Mitochondria-Targeted Nanoplatforms For Enhanced Photodynamic Therapy Against Hypoxia Tumor

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Research

Keywords: hypoxic tumor, 3-bromopyruvate, respiration inhibition, photodynamic therapy, nanomedicine

Posted Date: August 6th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-757623/v1>

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Version of Record: A version of this preprint was published at Journal of Nanobiotechnology on December 1st, 2021. See the published version at <https://doi.org/10.1186/s12951-021-01196-6>.

Abstract

Background: Photodynamic therapy (PDT) is a promising therapeutic modality that can convert oxygen into cytotoxic reactive oxygen species (ROS) *via* photosensitizers to halt tumor growth. However, hypoxia and the unsatisfactory accumulation of photosensitizers in tumors severely diminish the therapeutic effect of PDT. In this study, a multistage nanoplatform is demonstrated to overcome these limitations by encapsulating photosensitizer IR780 and oxygen regulator 3-bromopyruvate (3BP) in poly (lactic-co-glycolic acid) (PLGA) nanocarriers.

Results: The as-synthesized nanoplatforms penetrated deeply into the interior regions of tumors and preferentially remained in mitochondria due to the intrinsic characteristics of IR780. Meanwhile, 3BP could efficiently suppress oxygen consumption of tumor cells by inhibiting mitochondrial respiratory chain to further improve the generation of ROS. Furthermore, 3BP could abolish the excessive glycolytic capacity of tumor cells and lead to the collapse of ATP production, rendering tumor cells more susceptible to PDT. Successful tumor inhibition in animal models confirmed the therapeutic precision and efficiency. In addition, these nanoplatforms could act as fluorescence (FL) and photoacoustic (PA) imaging contrast agents, effectuating imaging-guided cancer treatment.

Conclusions: This study provides an ideal strategy for cancer therapy by concurrent oxygen consumption reduction, oxygen-augmented PDT, energy supply reduction, mitochondria-targeted/deep-penetrated nanoplatforms and PA/FL dual-modal imaging guidance/monitoring. It is expected that such strategy will provide a promising alternative to maximize the performance of PDT in preclinical/clinical cancer treatment.

Introduction

Photodynamic therapy (PDT) is an evolving and promising therapeutic modality for its non-invasiveness, high selectivity, and low systemic toxicity compared to traditional chemotherapy and radiotherapy [1]. Encouragingly, PDT has proven clinically to be an efficient therapeutic option for the treatments of esophageal cancer, lung cancer, and brain cancer, *etc.* [2]. PDT usually involves three elements including a tumor-localizing photosensitizer (PS), oxygen, and light irradiation at a certain wavelength [3, 4]. Upon excitation, the PS transmits the energy from light to oxygen molecules in tumor microenvironment, consequently producing reactive oxygen species (ROS) which kill tumor cells directly *via* inducing apoptosis and/or necrosis [5, 6].

The therapeutic effect of PDT largely depends on the amount of oxygen in the tumor. However, most solid tumors are in a state of hypoxia, namely an inadequate oxygen supply ($pO_2 < 1.3\%$), which is mainly attributed to the deteriorating microenvironments, disturbed microcirculation, and aberrant tumor propagation [7, 8]. Clinical studies have proven that hypoxia is one of the main features that lead to the resistance to radiotherapy and chemotherapy [9–11]. So as to PDT, it is well known that PDT requires the presence of oxygen [6, 8, 12]. To make matters worse, these hypoxic cells are reported to be even more

resistant to ROS than aerobic cells [13, 14]. To solve this problem, some strategies have been designed to alleviate hypoxia in tumor environment, many of which are developed to directly deliver oxygen molecules (O_2) or hydrogen peroxide catalysts to tumor tissues with nanocarriers [12, 15–18]. Although with preliminary successes, there still remains insufficiencies, such as limited oxygen loading capacity, premature oxygen release and low oxygen production efficiency [19–21]. Bearing these in mind, aiming at the reduction of oxygen consumption is an insightful bypass to discourage tumor hypoxia.

