles with a particle size of 70 ∼ 200 nm are most suitable for tumor treatment, as are satisfactory for passive tumor targeting due to enhanced permeability and retention (EPR) effect.28 Also, as shown in Figure 2d,e, the DLS measurement further indicated that LBBr2 NPs and LBCl2 NPs presented relatively uniform size dispersion in deionized water, with an average size of 75 ± 1.6 and 85 ± 2.4 nm. Colloidal stability of nanoparticles is a crucial requirement for their systemic intravenous administration.29 Under the effect of increasing permeability and retention rate (EPR), micelles had obvious advantages in extending the blood circulation time and increasing the permeability of drugs in tumor tissues.30 Simultaneously, as shown in Figure 2f, LBBr2 NPs and LBCl2 NPs confirmed good dispersion stability in deionized water without any aggregation and sedimentation for 15 days, indicating that LBBr2 NPs and LBCl2 NPs had monodisperse stability in the environment and did not agglomerate after a period. These data suggested that LBBr2 NPs and LBCl2 NPs possessed a suitable stability profile for further investigation.

Figure 1. Characterization of BDPBr2 and BDPCl2 and LBPNPs. (a) UV−vis absorbance of BDPBr2 and BDPCl2 in THF. (b) Normalized emission spectra of BDPBr2 and BDPCl2 in THF. (c) UV−vis absorbance of LBBr2 NPs and LBCl2 NPs in deionized water. (d) Normalized emission spectra of LBBr2 NPs and LBCl2 NPs in deionized water. The excitation wavelength is 660 nm. (e) Transient fluorescence spectra of LBBr2 NPs and LBCl2 NPs in deionized water. The excitation wavelength is 660 nm. (f) UV−vis absorbance of lenvatinib in deionized water.
micelle concentration value and adaptable compositions, sizes, and surface properties, is potentially suitable for encapsulating lenvatinib and halogenated BODIPY. The above results present that in our designed nanoparticles, the core−shell structure of Pluronic F127 comprises a hydrophilic surface layer for stabilizing in blood circulation and a hydrophobic interior core for loading lenvatinib and halogenated BODIPY.

To investigate the drug loading efficiency of LBBr2 NPs and LBCl2 NPs, lenvatinib and halogenated BODIPY loading rates were calculated according to the standard absorption curves as shown in Figures S2−S4. The calculated loading rates of lenvatinib for LBBr2 NPs and LBCl2 NPs were 11.8 and 10.2%, respectively, and the loading rates of halogenated BODIPY for LBBr2 NPs and LBCl2 were 5.8 and 4.9%.

2.2. Drug Release in Different pH Environments and Singlet Oxygen Detection. Vigorous metabolism in tumor tissues promotes the glucose consumption and lactic acid accumulation, and therefore, the pH conditions in tumor tissues are frequently 0.5−1.0 units lower compared with healthy tissues. As the pH values vary between different tissues and cellular compartments, pH responsiveness has become one of the most extensively exploited triggers. The environment of cancer tissues is more acidic (pH 6.5−6.8) than that of blood and normal tissues (pH 7.0−7.4), while the pH value in cellular endo/lysosomes is even lower (pH 4.5−5.0). Figure 3a,b shows the lenvatinib release curves of LBBr2 NPs and LBCl2 NPs in different pH PBS solutions. After 48 h, the cumulative release of lenvatinib reached 19.4 and 18.4% at pH 7.4 in PBS, respectively. When the pH was 6.8, the release rate of lenvatinib reached 33.2 and 32.6%, respectively. Under the acidic solution of pH 5.4, the release rate of lenvatinib reached 88.5 and 82.4%. With decreasing pH, the accumulative release of lenvatinib increased, demonstrating the excellent pH-responsive property of LBPNPs. The results implied that the core−shell structured micelles were degraded to release lenvatinib and halogenated BODIPY at low pH conditions. Thus, the pH-responsive property of LBPNPs would benefit the accumulation of drugs at tumor sites and enhance the antitumor efficacy of LBPNPs.

