All-in-One Phototheranostics: Single Laser Triggers NIR-II Fluorescence/Photoacoustic Imaging Guided Photothermal/Photodynamic/Chemo Combination Therapy

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Development of single near-infrared (NIR) laser triggered phototheranostics for multimodal imaging guided combination therapy is highly desirable but is still a big challenge. Herein, a novel small-molecule dye DPP-BT is designed and synthesized, which shows strong absorption in the first NIR window (NIR-I) and fluorescence emission in the second NIR region (NIR-II). Such a dye not only acts as a dual-modal contrast agent for NIR-II fluorescence and photoacoustic (PA) imaging, but also serves as a combined therapeutic agent for photothermal therapy (PTT) and photodynamic therapy (PDT). The single NIR laser triggered all-in-one phototheranostic nanoparticles are constructed by encapsulating the dye DPP-BT, chemotherapy drug DOX, and natural phase-change materials with a folic acid functionalized amphiphile. Notably, under NIR laser irradiation, DOX can effectively release from such nanoparticles via NIR-induced hyperthermia of DPP-BT. By intravenous injection of such nanoparticles into Hela tumor-bearing mice, the tumor size and location can be accurately observed via NIR-II fluorescence imaging. However, low sensitivity and microscopic spatial resolution, which could hardly be achieved by NIR-II fluorescence imaging. Among the reported NIR-II fluorophores, small organic molecules NIR-II fluorophores have been regarded as a promising candidate for clinical imaging on the merits of superb biocompatibility, easily adjusted chemical structures, and optical properties. Despite significant improvements in NIR-II fluorescence imaging for diagnosis assessment, comprehensive tumor information at different spatial resolutions and scales could not effectively provided by only one imaging modality. Photoacoustic (PA) imaging is a hybrid imaging method that combines the superiorities of optical imaging and ultrasound imaging, providing deeper tissue penetration than NIR-II fluorescence imaging. Moreover, PA imaging can describe deep tumor profile with microscopic spatial resolution, which could hardly be achieved by NIR-II fluorescence imaging. However, low sensitivity and

1. Introduction

Intelligently devised multifunctional theranostic nanoplatforms are extremely urgent for the early diagnosis of diseased tissues and imaging-guided therapy. Recently, phototheranostics, which provide simultaneous diagnosis and therapy through light irradiation, have been validated as significant breakthrough toward cancer theranostics owing to their characteristics including noninvasiveness, high efficiency, as well as the precise controlled release of encapsulated substances both spatially and temporally. However, there still exist many barriers by using this strategy, including complex components, sophisticated construction, two or more different wavelength lasers as well as inadequate diagnostic accuracy or therapeutic efficacy. As a consequence, it is imperative to exploit highly efficient theranostic nanoplatforms, achieving ideal diagnostic accuracy and therapeutic efficacy under a single wavelength laser irradiation. Among the present photo-induced diagnostic techniques, fluorescence imaging in the second near-infrared optical window (NIR-II, 1000–1700 nm) has attracted tremendous attention owing to its irreplaceable merits over the conventional fluorescence imaging in the first NIR window (NIR-I, 650–900 nm). The fluorescence in the NIR-II window have highlighted the benefits of imaging at longer wavelengths, including deep penetration depth, high resolution, and sensitivity as well as the increased signal-to-background ratio by reducing photon scattering and tissue autofluorescence. Among the reported NIR-II fluorophores, small organic molecules NIR-II fluorophores have been regarded as a promising candidate for clinical imaging on the merits of superb biocompatibility, easily adjusted chemical structures, and optical properties. Despite significant improvements in NIR-II fluorescence imaging for diagnosis assessment, comprehensive tumor information at different spatial resolutions and scales could not effectively provided by only one imaging modality. Photoacoustic (PA) imaging is a hybrid imaging method that combines the superiorities of optical imaging and ultrasound imaging, providing deeper tissue penetration than NIR-II fluorescence imaging. Moreover, PA imaging can describe deep tumor profile with microscopic spatial resolution, which could hardly be achieved by NIR-II fluorescence imaging. However, low sensitivity and
limited applications in soft tissues are major barriers of this technique. Considering the merits of each imaging modalities, the integration of NIR-II fluorescence imaging and PA imaging can provide accurate and wealth information by compensating inherent limitations of each single imaging modality, thus improve the accuracy of cancer diagnosis. Consequently, the rational design and fabrication of an all-in-one phototheranostic for NIR-II fluorescence/PA dual-modal imaging guided cancer phototherapy is highly worthwhile to meet the demand for accurate diagnosis as well as precise and efficient cancer therapy.