Mitochondria-associated oxidative phosphorylation (OXPHOS) accounts for the predominant expense of oxygen [22–24]. Interruption on this loop can potentially suppress O_2 metabolism [25–28]. By introducing 3-Bromopyruvate (3BP), a small-molecule pyruvate mimetic and anticancer alkylating agent that has also been reported to be a biocompatible antitumor agent [29], mitochondrial respiratory chain will be blocked [30, 31]. Such perturbation in the electron flow induces the decrease in oxygen consumption and restores the oxygen level that counters hypoxia [32]. In the meantime, hexokinase, catalyzing the essential first step of glycolysis, is also an important target of 3BP [32, 33]. 3BP has pronounced inhibitory effects on multiple metabolic interactions, especially on the excessive glycolysis mediated by the over-expressed hexokinase type II (HK-II), which will lead to the collapse of adenosine triphosphate (ATP) production [33, 34]. The starvation caused by 3BP will malnourish tumor cells and turn them into less defended therapeutic measures [35, 36]. Furthermore, the mitochondrial HK-II (mt-HK-II) that tightly integrated with voltage dependent anion channel (VDAC) on the outer membrane of mitochondria is of great significance to maintain mitochondrial membrane potential [37, 38]. Thus 3BP-induced inhibition of mt-HK-II can cause dissipation of mitochondrial membrane potential and facilitate the mitochondrial apoptotic pathway [32, 39, 40]. The O_2 consumption suppression, starvation and enhanced mitochondria-associated apoptosis simultaneously render cancer cells more susceptible towards PDT. However, to properly design a carrier allowing on-target delivery remains a critical challenge.

To meet this need, in this study, we rationally constructed a core/shell structured poly(lactic-co-glycolic acid) (PLGA) nanoplatfrom, with 3BP encapsulated in the core and IR780 loaded in the shell (designated as 3BP@PLGA-IR780), for enhanced PDT (Scheme 1). IR780, a lipophilic small molecular with characteristically considerable absorption and fluorescence in the near-infrared (NIR) wavelength region [41, 42], is used as a PS. These nanoplatfroms could selectively accumulate and penetrate deeply in tumor tissues endowed by the nature of IR780 [43–45]. Specially, 3BP@PLGA-IR780 can retain preferentially at intracellular mitochondria, presenting an excellent location for organelle-targeted PDT [12, 41, 46, 47]. Mitochondrion, a vital complex intracellular organelle, plays key roles in energy metabolism, production of ROS associated with oxidative stress and regulation of apoptosis [48, 49]. Thus, mitochondria have gained recognition as a viable subcellular target to enhance efficacy of anticancer treatments [12, 50]. To optimize therapeutic precision, we took advantage of the fluorescence (FL) and photoacoustic (PA) imaging capability of IR780 to monitor the tumor accumulation of these nanoplatfroms [42, 51]. Thus, highly efficient tumor therapy can be achieved by concurrent oxygen consumption reduction, oxygen-augmented PDT, energy supply reduction, mitochondria-targeted/deep-penetrated nanoplatfroms and PA/FL dual-modal imaging guidance. In practice, systematic *in vitro* and

in vivo evaluations have been conducted in this work to demonstrate the efficacy of 3BP@PLGA-IR780 for amplified PDT.

Materials And Methods

2.1 Materials and Reagents

IR780 iodide, 3BP, and 2',7'-dichlorofluorescein diacetate (DCFH-DA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). PLGA-PEG-3.4k was purchased from Xi'an Ruixi Biological Technology Co., Ltd (Xi'an, China). Cell-Counting Kit-8 (CCK-8), calcein AM, and pyridine iodide (PI) were obtained from Daigang Biological Engineering Ltd., Co. (Jinan, China). Dichloromethane (CHCl₂) was purchased from Chuandong Chemical Co. Ltd (Chongqing, China). Nuclear dye 4,6-Diamidino-2-phenylindole (DAPI), enhanced ATP assay kit and 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) were purchased from Beyotime Biotechnology (Shanghai, China). Singlet Oxygen Sensor Green (SOSG) and MitoTracker® Deep Red FM were purchased from Thermo Fisher Scientific (Invitrogen). All other reagents were of or above analytical grade and used as received without further purification.