ROS are the key cytotoxic substances during PDT, and singlet oxygen ($^{1}$O$_{2}$) is the most prominent ROS. The effective penetration depth of the laser light has a direct correlation with its wavelength, and visible light is more convenient to perform PDT; the present study selected 660 nm as the excitation wavelength in the nanoparticles. The singlet oxygen ($^{1}$O$_{2}$) generation ability of LBPNPs was measured by monitoring the photooxidation of 1,3-diphenyliso-benzofuran (DPBF) at 412 nm in DMSO irradiated by a 660 nm laser. As shown in Figure 3c, under 660 nm laser irradiation, the absorption peak of DPBF did not drop significantly. As shown in Figure 3d,e, the absorbance of the DPBF probe degraded much faster both in LBBr2 NPs and LBCl2 NPs in DMSO under 660 nm laser irradiation; their peak absorption dropped about 0.85 in 6 min, indicating that LBPNPs can generate singlet oxygen efficiently and demonstrated that LBPNPs can be an outstanding therapeutic agent for PDT.

2.3. ROS Generation of LBPNPs In Vitro. 2′,7′-Dichlorofluorescin diacetate (DCFH-DA) as a ROS detection probe can reflect the amount of ROS in the cells.
which has no fluorescence, passes through the cell membrane and enters into the cell freely and can be hydrolyzed by intracellular esterase to produce 2′,7′-dichlorodihydrofluorescein (DCFH). DCFH cannot penetrate the cell membrane so that the probe can stay in the cell. Under the oxidation of intracellular ROS, nonfluorescent DCFH produces fluorescent 2′,7′-dichlorofluorescein (DCF). Therefore, detecting the fluorescence of DCF can reflect the level of intracellular ROS. As shown in Figure 4a, Hep3B cells incubated with LBBr2 NPs and DCFH-DA only show weak green fluorescence in the control group without laser irradiation, indicating nearly no ROS generation that proved weak dark toxicity of LBBr2 NPs. However, the bright green fluorescence of DCF could be observed clearly in Hep3B cells under excitation of a 660 nm laser, which was ascribed to the high singlet oxygen quantum yield of BDPBr2, reflecting the outstanding ROS generation and phototoxicity of LBBr2 NPs in Hep3B cells. Similarly, LBCl2 NPs in Hep3B cells could generate ROS under 660 nm laser irradiation. As shown in Figure 4b, the same results also appeared in Huh7 cells. The results proved that laser irradiation further enhanced the tumor killing activity of LBPNPs in vitro.

2.4. In Vitro Cytotoxicity of LBPNPs. The key properties of photosensitizers that can be applied to PDT are their low dark cytotoxicity and high light cytotoxicity. To investigate the viabilities of LBPNPs, Hep3B cells and Huh7 cells were incubated with BDPBr2, BDPCl2, free lenvatinib, LBBr2 NPs, and LBCl2 NPs for 24 h, a standard 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay was applied. As shown in Figure 5a,b, when treated with BDPBr2 and BDPCl2 under laser irradiation, more than 90% Hep3B cells survived even in a high concentration of BDPBr2 and BDPCl2 of up to 30 μg/mL, indicating that separate photosensitizers BDPBr2 and BDPCl2 cannot be taken up by the cells to kill the tumor cells. There is no significant difference between free lenvatinib and LBBr2 NPs and LBCl2 NPs (equivalent to the dosage of lenvatinib) without laser irradiation. The results demonstrated that LBBr2 NPs and LBCl2 NPs were taken up by Hep3B cells and hydrolyzed in the acidic environment of the tumor cells and then released lenvatinib and halogenated BODIPY. Upon 660 nm laser irradiation (0.5 W/cm², 5 min), when treated with LBBr2 NPs and LBCl2 NPs (at a concentration of 15 μg/mL) for 24 h, 46 and 50% of the Hep3B cells are killed, which is much higher than...
The best photocytotoxicity compared with lenvatinib and BDPBr2 was observed. Strong phototoxicity with an IC50 of 16.6 μg/mL and 17.4 μg/mL under laser irradiation in Hep3B cells. In comparison, free lenvatinib showed a high IC50 of 41.2 μg/mL, implying the importance of synergistic actions of LBBr2 NPs and LBCl2 NPs.