To date, an amount of small-molecule dye-based organic nanomaterials with excellent NIR-II fluorescence and photothermal properties have been developed. Several of them also showed a favorable application in photothermal therapy (PTT). However, after PTT process, the residual cancer would be able to recrudesce due to the acquired thermal-resistance property of residual cancer cells, which may restrict the extensive application of PTT in cancer treatment. Fortunately, there are several other therapy modalities, which are helpful in overcoming the limitation of PTT. Photodynamic therapy (PDT) is another kind of noninvasive phototherapeutic approach, in which, the irradiation photosensitizers could generate cytotoxic reactive oxygen species (ROS, typically singlet oxygen \(1O_2\)) under light irradiation, which can lead to the death of cancer cell. Nevertheless, the PDT efficiency is seriously inhibited by the hypoxia microenvironment around the tumor. Combination therapy, which integrates the superiorities of two or more treatment modalities, has potential to dramatically enhance the antitumor efficacy and reduce the side effects. Consequently, the combination of PTT and PDT is desirable toward cancer treatment. However, the development of a substrate/molecule combining the function of NIR-II fluorescence/PA dual-modal imaging and PTT/PDT combination therapy is still a great challenge. In addition, as a conventional cancer therapy approach, chemotherapy technique was also integrated with PTT or PDT, and demonstrated the possibility to eradicate diffused microcarcinoma, give a long-term and reliable cancer treatment, and reduce the chances of the cancer coming back. On the contrary, PTT or PDT could active antitumor immunity and revert multidrug resistance, and thus improve the effect of chemotherapy. Especially, the development of NIR light-triggered chemotherapy systems has drawn increasing interest due to the facile controllability, high precision, and little damage to normal tissues, greatly reducing toxic side effect of chemotherapy. Therefore, the design and fabrication of NIR light-triggered phototheranostics for PTT/PDT/chemo combination therapy is expected as an innovative strategy to overcome the shortcomings of each therapy and achieve synergistic therapeutic effects.

Herein, we designed a novel diketoppyrrolopyrrole (DPP)-based small-molecule dye DPP-BT, which showed a single NIR laser triggered multifunctional properties. Such DPP-BT fluorophore could not only act as a NIR-II fluorescence/PA dual-modal imaging contrast agent, but also serve as a PTT/PDT combined therapeutic agent. The single NIR laser triggered phototheranostic nanoparticles, named as P(DPP-BT/DOX) NPs, were then prepared through coencapsulating hydrophobic DPP-BT and chemotherapy drug doxorubicin (DOX) in biocompatible organic phase-change material (PCM, mixture of lauric acid and stearic acid, eutectic point at 39 °C) with amphiphilic lecithin. To improve the therapy accuracy, the nanoparticles were functionalized with folic acid (FA) amphiphile (DSPE-PEG-FA) to realize cancer targeting property. Under the irradiation of a single NIR laser (730 nm), such nanoparticles could generate strong NIR-II fluorescence and PA signals, excellent photothermal and photodynamic effects, which could be used to in vivo tumor targeting NIR-II fluorescence/PA dual-modal imaging guided PTT/PDT combination therapy. Meanwhile, the loaded drug DOX could effectively release from the melted PCM matrix by NIR-induced photothermal effect of the DPP-BT, leading to synergistically enhance the overall tumor therapy efficacy (Scheme 1). Compared with previous theranostic nanoplatforms, this single NIR light triggers all-in-one phototheranostics contain the functionalities of dual-modal imaging and tri-modal combination therapy, which showed a great potential in theranostics of cancer.

2. Results and Discussion

2.1. Synthesis and Characterization of Small-Molecule NIR-II Dye

By integrating the electron-withdrawing benzothiadiazole (BT) moiety and DPP moiety together, a novel small-molecule NIR-II dye DPP-BT with markedly low energy gap was successfully synthesized (Figure 1a). This small-molecule NIR-II dye was prepared in a facile two-step reaction, which was confirmed by \(^1\)H NMR, \(^13\)C NMR, and MALDI-TOF-MS (Scheme S1, Supporting Information). Hydrophobic small-molecule NIR-II dye DPP-BT was then encapsulated with amphiphilic DSPE-mPEG5000 and lecithin to afford water-dispersed DPP-BT nanoparticles (DPP-BT NPs). The resulting DPP-BT NPs were fully characterized by transmission electron microscopy (TEM) and dynamic light scattering (DLS; Figure 1b). The average diameter of DPP-BT NPs was \(\sim\) 60 nm from TEM and DLS results. As well known, superior NIR absorption and NIR-II fluorescence emission are crucial to construct contrast agents for PA imaging and NIR-II fluorescence imaging. In Figure 1c, DPP-BT NPs in aqueous solution displayed excellent NIR-I absorption with a major peak at 686 nm. The absorption intensity of DPP-BT NPs in water increased linearly along with the rising of concentration, and the molar absorption coefficient of DPP-BT NPs at 730 nm was calculated about \(3.05 \times 10^4\) M\(^{-1}\) cm\(^{-1}\) (Figure S6, Supporting Information), which showed the outstanding light absorption capability of these NPs. In comparison with commercial small-molecule dye ICG and ICG NPs, DPP-BT NPs displayed outstanding photostability even after 60 min of continuous 730 nm laser irradiation, which made the DPP-BT favorable for biological application (Figure 1d). Meanwhile, DPP-BT NPs solution showed superb NIR-II emissions from 900 to 1300 nm with maximum emission wavelength at 1089 nm, displaying a large Stokes shift of 403 nm (Figure 1c). Such large Stokes shift could effectively decrease the interference from background fluorescence and excitation backscattered light, improving the signal fidelity. Subsequently, the fluorescence quantum yield of DPP-BT NPs in water was estimated...
about 0.42% by using IR 1061 as a reference (Figure S7, Supporting Information). Therefore, DPP-BT provides a promising potential to serve as a NIR-II fluorescent probe.