2.2 Synthesis of 3BP@PLGA-IR780

3BP@PLGA-IR780 were synthesized *via* a water-oil-water double emulsion protocol [52]. Briefly, 50 mg of PLGA and 1 mg of IR780 were dissolved in 2 mL of dichloromethane (CHCl₂), and 3BP (50 mg) dissolved in PBS (200 µL) was added to the above solution. The polymeric solution was emulsified with an ultrasonic processor (Sonics & Materials Inc., Danbury, CT, USA) operating at a power intensity of 100 W for 3 min (on 5 s, off 5 s). Then, 9 mL of polyvinyl acetate (PVA) solution was added into the formed emulsion. Then, the emulsion was further subjected to a probe sonication (50 W, on 5 s, off 5 s). Finally, 10 mL of isopropyl alcohol was added to the above solution, and the mixture was stirred mechanically to evaporate the CHCl₂ completely. The fabricated 3BP@PLGA-IR780 nanoplateforms were collected and stored at 4 °C for further use. The same procedure was made to other nanoplateforms, but differentiating the drug.

2.3 Characterizations of 3BP@PLGA-IR780

The morphology of 3BP@PLGA-IR780 was observed by transmission electron microscopic (TEM) (Hitachi H-7600, Japan) and scanning electron microscopic (SEM) (JEOL JSM-7800F, Japan). A laser particle size analyzer system (Nano, ZS90, Malvern instrument Ltd) was used to determine the size distribution and surface zeta potential of 3BP@PLGA-IR780. UV-vis–NIR absorption spectra of IR780 at different concentrations (0.125, 0.25, 0.5, 1.0, 2.0, 3.0, 4.0, and 5.0 µg/mL), and 3BP at different concentrations (0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL) were recorded using the SpectraMax Paradigm Multi-Mode Microplate Reader (Molecular Devices). The Drug Loaded Efficiency (LE) was then calculated by the following equation.

Drug Loaded Efficiency = (Initial Weight of Drug – unloaded Drug) / Initial weight of Drug

2.4 Cell Culture and 4T1 Tumor-Bearing Mice Model

Murine breast cancer line 4T1 cells were obtained from Shanghai Zhongqiaoxinzhou Biotechnology Co., Ltd (Shanghai, China). All-female BALB/c nude mice and *Kunming* mice were aged 4–6 weeks were purchased from Chongqing Medical University. All the experiments and procedures were performed under guidelines approved by the Institutional Animal Care and Use Committee of Chongqing Medical University. To establish 4T1 tumor-bearing mice models, 1×10^6 4T1 cells were suspended in 100 μ L serum-free medium, which was then injected subcutaneously to the flanks of the nude mice.

2.5 Intracellular Uptake and Mitochondria Location of 3BP@PLGA-IR780

The intracellular uptake of 3BP@PLGA-IR780 in 4T1 cells was detected by confocal laser scanning microscope (CLSM) (Nikon A1, Japan) and flow cytometry (CytoFLEX, USA). Briefly, 4T1 cells were seeded in a CLSM-specific dish at a density of 1×10^5 cells per well. After 24 h of incubation, the culture medium was replaced with a serum-free medium containing DiI labeled 3BP@PLGA or 3BP@PLGA-IR780. After different intervals of incubation (0.5 h, 1 h, 2 h, and 4 h), the cells were washed with PBS three times, fixed with 4% polyformaldehyde, and stained with DAPI. Then, the cells were observed by CLSM. Additionally, the quantitative analysis of intracellular uptake was further analyzed with flow cytometry. After 4 h of incubation with DiI-labeled 3BP@PLGA-IR780 or 3BP@PLGA, 4T1 cells were sequentially stained with MitoTracker®Deep Red FM for another 30 min. Then the cells were rinsed with PBS and observed with CLSM.