As indicated in Figure 5c,d, the same results can be observed in Huh7 cells. Overall, LBBr2 NPs and LBCl2 NPs showed the best photocytotoxicity compared with lenvatinib and BDPBr2 and BDPCl2, confirming the advantages of the combination of PDT and chemotherapy.

2.5. LBPNPs Promoted the Apoptosis of Liver Cancer.
Both apoptosis and autophagy can occur after photodamage. Based on the above intracellular tests, the flow cytometry assay and western blot were used to deeply investigate the synergistic therapeutic efficiency of LBBr2 NPs and LBCl2 NPs on HCC cells. The apoptotic and necrotic cell distribution were detected by using an Annexin V PE+/7-ADD kit as the probe. Annexin V PE+/7-ADD— cells are referred as early-stage apoptotic cells, while cells in the Annexin V PE+/7-ADD+ region are regarded as late-stage apoptotic cells, and Annexin V PE−/7-ADD− are living cells. As shown in Figure 6a,b, Hep3B cells treated with BDPBr2 (15 μg/mL) showed a low rate of apoptosis and necrosis (1.26 and 2.31% for early- and late-stage apoptosis, respectively) under 660 nm laser irradiation, mainly due to the poor water solubility of BDPBr2 and it could not be taken up by Hep3B cells. Hep3B cells incubated with LBBr2 NPs demonstrated a 30.4% rate of apoptosis (11.2 and 19.2% rate of early- and late-stage apoptosis, respectively) without illumination, and there is no significant difference compared to lenvatinib (12.2 and 15.1% rate of early- and late-stage apoptosis, respectively). However, under 660 nm laser irradiation, Hep3B cells showed a larger amount (47.8%) of apoptosis than that without illumination (30.4%), mainly due to the PDT of BDPBr2. Meanwhile, the above results showed that the LBBr2 NPs could be taken up by Hep3B cells, and Pluronic F127 was hydrolyzed in the acidic environment of tumor cells to release lenvatinib and BDPBr2. Without laser irradiation, BDPBr2 could not play the role of killing the tumor cells, but the released lenvatinib could kill tumor cells. Under light conditions, BDPBr2 in PDT, kills tumor cells and synergistically with lenvatinib enhances the therapeutic effect. These observations demonstrated that the inhibitory effect on proliferation induced by LBBr2 NPs was due to the promotion of apoptosis. We observed the same results in Huh7 cells when treated with LBBr2 NPs (Figure 6a,b). The same methods were used to prove the effect of LBCl2 NPs on the apoptosis of Hep3B and Huh7 cells (Figure 6a,b). These results were in good agreement with the results from MTT assay.

To clarify the molecular mechanism by which LBBr2 NPs and LBCl2 NPs inhibited the proliferation of liver cancer cells, the downstream apoptotic pathway was further studied by means of immunoblotting. We detected the expression of some apoptosis-related proteins, mainly on caspase-3, caspase-9, and the Bcl-2 family. Caspase-3 has been shown as one of the most important cell executioners for apoptosis; therefore, we first detected the expression of caspase-3 in Hep3B cells when incubated with BDPBr2 under illumination, free lenvatinib, and LBBr2 NPs under illumination and without illumination conditions. As shown in Figure 7a, upon 660 nm laser irradiation, when treated with BDPBr2, Hep3B hardly expressed caspase-3, which directly demonstrated that BDPBr2 almost had no killing effect on Hep3B cells. The result indicated that separate photosensitizer-BDPBr2 cannot be taken up by Hep3B due to its poor biocompatibility and water solubility, which will not cause liver cancer cell apoptosis. However, when Hep3B cells were incubated with LBBr2 NPs in the absence of light, the expression of caspase-3 protein was not significantly different from that of free lenvatinib. Under light conditions, the expression of caspase-3 protein increased significantly, indicating that LBBr2 NPs can be taken up by Hep3B cells and promoted the apoptosis of Hep3B cells. Under this condition, the released BDPBr2 had a PDT effect and produced ROS, promoting the expression of apoptotic proteins and had a synergistic therapeutic effect with lenvatinib, thereby killing tumor cells. It also proved the phototoxicity and weak dark toxicity of LBBr2 NPs.