2.2. Photothermal and Photodynamic Properties of DPP-BT NPs

In view of the excellent NIR-I absorption of DPP-BT, which makes DPP-BT NPs to be suitable for tumor PTT, the photothermal conversion capabilities of the NPs in water were systematically investigated. As expected, the temperature of the aqueous dispersed DPP-BT NPs could be effectively increased under 730 nm laser irradiation in concentration-dependent manner (Figure 2a). With increasing the concentration from 20 to 100 µg mL⁻¹, the temperature changes of the solution could rise from 21.8 to 43.6 °C upon 730 nm laser irradiation in 10 min (1.0 W cm⁻²), which is sufficient to ablate cancer cells. Whereas only insignificant temperature increase was found for pure water under the same conditions. The temperature changes of various solutions were also confirmed by the infrared (IR) thermal images (Figure 2c). The relationship between temperature change of DPP-BT NPs and laser power density was then investigated. As presented in Figure 2b, the temperature of NPs solution rose rapidly as the power density increase, verifying that the temperature increase was linearly related to the power density. When the NPs were irradiated with 730 nm laser for five on-and-off cycles, no appreciable reduction of temperature elevation was discovered, suggesting the superior photothermal stability of such DPP-BT NPs (Figure S8, Supporting Information). In addition, to further evaluate the conversion of photo energy into thermal energy, the photothermal conversion efficiency (η) of DPP-BT NPs was measured to be 50.0% (Figure S9, Supporting Information), which is extremely higher in comparison with previously reported PTT agents. All these results clearly indicated that such NPs are promising PTT agents with outstanding photothermal conversion performance.

Subsequently, the ROS production capability of these NPs upon 730 nm laser irradiation was examined by monitoring the UV–vis spectrum change of 1,3-diphenylisobenzofuran (DPBF, an excellent 1O₂ probe), whose absorption weakening upon irreversibly react with 1O₂. The absorption intensity of DPBF at 414 nm in the presence of DPP-BT NPs reduced sharply as the laser power density increased (Figure 2d and Figure S10, Supporting Information). Especially, the absorption intensity decreased 66.3% within
16 min under laser irradiation (1.0 W cm\(^{-2}\)), suggesting that the \(^{1}\text{O}_2\) could efficiently generate. Meanwhile, the characteristic \(^{1}\text{O}_2\) emission at about 1270 nm of DPP-BT NPs under 730 nm laser irradiation was obtained (Figure 2e). The \(^{1}\text{O}_2\) quantum yield of DPP-BT NPs in water was deemed about 27.3% with methylene blue (MB) as a standard (Figure S11, Supporting Information). Moreover, the intracellular ROS generation was further assessed using non-fluorescent 2',7'-dichlorofluorescein diacetate (DCFH-DA) as a probe, which can be rapidly converted into green fluorescent 2',7'-dichlorofluorescein (DCF) by ROS. After laser irradiation of the Hela cells incubated with NPs and DCFH-DA, a bright green fluorescence of DCF can be clearly observed, while there was no obvious fluorescence in the control experiments (Figure 2f and Figure S12, Supporting Information). This observation indicated the capability of DPP-BT NPs for light-induced generation of ROS inside cancer cells. According to the above investigations, the single small molecular DPP-BT can act as a promising candidate for PTT/PDT combination therapy under a single 730 nm laser irradiation.

### 2.3. Drug Encapsulation and Its NIR-Triggered Controllable Release

After the confirmed photothermal effect of DPP-BT, DOX- and DPP-BT-coencapsulated PCM NPs were elaborately fabricated for NIR-triggered drug release. In order to improve the therapy accuracy, FA functionalized amphiphile (DSPE-PEG-FA) was modified in the nanoprecipitation process. In brief, the mixture solution of lauric acid and stearic acid (4:1 by weight, natural PCM with eutectic point at 39 °C)[18] were mixed together with DPP-BT and DOX, and the above solution was added dropwise to the preheated aqueous solution of lecithin and DSPE-PEG-FA under magnetic stirring to produce nanoparticles via the self-assembly processes. Next, the suspension was stored at about 4 °C, making the formed nanoparticles quickly solidify to afford excellent biocompatible and water-dispersed P(DPP-BT/DOX) NPs. Moreover, P(DPP-BT) NPs that just without DOX compared with P(DPP-BT/DOX) NPs were obtained according to the above method. After loading DOX, the solution showed a clear color change from bluish green to brown (Figure S13, Supporting Information). As presented in the TEM image, the P(DPP-BT/DOX) NPs were monodispersed spheres with average diameter of ~100 nm, which was close to the DLS result (Figure 3a). From the absorption spectra, we confirmed that both DPP-BT and DOX were successfully loaded into the formed NPs (Figure 3b). The loading efficiency was measured to be 51.2% for DPP-BT and 45.7% for DOX, respectively. More significantly, compared with P(DPP-BT) NPs, the maximum absorption peak of P(DPP-BT/DOX) NPs was red-shifted from 700 nm to 730 nm, which may be ascribed to the interaction between DPP-BT and DOX, leading to it significantly overlap with the 730 nm NIR laser.
The fluorescence spectrum of P(DPP-BT/DOX) NPs showed a maximum fluorescence peak at 1076 nm (Figure 3b). Moreover, the as-prepared P(DPP-BT/DOX) NPs were relatively stable in water, PBS solution, or FBS solution for 5 weeks without any noticeable precipitation (Figure 3c).