2.6 Mitochondrial Membrane Potential and ATP Measurement

Mitochondrial membrane potential was analyzed with JC-1. 4T1 cells were cultured in confocal cell-culture dishes as mentioned above. 3BP@PLGA-IR780 and 3BP@PLGA were added in culture media (PLGA = 200 μ g/mL), and the cells were further incubated for 24 h. The mitochondria were then stained with JC-1 for another 20 min. Cells were washed twice with PBS prior to CLSM imaging. Cellular ATP levels were detected with an Enhanced ATP Assay Kit performed according to the standard protocol.

2.7 Dissolved Oxygen Measurement

4T1 cells were seeded in 12-well plate (1.2×10^5 /well) and incubate overnight. These cells were treated with corresponding concentrations of drugs, and then 1 mL of liquid paraffin oil was added to each well. After incubating for 4 h in a 37°C incubator, the oxygen content in the culture medium was measured with a dissolved oxygen meter (Mettler Toledo, Switzerland).

2.8 Intracellular ROS Generation and *In Vitro* Cytotoxicity of 3BP@PLGA-IR780

For the detection of intracellular ROS, pre-cultured 4T1 cancer cells were incubated with 3BP@PLGA-IR780 or 3BP@PLGA for 4 h (PLGA = 200 μ g/mL). Subsequently, these cells were incubated with ROS probe DCFH-DA solution, followed by CLSM observation. In addition, the fluorescence intensities of each group were measured by flow cytometry.

To test the therapeutic effects of 3BP@PLGA-IR780, 4T1 cells were seeded in a 96-well plate (1×10^4 cells per well) and incubated for 24 h. Then, these 4T1 cells were subjected to following treatments: (i) control (PBS), (ii) Laser only (1.0 W/cm², 5 min), (iii) 3BP@PLGA, (iv) 3BP@PLGA-IR780, (v) PLGA-IR780 + Laser

(1.0 W/cm², 5 min), and (vi) 3BP@PLGA-IR780 + Laser (1.0 W/cm², 5 min). The corresponding PLGA concentrations were 200 µg/mL. All PDT treatments were conducted in ice-bath to cool down to exclude the photothermal effect. Finally, the cell viabilities were evaluated using a standard CCK-8 kit. Besides, flow cytometry was also employed to analyze cell apoptosis induced by different treatments. To discriminate live and dead cells, the treated cells were costained with calcein-AM and PI for the CLSM observation. The red fluorescence represents dead cells, while green fluorescence means alive cells.

2.9 *In Vivo* Biodistribution (FL Imaging) of 3BP@PLGA-IR780

For the detection of *in vivo* biodistribution of 3BP@PLGA-IR780, mice bearing 4T1 tumors were intravenously injected with 3BP@PLGA-IR780 saline solution (200 µL, the corresponding PLGA concentration was 10 mg/mL), *in vivo* FL imaging at varied time points was acquired with a Living Imaging System. 24 h after injection, mice were sacrificed to collect the tumor tissue and major organs for frozen section examination.

2.10 Deep Penetration of 3BP@PLGA-IR780

The deep penetration capability of 3BP@PLGA-IR780 was evaluated in 3D tumor spheroid models. To establish the 3D tumor models, 4T1 cells (5×10^4) were seeded in spheroid microplates for 7 days. The established 3D spheroids were treated with DiI-labeled 3BP@PLGA-IR780 or 3BP@PLGA. After co-culturing for 12 h, the tumor spheroids were rinsed twice with PBS and then analyzed by CLSM.

For the evaluation of *in vivo* penetration, 4T1 tumor-bearing mice were treated with DiI-labeled 3BP@PLGA-IR780 or 3BP@PLGA, and the transplanted tumors were harvested 24 h post-injection. The tumor masses were sliced and scanned by tomography. In addition, some tumor nodules were dissected for frozen-section examination, and the anti-CD31 antibody was used to stain blood vessels for CLSM observation.