Next, we explored the upstream key apoptosis-related protein—cysteine aspartate-specific protease (caspase-9).
Hep3B cells incubated with BDPBr2 hardly expressed caspase-9 protein under light conditions, but when incubated with LBBr2 NPs under light conditions, the expression of caspase-9 increased significantly. There was no significant difference in the expression of caspase-9 between free lenvatinib and LBBr2 NPs without illumination. These results indicated caspase-9 can subsequently activate caspase-3 and then further lead to apoptosis, and upon laser irradiation, LBBr2 NPs enhanced the antitumor activity by increasing intracellular ROS levels and subsequently inducing the activation of caspase-3-related apoptotic pathways.

Additionally, the Bcl-2 family as the most crucial apoptosis proteins can better detect the extent of apoptosis. Bcl-2 and Bcl-XL are antiapoptotic proteins in the Bcl-2 gene family, Bax has a proapoptotic effect in the Bcl-2 gene family, while Bcl-2 can form dimers with proapoptotic Bax. If the relative amount of Bax is higher than Bcl-2, the number of Bax homodimers will increase, thereby promoting cell death. As shown in Figure 7a, the Hep3B cells did not express the Bax protein but expressed the Bcl-2 and Bcl-XL protein significantly when incubated with BDPBr2, irradiated by a 660 nm wavelength laser. However, compared to the treatment with lenvatinib alone, when incubated with LBBr2 NPs with illumination, Hep3B cells expressed more Bax protein and downregulated the expression of Bcl-2 and Bcl-XL proteins. All these results clearly expounded the underlying mechanism of the synergistic effects of lenvatinib and BDPBr2 in LBBr2 NPs with laser irradiation: the elevation of ROS strengthened the permeability of mitochondrial membranes; thus, the Bcl-2 family was released into the cytoplasm, which induced a biochemical cascade of caspase leading to apoptosis. As represented in Figure 7b, the same experimental results can be observed in Huh7 cells.

3. CONCLUSIONS

In summary, we have designed self-assembly nanoparticles (LBPNPs), which were encapsulated by amphiphilic polymer F127, for longer blood retention time and preferable tumor accumulation through the reprecipitation approach, which has shown excellent biocompatibility. LBPNPs had a desirable morphology, homogeneous diameter distribution, and optimal residual charge, indicating that there was no formation of agglomerates or physicochemical destabilization of samples during their shelf life. LBPNPs can generate singlet oxygen under laser irradiation when triggered by the acidic tumor microenvironment, which could improve the molecular-targeted therapy of lenvatinib via ROS induction. By measuring the toxicity, LBPNPs could significantly inhibit the growth of liver cancer cells through effectively combining PDT with chemotherapy. By detecting the expression of apoptotic proteins and flow cytometry analysis, LBPNPs can promote the apoptosis of liver cancer cells, thereby inhibiting the growth...
of liver cancer. This study proved the effectiveness of PDT combined with chemotherapy for liver cancer. Moreover, BODIPY, as a kind of photosensitizer, can be designed to combine with enzymes or peptides and molecular-targeted drugs to fabricate multifunctional nanomedicine platforms for future clinical application. Considering that both lenvatinib and BODIPY have been widely used in clinical application, the rationally designed LBPNP nanodrug would further benefit...
more HCC patients. In addition, the nanoparticles (LBPNPs) had potential for bioimaging application and may be useful for cancer diagnosis in the future.