Subsequently, the NIR-triggered DOX release from P(DPP-BT/DOX) NPs was investigated under 730 nm laser irradiation. Under the irradiation of NIR laser, the temperature is raised above the eutectic point of PCM matrix, and the drugs could quickly release from the melted PCM matrix in fluid state. In Figure 3d, the DOX release behavior exhibited obvious “on–off” release feature responding to NIR laser irradiation. However, no significant release was found in the control group without laser irradiation, demonstrating that the DOX release only triggered by NIR induced photothermal effect of DPP-BT. To further confirm the NIR-triggered release property, intracellular DOX release behavior was then studied. After incubating tumor cells with P(DPP-BT/DOX) NPs, the release amount of DOX could be directly detected by monitoring the fluorescence intensity of DOX. As presented in Figure 3e, the intensity of intracellular red fluorescence increased with the increase of irradiation time. Meanwhile, after irradiation with 730 nm laser for 10 min, red fluorescence also could be found in the nuclei, indicating that the loaded DOX could effectively release from the P(DPP-BT/DOX) NPs and entered into the nuclei for killing cancer cells. Moreover, the release profile of DPP-BT from P(DPP-BT/DOX) NPs was also investigated (Figure 3d). The fluorescence intensity of DPP-BT release from P(DPP-BT/DOX) NPs gradually increased up 730 nm laser irradiation (Figure 3f), maybe due to the change of aggregate state of DPP-BT after release from P(DPP-BT/DOX) NPs, making these NPs to be powerful NIR-II fluorescent agent. These results clearly shown that these PCM NPs are excellent candidates for NIR-triggered drug release.

2.4. In Vitro Targeting Ability and Cytotoxicity of P(DPP-BT/DOX) NPs

Since FA can act as an effective tumor targeted ligand, the cells targeting ability of P(DPP-BT/DOX) NPs was systematically investigated by fluorescence imaging in vitro, where Hela cancer cells (overexpressing FA receptors) and NIH3T3 normal fibroblast cells (lacking FA receptor) were chosen as cells model. As demonstrated in Figure 4a, strong DOX fluorescence could be clearly discovered after 12 h of incubation in the Hela cancer cells that treated with FA functionalized P(DPP-BT/DOX) NPs, due to the over-expressing FA receptors on Hela cells. In comparison, the control groups (Hela cancer cells treated with P(DPP-BT/DOX) NPs without FA motif and NIH3T3 normal cells treated with P(DPP-BT/DOX) NPs W/WO FA motif) were all showed quite weak DOX fluorescence (Figure 4b–d). According to the remarkable difference in fluorescence images, it can be confirmed that FA functionalized P(DPP-BT/DOX) NPs have the excellent targeting ability toward Hela cells.

In order to realize biomedical and clinical applications in vivo, the biocompatibility of as-prepared NPs against NIH3T3 normal cells was assessed by MTT assay. The
Figure 3. a) DLS data (polydispersity: 0.18) and TEM result (inset) of P(DPP-BT/DOX) NPs. b) UV–vis–NIR spectra of free DOX, P(DPP-BT) NPs, and P(DPP-BT/DOX) NPs, and fluorescence spectrum of P(DPP-BT/DOX) NPs. c) Stability of the P(DPP-BT/DOX) NPs in different mediums. d) NIR-triggered DOX and DPP-BT release curves from P(DPP-BT/DOX) NPs. e) Time-course intracellular DOX release upon irradiation with 730 nm laser (0.5 W cm⁻²). Scale bars: 20 µm. f) Fluorescence spectra of DPP-BT release from P(DPP-BT/DOX) NPs upon irradiation with 730 nm laser (0.5 W cm⁻²).