2.11 PA Imaging of 3BP@PLGA-IR780

To investigate the performance of 3BP@PLGA-IR780 as a PA contrast agent, both *in vitro* and *in vivo* experiments were conducted on a Vevo LAZR System (Visual Sonics Inc., Canada). Firstly, 3BP@PLGA-IR780 suspension was stimulated by a PA laser with excitation wavelength ranging from 680 nm to 970 nm. Then, PA values of different concentrations of 3BP@PLGA-IR780 (the corresponding IR780 concentrations were 10, 20, 30, 40, 50 µg/mL) were measured, and corresponding PA images were attained. To evaluate the *in vivo* PA performance of 3BP@PLGA-IR780, 4T1 tumor-bearing mice were intravenously injected with 3BP@PLGA-IR780 solution. The tumor PA images were collected at different time points (0, 1, 2, 4, 6, 24, and 48 h).

2.12 Detection of Tumor Hypoxia Status

To detect the hypoxia status of the 4T1 tumor, tumor-bearing mice were randomly divided into three groups and injected with saline, 3BP@PLGA, and 3BP@PLGA-IR780, respectively. At 24 h post-injection, the tumor masses were collected and sliced for the detection of HIF-1α expression. Meanwhile, the

oxygenated hemoglobin level of tumors of each group were monitored by a PA imaging in oxy-hem mode. The average oxyhemoglobin saturation within tumors was analyzed by testing the ratio of oxygenated hemoglobin and deoxygenated hemoglobin. Furthermore, western blot analysis was operated to assess the expression of HIF-1 α in tumors.

2.13 In Vivo Synergistic Tumor Therapy

To evaluate the *in vivo* PDT efficacy of 3BP@PLGA-IR780, thirty 4T1 tumor-bearing mice were randomly separated into five groups as follows: (i) Control (intravenous injection of saline), (ii) Laser only (1.0 W/cm², 5 min), (iii) 3BP@PLGA (intravenous injection of 3BP@PLGA), (iv) 3BP@PLGA-IR780 (intravenous injection of 3BP@PLGA-IR780), (v) PLGA-IR780 + Laser (intravenous injection of PLGA-IR780 + laser exposure), and (vi) 3BP@PLGA-IR780 + Laser (intravenous injection of 3BP-PLGA-IR780 + laser exposure). The corresponding PLGA concentrations were 10 mg/mL and the injection volume was 200 μ L.

To exclude the photothermal effect, the laser irradiation was implemented for 40 s and subsequently followed by intervals to keep the tumor temperature under 42°C. The above irradiation was repeated for 15 cycles. The tumor volumes and bodyweight of mice were monitored every other day. At the end of treatments, the tumor tissues were dissected, weighed, and photographed. The main organs (heart, liver, spleen, lung, and kidney) and the tumor tissues were harvested and fixed in a 4% paraformaldehyde solution for histopathological analysis.

2.14 Biosafety Assessment

The biosafety assessment of 3BP@PLGA-IR780 was implemented on healthy Kunming mice. The mice were intravenously injected with 3BP@PLGA-IR780 (The corresponding PLGA concentrations were 10 mg/mL and the injection volume was 200 μ L), while the untreated mice were used as the control. The blood of the mice was collected at various time points (7 d, 15 d, and 30 d) for blood cell analysis and biochemical assays. Meanwhile, the major organs of the mice were collected and stained with Hematoxylin-eosin (H&E).

2.15 Statistical Analysis

All statistical analyses were performed with SPSS 20.0 software. Data were presented as mean $\pm$ standard deviation. The significance of the data is analyzed according to a Student's t-test: *P < 0.05, **P < 0.01, ***P < 0.001.