4. EXPERIMENTAL SECTION

4.1. Materials and Apparatus. Lenvatinib mesylate was purchased from Xi’an Qiyue Biotechnology Co. Ltd. Pluronic F127, triethylamine, and THF were obtained from Shanghai Aldrich Corporation. Nuclear stains 4,6-diamidino-2-phenylindole (DAPI) and MTT were supplied by Shanghai Solarbio Co. Ltd. and 100 IU/mL penicillin and 100 μg/mL streptomycin (Invitrogen).

4.2. Cell Lines. The human HCC cell lines Hep3B and Huh7 were obtained from the Shanghai Institutes for Biological Sciences Cell Center (China). In a humidified atmosphere incubator containing 5% CO₂ at 37 °C, the cell lines were maintained in DMEM (Gibco) supplemented with 10% FBS and 100 IU/mL penicillin and 100 μg/mL streptomycin (Invitrogen).

4.3. Synthesis of 3,7-Dibromo-5,5-difluoro-10-Mesityl-4,5-5,5'-dipyrrrolo-[1,2-c:2',1'-f] [1,3,2]-diazaborinine (Compound 1) and 3,7-Dichloro-5,5-difluoro-10-Mesityl-4,5-5,5'-dipyrrrolo-[1,2-c:2',1'-f] [1,3,2]-diazaborinine (Compound 2). Compound 1 and Compound 2 were synthesized according to the method reported in the literature.33–35 2,2′-(Mesitylmethylene)bis(1H-pyrrole) (2.64 g,1 mmol) was dissolved in dichloromethane (DCM,100 mL), and THF was employed to elute the solution. The crude product was further purified by chromatography on silica gel eluting with DCM/n-hexane (1:2) to collect the first yellow-green fluorescent band (884.4 mg, 1.89 mmol, yield: 50.1%) (Compound 1). The same method was used to prepare Compound 2 (787.9 mg, 2.08 mmol, yield: 55.0%).

Compound 1: 1H NMR (500 MHz; CDCl₃): δ = 2.11 (s, 6H, Me-H), 2.38 (s, 3H, Me-H), 6.48 (d, J = 3.5 Hz, 2H, pyrrole-H), 6.7 (d, J = 3.5 Hz, 2H, pyrrole-H), 6.97 (s, 2H, Ar-H) ppm. HR-MS (MALDI-TOF-MS): m/z = 467.926, calcd for (C₃₉H₃₅N₃O)⁺ = 467.964 ([M⁺]) (Figure S1a,b).

Compound 2: 1H NMR (500 MHz; CDCl₃): δ = 2.13 (s, 6H, Me-H), 2.39 (s, 3H, Me-H), 6.5 (d, J = 3.5 Hz, 2H, pyrrole-H), 6.71 (d, J = 3.5 Hz, 2H, pyrrole-H), 6.8 (s, 2H, Ar-H) ppm. HR-MS (MALDI-TOF-MS): m/z = 378.031, calcd for (C₃₉H₃₅N₃O)⁺ = 378.070 ([M⁺]) (Figure S1c,d).

4.4. Preparation of LBPNPs. To prepare LBPNPs (LBB₃ NPs/LBCl₂ NPs), 5 mg of lenvatinib mesylate was dissolved in 5 mL of deionized water; then, 0.2 mL triethylamine was added to the solution under sonication for 5 min. Then, centrifugal precipitation was carried out to remove water. THF (1 mL) was added dropwise into the precipitate and then 1 mg Compound 2/Compound 3 was added to the mixed solution. Then, the solution was added dropwise into a 30 °C water solution of Pluronic F127 (1 mg/mL) under medium-speed magnetic stirring. THF was removed at a temperature of 50 °C by vacuum suction. The residual solution was filtered with a 220 nm filtration membrane to remove unencapsulated Compound 2/Compound 3 and lenvatinib. Consequently, LBB₃ NPs/LBCl₂ NPs were generated.