Figure 4. Fluorescence imaging of (a) Hela cells treated with FA functionalized P(DPP-BT/DOX) NPs, b) Hela cells treated with P(DPP-BT/DOX) NPs without FA motif, c) NIH3T3 cells treated with FA functionalized P(DPP-BT/DOX) NPs, and d) NIH3T3 cells treated with P(DPP-BT/DOX) NPs without FA motif. Scale bars: 40 µm. e) Biocompatibility assays of as-prepared materials against NIH3T3 normal cells. f) Cytotoxicity assays of as-prepared materials against Hela tumor cells with or without laser irradiation (*p < 0.05, **p < 0.01). g) Live/dead assays of Hela cells treated with P(DPP-BT) NPs or P(DPP-BT/DOX) NPs with or without irradiation. Scale bars: 100 µm.
viability of NIH3T3 cells showed no significant difference as the P(DPP-BT/DOX) NPs or P(DPP-BT) NPs concentration increased, convincingly revealing the outstanding biocompatibility of these NPs for biomedical and clinical applications (Figure 4e). The performance of as-prepared NPs for combination therapy against Hela cancer cells in vitro was further examined. In Figure 4f, no evident toxic effects of P(DPP-BT) NPs and P(DPP-BT/DOX) NPs were observed without laser irradiation. By contrast, after irradiation with 730 nm laser, remarkable cell apoptosis was discovered for P(DPP-BT) NPs by the integration of PTT and PDT effect. More importantly, in comparison with P(DPP-BT) NPs, higher cytotoxicity could be found for P(DPP-BT/DOX) NPs because of the loaded DOX could effectively release by NIR-triggered photothermal effect of DPP-BT for synergistically enhance the overall tumor therapy efficacy. The synergistic therapeutic strategy was further visually evaluated using live/dead assay, where green-emissive calcein AM and red-emissive propidium iodide (PI) were used to stain live and dead cells, respectively. In Figure 4g, a markedly higher level of cell death showed by red fluorescence was clearly observed in the P(DPP-BT/DOX) NPs group than that of in the P(DPP-BT) NPs group after irradiation with 730 nm laser. Meanwhile, in all comparison groups, Hela cells treated with P(DPP-BT) NPs and P(DPP-BT/DOX) NPs groups without laser irradiation exhibited widespread green fluorescence, suggesting negligible cell damage. These observations from MTT and live/dead assays distinctly demonstrated that the P(DPP-BT/DOX) NPs could serve as promising nanoagents for tumor targeting PTT/PDT/chemo combination therapy.

2.5. In Vitro Fluorescence and Photoacoustic Properties of P(DPP-BT/DOX) NPs

Encouraged by the outstanding optical properties of P(DPP-BT/DOX) NPs, their NIR-II fluorescence properties were first tested using an NIR-II fluorescence imaging system. Figure 5a showed that the NIR-II signal intensity of the P(DPP-BT/DOX) NPs under excitation at 730 nm was a function of NPs concentration. As expected, the NIR-II signals exhibited excellent linear relationship with NPs concentration. In addition, the NIR-II fluorescence imaging penetration depth of P(DPP-BT/DOX) NPs under chicken-breast tissue could be reached up to 10 mm (Figure S14, Supporting Information), which is much higher than that observed from NIR-I agents.[7e] The NIR-II fluorescence imaging in cellular level was further investigated. The fluorescence intensity of Hela cells incubated with P(DPP-BT/DOX) NPs under chicken-breast tissue could be reached up to 10 mm (Figure 5b). The PA properties of P(DPP-BT/DOX) NPs were subsequently evaluated in vitro. A strongest PA signal at 730 nm was distinctly observed from the PA spectrum, which is in
good agreement with its UV–vis–NIR absorption spectrum (Figure S15, Supporting Information). From the PA mapping of P(DPP-BT/DOX) NPs at different concentrations, the strength of PA signals and the brightness of PA images under excitation at 730 nm are linearly proportional to the NPs concentration (Figure 5c). Furthermore, the conclusions from PA imaging assay in Hela cells were similar to the above-mentioned results (Figure 5d). All these investigations indicated that the P(DPP-BT/DOX) NPs are efficient NIR-II fluorescence/PA dual-modal imaging probes.

2.6. In Vivo NIR-II Fluorescence Imaging and PA Imaging

Subsequently, the dual-modal imaging capability of P(DPP-BT/DOX) NPs in vivo was evaluated. The performance of P(DPP-BT/DOX) NPs in NIR-II fluorescence imaging was first studied by tail vein injection of P(DPP-BT/DOX) NPs into Hela tumor-bearing mice. As shown in Figure 6a, b, the NIR-II signals from P(DPP-BT/DOX) NPs gradually increased in the tumor region and reached a maximum at 24 h postinjection, increasing by 9.4-fold in comparison with that before injection. After that, the NIR-II signals began to decline owing to metabolism. Hence, 24 h postinjection was selected as the optimal time for cancer diagnosis and treatment owing to the maximal accumulation of P(DPP-BT/DOX) NPs in the tumor. Moreover, the major organs and tumor were gained after 72 h injection of P(DPP-BT/DOX) NPs for ex vivo imaging. The NIR-II fluorescence images disclosed that P(DPP-BT/DOX) NPs were preferentially distributed in tumor, liver, and spleen than heart, lung, and kidney, suggesting the nanoparticles were metabolized mainly through hepatobiliary system (Figure S16, Supporting Information). The capability of P(DPP-BT/DOX) NPs to produce PA signals in vivo was also investigated in Hela tumor-bearing mice. The changes of PA signals in the tumor regions with different injection times were in good agreement with the NIR-II fluorescence imaging results. As shown in Figure 6c, d, the PA signals progressively enhanced in early time and displayed maximal retention at 24 h postinjection, increasing by 10.5-fold compared with that before injection. Moreover, the uniform distribution of P(DPP-BT/DOX) NPs inside the tumor was clearly observed at 24 h postinjection (Figure 6c), suggesting the deep penetrating ability of P(DPP-BT/DOX) NPs. Specially, compared with NIR-II fluorescence imaging, PA imaging has microscopic imaging capability with spatial resolution, offering a complementary imaging modality to acquire comprehensive information of tumor. Therefore, the integration of NIR-II fluorescence and PA imaging holds great promise to offer both macroscopic ultrasensitivity and microscopic spatial resolution for tumor diagnosis and imaging-guided therapy.