Results And Discussion

3.1 Synthesis and Characterization of 3BP@PLGA-IR780

3BP@PLGA-IR780 was prepared *via* a double-emulsion in absence of light, with hydrophobic 3BP encapsulated inside the core and lipophilic IR780 in the lipid bilayer. Only 3BP was added in synthesis of

3BP@PLGA. Typical SEM and TEM images showed that the as-prepared 3BP@PLGA-IR780 nanoplateforms presented a spherical structure, with an average diameter around 250 nm (Fig. 1a and 1b). Measured by dynamic light scattering (DLS), the average hydrodynamic diameter was 252.8 ± 61.76 nm, with a polydispersity index (PDI) of 0.159 (Fig. 1c). And the zeta potential of 3BP@PLGA-IR780 nanoplateforms was -16.2 ± 6.08 mV, which was desirable for *in vivo* application (Fig. 1d) [53]. The UV-vis-NIR spectra of IR780 and 3BP exhibited a concentration-dependent manner, with obvious characteristic bands at 789 nm and 326 nm (**Fig. S1** and **S2**). The loading efficiencies of IR780 and 3BP in 3BP@PLGA-IR780 were calculated to be $86.86 \pm 5.22\%$ and $12.60 \pm 2.58\%$, respectively.

Since the prepared nanoplateforms were designed for boosting PDT efficiency, which was closely correlated with ROS generation, *in vitro* ROS generation of 3BP@PLGA-IR780 was demonstrated by a singlet oxygen sensor SOSG fluorescence probe. At a fixed IR780 concentration of 10 $\mu\text{g/mL}$, the fluorescence intensities increased with prolonged laser irradiation duration, indicating the excellent ROS generation of 3BP@PLGA-IR780 (Fig. 1e). Moreover, the fluorescence intensity changes demonstrated concentration-depend manner at various concentrations of 3BP@PLGA-IR780 within the same irradiation duration (Fig. 1f). The changes in SOSG fluorescence intensities indicated the potential of 3BP@PLGA-IR780 as a dependable PS to mediate PDT against cancer.

3.2 Intracellular Uptake of 3BP@PLGA-IR780

Considering that these nanoplateforms may confront tremendous challenges including cell phagocytosis, which is detrimental to the subsequent therapeutic efficacy, in this work, we investigated the intracellular uptake behavior of 3BP@PLGA-IR780. 4T1 cells were incubated with Dil-stained 3BP@PLGA-IR780 or 3BP@PLGA suspension and subsequently observed by CLSM. As shown in Fig. 2a, intensive red fluorescence of 3BP@PLGA-IR780 was observed in 4T1 cells and increased significantly over incubation time, while relatively weaker red fluorescence was detected in the absence of IR780. Similarly, the results of flow cytometry analysis were consistent with CLSM images that the red fluorescence intensities in cells treated with 3BP@PLGA-IR780 were much stronger than that in the 3BP@PLGA group (Fig. 2b). The results suggested that IR780 played a key role in enhancing the intracellular uptake of the nano-systems and facilitating the accumulation of nanoplateforms in tumor tissue.

3.3 Mitochondria-Targeting Capability of 3BP@PLGA-IR780 and Synergetic Effects of 3BP

3BP is a highly effective mitochondrial-targeted anti-tumor drug, but the clinical application is limited to some degree due to the insufficient delivery and subsequent side-effects [29]. These multi-functional 3BP@PLGA-IR780 nanoplateforms offer an intriguing approach. IR780 has the unique ability to target tumor cells and mitochondria, not only enrich IR780-based nanoplateforms in the targeted region, but also enhances the focus of 3BP on mitochondria. The difference of subcellular localization of 3BP@PLGA-IR780 and 3BP@PLGA can be seen from Fig. 3a. As expected, 3BP@PLGA-IR780 were mainly internalized and retained in mitochondria after 4 h of incubation. In contrast, relatively poor accumulation of 3BP@PLGA in mitochondrial regions was observed (Fig. 3a), implicating the indispensable contribution

of IR780 in the mitochondrial-targeting behavior. Mitochondrion is a vital cell organelle, and any damage or interruption on mitochondria can be lethal to cells. The mitochondria-targeting of 3BP@PLGA-IR780 may represent a promising approach in this regard [12, 38, 50, 54].