4.5. Characterization of LBPNPs. Different techniques were used to characterize the BDBr₂, BDPCl₂ and LBB₃ NPs and LBCl₂ NPs. UV–vis–NIR absorption spectra were...
recorded by a UV-3600 Shimadzu UV−vis−NIR spectrometer (Shimadzu, Japan). Fluorescence spectra were recorded by an F-4600 spectrofluorophotometer (Hitachi, Japan). Hydrodynamic diameters and zeta potentials of LBPNPs were measured by Mastersizer 3000. (Zeta/DLS, Malvern, England). Characterizations of sample morphology were performed on an S-4800 field-emission scanning electron microscope (FESEM, Hitachi S4800, Japan) and a transmission electron microscope (Joel Jem-2010F, Japan). Taking the methoxyfluoroboron pyrrole BDPOMe (BDPOMe, Φ = 0.30/Me CN) reported in the literature as the reference standard, the reference method was used to determine and calculate relative fluorescence quantum yields of BDPBr2, BDPCl2, LBBr2 NPs, and LBCl2 NPs. Time-correlated single photon counting was used to determine the fluorescence lifetime of LBBr2 NPs and LBCl2 NPs.

4.6. Loading Content, Drug Release, and Singlet Oxygen Detection of LBPNPs. The drug loading content of in LBBr2 NPs and LBCl2 NPs was measured by a UV−vis spectrophotometer.

LBPNPs (10 mL, 200 μg/mL, equivalent to the concentration of lenvatinib) in a dialysis bag (3 KDa) were steeped into 50 mL of PBS solution (pH 7.4/pH 6.8/pH 5.0, 37 °C) under stirring. Triggered by a 660 nm laser (0.5 W/cm²), 3 mL of PBS solution was extracted, and another 3 mL of fresh PBS solution was added every time point. The concentration of released lenvatinib was recorded by UV−vis spectrophotometry. 1,3-Diphenylisobenzofuran (DPBF) was employed for detecting ROS production by LBPNPs. The mixture of DPBF and LBPNPs in DMSO was irradiated by a 660 nm laser for 6 min. The decrease of DPBF was monitored by a UV−vis spectrometer at different time points over 6 min.

4.7. Intracellular ROS Induction of LBPNPs In Vitro. For detection of ROS, Hep3B cells and Huh7 cells were seeded in 96-well plates which were incubated for 24 h and added 0.1 mL of LBBr2 NPs at 15 μg/mL concentration. One of them was irradiated with a 660 nm laser for 5 min (0.5 W/cm²). The other group was kept without illumination, and then DCFH-DA was added and incubated for 30 min. Then, the cells were washed three times with a serum-free medium, and then DAPI was added to stain the nucleus. Ultimately the production of ROS was observed in cells with a fluorescence microscope. The same method was used to detect ROS of LBCl2 NPs in Hep3B cells and Huh7 cells.

4.8. Cytotoxicity Assay. The main property of an ideal nanophotosensitizer applied is its low level of dark toxicity— the photosensitizer drug should be nontoxic in the absence of light irradiation. Therefore, to determine the best dose-response of nanoparticles that would not affect liver cancer cells in the absence of photoactivation, MTT assay was carried out. At the same time, the MTT method was used to verify the synergistic effect of LBPNP PDT and chemotherapy. Hep3B cells were seeded at 1 × 10⁴ cells per well in 96-well plates; after incubation for 24 h, the medium was replaced with 0.1 mL of BDPBr2, free lenvatinib, and LBBr2 NPs at different concentrations (0, 5, 10, 15, 20, 25, and 30 μg/mL). After being treated with 24 h, the cells of free Lenvatinib, one of LBBr2NPs groups were not illumination, and BDPBr2 and the other of LBBr2NPs groups were irradiated with 660 nm laser (0.5 W/cm²) for 5 min. Then, the cells were incubated at 37 °C for 24 h. For MTT assay, the medium was replaced with MTT solution (0.01 mL, 0.5 mg/mL) and kept for another 4 h. Subsequently, the supernatant was carefully removed and DMSO (0.15 mL) was added. The absorbance at 492 nm was measured by Bio-Tek microplate reader. The toxicity of LBBr2 NPs in Huh7 cells was obtained in the same way. Similarly, the phototoxicity and dark toxicity of LBCl2 NPs in Hep3B cells and Huh7 cells were also tested by the above methods.