2.7. In Vivo Combination Therapy

Motivated by the efficient and homogenous tumor accumulation of the nanoagents, the single NIR laser induced PTT/PDT/chemo combination therapy of P(DPP-BT/DOX) NPs was assessed in vivo with Hela tumor-bearing mouse model. To visually verify the drug release in vivo by NIR-triggered
photothermal effect, IR thermal imaging of P(DPP-BT/DOX) NPs in vivo was first investigated under irradiation with 730 nm laser (1.0 W cm$^{-2}$) for 10 min after 24 h postinjection (maximum accumulation time). In the group treated with P(DPP-BT/DOX) NPs, hyperthermia was quickly generated in tumor region and reached up to around 54 °C within 4 min, while negligible temperature change was noted in the control group, demonstrating that P(DPP-BT/DOX) NPs could act as superb photothermal agents to produce hyperthermia in vivo to efficiently release DOX and kill tumor cells (Figure 7).

To evaluate the PTT/PDT/chemo combination therapeutic effect, mice with the same Hela tumor model were stochastically divided into five groups and given following treatments: 1) PBS, 2) P(DPP-BT) NPs, 3) P(DPP-BT/DOX) NPs, 4) P(DPP-BT) NPs + laser, and 5) P(DPP-BT/DOX) NPs + laser. After 24 h postinjection of NPs, the tumor areas of the mice were exposed to 730 nm laser (1.0 W cm$^{-2}$) for 10 min. The tumor sizes were monitored during the treatment process to qualitatively evaluate the combination therapeutic effect. As shown in Figure 7b,c, the tumor volumes of P(DPP-BT) NPs or P(DPP-BT/DOX) NPs injected mice without laser irradiation grew steadily similar to that of control group, suggesting no anticancer efficacy of both P(DPP-BT) NPs and P(DPP-BT/DOX) NPs in such a situation. After laser irradiation, P(DPP-BT) NPs could suppress tumor growth due to the effect of PTT and PDT, but could not immediately diminish the tumor size. In contrast, under the same conditions, the combinational PTT/PDT/chemo treatment of P(DPP-BT/DOX) NPs groups showed much more efficient tumor inhibition than that of P(DPP-BT) NPs groups, confirming the higher synergetic therapeutic efficiency of P(DPP-BT/DOX) NPs in vivo. In addition, no apparent body weight loss or difference was discovered for all the groups, demonstrating the insignificant side effects of P(DPP-BT/DOX) NPs in vivo (Figure 7d).

To further evaluate the toxicity of P(DPP-BT/DOX) NPs in vivo, the major organs of mice (heart, liver, spleen, lung, and kidney) were also performed using hematoxylin and eosin (H&E) staining after therapy. As presented in Figure 8, no obvious lesions and side effects were detected in these organs. In contrast, noticeable cell apoptosis and necrosis could be found in the tumor treated with P(DPP-BT/DOX) NPs and laser (Figure S17, Supporting Information), further indicating the insignificant cytotoxicity of P(DPP-BT/DOX) NPs to normal tissues and remarkable damage to tumor cells.

Figure 7. In vivo combination therapy. a) IR thermal images of Hela-tumor-bearing mice under 730 nm laser irradiation after PBS, P(DPP-BT) NPs or P(DPP-BT/DOX) NPs injection. b) Tumor growth profiles of different groups of mice (*p < 0.05; **p < 0.01). c) Tumor images of different groups of mice after 14 days therapy. d) Body weight curves of different groups of mice.
3. Conclusion

In conclusion, a novel small-molecule dye DPP-BT with excellent NIR-I absorption and NIR-II fluorescence emission was successfully synthesized. The resulted small-molecule dye could not only act as a NIR-II fluorescence/PA dual-modal imaging contrast agent, but also serve as a PTT/PDT combined therapeutic agent. Based on the promising properties of DPP-BT, the single NIR laser triggered all-in-one phototheranostic nanoparticles P(DPP-BT/DOX) NPs were effectively constructed by encapsulating dye DPP-BT, chemotherapy drug DOX, natural PCM matrix with FA-functionalized amphiphile and amphiphilic lecithin, which showed outstanding biocompatibility, high photostability, and specific tumor targeting. Under a single NIR laser irradiation, effectively release of DOX from such NPs could be observed due to the NIR-induced photothermal effect of the DPP-BT. By tail vein injection of P(DPP-BT/DOX) NPs into Hela tumor-bearing mice, the tumor size and location could be accurately observed via NIR-II fluorescence/PA dual-modal imaging, which provides real-time monitoring of the therapeutic treatment and avoids unwanted damage to healthy tissue. From in vitro and in vivo therapy experiments, such NPs simultaneously presented remarkable antitumor efficacy through PTT/PDT/chemo combination therapy, which was triggered by a single NIR light. Furthermore, no noticeable toxic side effects of such NPs to normal organs were observed from histological examination. Overall, the single NIR laser triggered all-in-one phototheranostics developed in this work show irreplaceable advantages in precise cancer theranostics.

4. Experimental Section

Materials: (E)-3-(7-Bromobenzo[1,2,5]thiadiazol-4-yl)-2-cyanoacrylic acid and 2,5-bis(2-octyldodecyl)-3,6-bis[5-(trimethylstannyl)thiophene-2-yl]-2,5-dihydropyrrolo[3,4-c]pyrrole-1,4-dione were bought from Derthon Optoelectronic Materials Co., Ltd. DSPE-mPEG5000, lecithin, and DSPE-PEG-FA (MW:5000) were bought from Shanghai Yare Biotech, Inc. Lauric acid, stearic acid, and doxorubicin were obtained from Sigma–Aldrich. Calcein AM/PI stain kit was bought from Nanjing KeyGen Biotech Co. Ltd.

Preparation of DPP-BT NPs: The synthetic processes of DPP-BT are exhibited in Scheme S1, Supporting Information. DPP-BT in THF (1.0 mg mL\(^{-1}\), 1 mL) was quickly added into the mixed aqueous solution (4 mL H\(_2\)O) of DSPE-mPEG5000, lecithin, and DSPE-PEG-FA (MW:5000) were bought from Shanghai Yare Biotech, Inc. Lauric acid, stearic acid, and doxorubicin were obtained from Sigma–Aldrich. Calcein AM/PI stain kit was bought from Nanjing KeyGen Biotech Co. Ltd.

Photothermal Measurement of DPP-BT NPs: The temperature changes of DPP-BT NPs with different concentrations (0, 20, 40, 60, 80, and 100 µg mL\(^{-1}\)) under 730 nm laser irradiation (1 W cm\(^{-2}\)) were acquired by an IR image camera (FLIR E50; Estonia). In addition, 80 µg mL\(^{-1}\) DPP-BT NPs under irradiation with 730 nm laser at various power densities (0.25, 0.5, 0.75, and 1.0 W cm\(^{-2}\)) were also studied.

Photodynamic Measurement of DPP-BT NPs: DPBF in ethanol (2 × 10\(^{-5}\) mol L\(^{-1}\)) was added into DPP-BT NPs aqueous solution (1 × 10\(^{-5}\) mol L\(^{-1}\)) and lecithin (10 mg) under sonication. Then, THF was dislodged via argon blowing at surface of solution. Water-dispersed DPP-BT NPs were obtained after filtration.

Photothermal Imaging of DPP-BT NPs: The temperature changes of DPP-BT NPs with different concentrations (0, 20, 40, 60, 80, and 100 µg mL\(^{-1}\)) under 730 nm NIR laser (1 W cm\(^{-2}\)) were acquired by an IR image camera (FLIR E50; Estonia). In addition, 80 µg mL\(^{-1}\) DPP-BT NPs under irradiation with 730 nm laser at various power densities (0.25, 0.5, 0.75, and 1.0 W cm\(^{-2}\)) were also studied.
Preparation of P(DPP-BT/DOX) NPs: In order to obtain PCM NPs for NIR-triggered drug release, DSPE-PEG-FA (5 mg) and lecithin (10 mg) were dissolved in 5 mL ultrapure water and heated to 50 °C. The mixture of lauric acid and stearic acid (4:1 by weight, 2 mL, 4 mg mL−1 in THF), DPP-BT solution (2 mL, 2 mg mL−1 in THF) and DOX solution (1 mL, 2 mg mL−1 in DMSO) were mixed together and added dropwise into the preheated phospholipid solution. Next, the suspension was stored at about 4 °C for 10 min to solidify the fatty acid. Then, the solution was warmed up to ambient temperature. The organic solvents and unloaded molecules were removed by ultrafiltration (cutoff, 10 kDa, 10 000 rpm) for three times to obtain FA functionalized P(DPP-BT/DOX) NPs. Moreover, P(DPP-BT) NPs that just without DOX compared with P(DPP-BT/DOX) NPs were obtained according to the above method.