4.9. Flow Cytometry. To measure the apoptosis of liver cancer cells, a total of 5 × 10⁴ Hep3B/Huh7 cells were seeded into 6-well plates. Then, the cells were incubated with BDPBr2, BDPCl2, free lenvatinib, LBBr2 NPs, and LBCl2 NPs for 24 h. Then, BDPBr2, BDPCl2, LBCl2 NPs, and LBBr2 NPs were irradiated with a 660 nm laser (0.5 W/cm²) for 5 min. Next, the cells were stained with the Annexin V-PE/7-AAD Apoptosis Detection Kit (Beijing Solarbio Science & Technology Co., Ltd). The samples were analyzed using FACS, and the data were processed using FlowJo.

4.10. Western Blot. Hep3B cells and Huh7 cells were seeded at 1 × 10⁵ cells per well in a 6-well plate; the cells were incubated for 24 h with BDPBr2, BDPCl2, free lenvatinib, LBBr2 NPs, and LBCl2 NPs (lenvatinib concentration was 15 μg/mL). BDPBr2, BDPCl2, LBBr2 NPs, and LBCl2 NPs were irradiated with 660 nm laser (0.5 W/cm²) for 15 min, and at the same time, LBBr2 NPs and LBCl2 NPs were kept without illumination. Then, they were incubated for 24 h, and the cells were collected after trypsinization. Cell samples were lysed by RIPA lysis buffer (Beyotime, China), which contained a cocktail of phosphatase inhibitors (Roche Applied Science) and a cocktail of protease inhibitors (Roche Applied Science, Switzerland). Total proteins were extracted from Hep3B/Huh7 cells according to the manufacturer’s instruction. Proteins were resolved using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) (Millipore, Massachusetts), which were blocked with a QuickBlock Blocking Buffer (Vazyme) for western blotting. Then, PVDF membranes were incubated overnight at 4 °C with the appropriate primary antibodies caspase-3 antibody (1:1000; AC030, Beyotime), caspase-9 antibody (1:1000; AC062, Beyotime), Bcl-2 antibody (1:1000; AB112, Beyotime), Bcl-xl antibody (1:1000; AB126, Beyotime), Bax antibody (1:1000; AB026, Beyotime), and anti-GAPDH mouse mAb (1:1000, TA-08, Servicebio). Primary antibodies were diluted in a primary antibody diluent according to the manufacturer’s instructions. After incubating with primary antibodies, the blots were washed with TBST and incubated for 1 h at room temperature with the secondary antibody tagged with horseradish peroxidase. Finally, the antibody-labeled proteins were detected using an electrochemiluminescent (ECL) system. GADPH was used as the internal control.

4.11. Statistical Analysis. All experiments were repeated at least three times. Normalization was employed for data-processing. Data analysis was performed with GraphPad Prism8 software and Origin 2021 software using unpaired two-tailed Student’s t-tests. Sample size (n) and mean ± standard deviation was adapted for presentation of results.

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c01346.

Characterization of BDPBr2 and BDPCl2 and UV−vis−NIR absorbance spectra of lenvatinib, BDPBr2, and BDPCl2 at different concentrations (PDF)
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Notes

The authors declare no competing financial interest.

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