NIR-triggered DOX Release from P(DPP-BT/DOX) NPs: The P(DPP-BT/DOX) NPs in deionized water was exposed to a 730 nm NIR laser (0.8 W/cm²). At indicated time points, the release profiles of DOX were monitored using a fluorescence spectrophotometry. To further confirm the NIR-triggered release property, intracellular DOX release behavior was then studied. Hela tumor cells were incubated with P(DPP-BT/DOX) NPs for 12 h, and then irradiated with a 730 nm NIR laser (0.5 W cm⁻²). The fluorescence density of DOX (Ex/Em = 488 nm/590 nm) was monitored using a CLSM at 0, 5, and 10 min, respectively.

In vitro targeting ability of P(DPP-BT/DOX) NPs: Hela tumor cells and NIH3T3 normal cells (1 × 10⁵ cells per well) were selected as cell models to confirm the tumor targeting of FA functionalized P(DPP-BT/DOX) NPs and cultured in DMEM medium with 10% fetal bovine serum and 1% streptomycin-penicillin at 37 °C in 5% CO₂ for 24 h. Then, cells were divided into four groups: 1) Hela cells treated with FA functionalized P(DPP-BT/DOX) NPs, 2) Hela cells treated with P(DPP-BT/DOX) NPs without FA motif, 3) NIH3T3 cells treated with FA functionalized P(DPP-BT/DOX) NPs, and 4) NIH3T3 cells treated with P(DPP-BT/DOX) NPs without FA motif. After 12 h incubation, all of the cells were washed three times with PBS and then observed using a CLSM.

Cell Viability Assay: In vitro cytotoxicity of P(DPP-BT) NPs and P(DPP-BT/DOX) NPs against NIH3T3 cells and Hela cells were evaluated by MTT assay. Two kinds of cells were cultured with complete culture medium in a 96-well plate (1 × 10⁴ cells per well) at 37 °C for 24 h. Then, NIH3T3 cells were cultured in fresh medium with P(DPP-BT) NPs and P(DPP-BT/DOX) NPs. Hela cells were cultured in fresh medium with P(DPP-BT) NPs and P(DPP-BT/DOX) NPs, and divided into two groups: 1) without irradiation and 2) with 730 nm laser irradiation (0.8 W cm⁻², 5 min). After 12 h of co-cultivation, MTT (20 µL) was added into each well and further culturing for another 3 h. Next, the formed formazans were dissolved with DMSO after removed the supernatant, and the absorbance was monitored by microplate reader with untreated cells as control.

Live and Dead Cell Assay: Hela cells were treated with the same ways as above and co-stained with 1 µM Calcein AM (for live cells) and 4 µM PI (for dead cells) for 10 min. Fluorescence images of cells were obtained under an inverted fluorescence microscope.

In Vivo NIR-II Fluorescence Imaging and PA Imaging: For NIR-II imaging, the fluorescence imaging of P(DPP-BT/DOX) NPs with different concentrations (0.1, 0.2, 0.4, 0.6, 0.8, and 1 mg mL⁻¹) was recorded by an NIR-II fluorescence imaging instrument. For PAI, P(DPP-BT/DOX) NPs with different concentrations (0.25, 0.5, 0.75, and 1 mg mL⁻¹) were added into 600 µL agar tubes for PA signal detection using a PA instrument (Nexus 128, Endra Inc.).

In Vivo NIR-II Fluorescence Imaging and PA Imaging: For in vivo NIR-II imaging, after tail vein injection of P(DPP-BT/DOX) NPs (200 µL, 1 mg mL⁻¹) into Hela tumor-bearing mice, the real-time NIR-II imaging was acquired using a home built NIR-II fluorescence imaging instrument with an excitation wavelength of 808 nm at 0, 6, 12, 24, 48, and 72 h postinjection. For in vivo PA imaging, the mice were monitored by a PA instrument (Nexus 128, Endra Inc.) with a laser wavelength of 730 nm at indicated time points after injection.

In Vivo Combination Therapy: All animal experiments were performed with the permission of the Animal Ethics Committee of Simcere BioTech Corp., Ltd., according to the guidelines approved by Jiangsu Administration of Experimental Animals. In order to investigate the combination therapy effect of P(DPP-BT/DOX) NPs in vivo, Hela tumor-bearing mice were stochastically divided into five groups and given following treatments: 1) PBS, 2) P(DPP-BT) NPs (1 mg mL⁻¹, 200 µL), 3) P(DPP-BT) NPs (1 mg mL⁻¹, 200 µL) + laser, 4) P(DPP-BT/DOX) NPs (1 mg mL⁻¹, 200 µL), and 5) P(DPP-BT/DOX) NPs (1 mg mL⁻¹, 200 µL) + laser. After 24 h tail vein injection of NPs, the tumor areas of the mice were exposed to a 730 nm laser (1 W cm⁻²) for 10 min. Then, the tumor volume and body weight of the mice were recorded every 2 days within 14 days. After therapy, the mice were dissected, and the final tumor and major organs were further studied by H&E staining.

Statistical analysis: Statistical analysis was evaluated by unpaired Student’s t-test.

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

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Conflict of Interest
The authors declare no conflict of interest.

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