Effects of 3BP on mitochondria mainly include the inhibition of mitochondrial HK-II and the interference with the mitochondrial respiratory chain to cause mitochondrial dysfunction, which further leads to over-increased membrane permeability and induces apoptosis following mitochondrial pathways [32]. HK-II is an important target of 3BP [32]. In addition to directly inhibiting the activity of HK-II, 3BP can also reduce the expression of HK-II [55]. The Western blot test results showed that the expression of HK-II decreased significantly after co-incubation with 3BP@PLGA or 3BP@PLGA-IR780, and the addition of IR780 made the expression of HK-II drop more significantly (Fig. 3b). The blocking effect to the HK-II on the outer mitochondrial membrane can further cause the separation of HK-II and VDAC, resulting in the shedding of HK-II from the outer mitochondrial membrane, which in turn causes a loss in the mitochondrial membrane potential and an increase in the permeability of the mitochondrial outer membrane.[56] JC-1 was applied as a fluorescence probe in tumor cells to reflect the change of mitochondrial membrane potential. Cells after different treatments were shown in Fig. 3c, compared to the control group, tumor cells treated with 3BP@PLGA group and 3BP@PLGA-IR780 showed significantly weak red fluorescence and strong green signal. It indicated a pronounced decline in mitochondrial membrane potential. In the meantime, 3BP can also perturb mitochondria-associated OXPHOS, which is predominant for O_2 consumption, by mainly inhibiting the activity of succinate dehydrogenase (SDH) on Complex II of mitochondria [30, 31, 55]. The disfunction of mitochondrial respiratory chain can lead to a plummeting of oxygen consumption. Oxygen electrode was employed to detect the oxygen consumption of 4T1 cells as a function of time (Fig. 3d). Results showed that compared to the control group, 3BP@PLGA reduced oxygen consumption of tumor cells, while 3BP@PLGA-IR780 achieved better inhibition effects (Fig. 3e).

Both the blockade of mt-HK-II and the interference of mitochondrial respiration can lead to severe mitochondrial dysfunction, and further induce the apoptosis of the mitochondrial pathway [39, 56]. The western blot results are shown in Fig. 3f. It can be seen that apoptotic correlation factors including Cyt c and Caspase-3 showed high expression. The results also displayed significant elevation in the ratio of Bax/Bcl-2 (Fig. 3f). The results further confirmed that 3BP activated the mitochondrial apoptotic signaling pathway. Based on the *in vitro* assessments, it is undoubtedly that 3BP showed reliable and efficient anti-tumor effects by functioning on mitochondria. More importantly, the therapeutic effect can be significantly improved with the assistance of IR780, enabling a mitochondria-targeted anti-tumor activity.

3.4 Intracellular ROS Generation and *In Vitro* Synergistic Therapeutic Effects of 3BP@PLGA-IR780

Given the encouraging ROS generation of 3BP@PLGA-IR780 in the aqueous solution, the ROS generation at the cellular level was further evaluated with a molecular probe DCFH-DA. After NIR laser irradiation ($\lambda = 808\text{nm}$, 1.0 W/cm^2) for 5 min, 4T1 cells in the PLGA-IR780 + Laser group emitted bright green

fluorescence, which represented the consequential production of ROS (Fig. 4a). In addition, the fluorescence intensity in 3BP@PLGA-IR780 + Laser group was higher than that in the PLGA-IR780 + Laser group, which resulted from the indispensable contribution of 3BP in relieving tumor hypoxia. Thus, this unique advantage of 3BP is expected to augment PDT efficacy. The quantitative results measured by flow cytometry were similar to CLSM observation that the strongest fluorescence intensity (67.81%) appeared in the group of 3BP@PLGA-IR780 + Laser (Fig. 4b). In contrast, almost no obvious fluorescence was observed in the groups of Laser Only, 3BP@PLGA and 3BP@PLGA-IR780 (2.15%, 6.20% and 7.40%, respectively). Such difference reveals that the assistance of 3BP in 3BP@PLGA-IR780 considerably improved the production of ROS in tumor cells.

Following the investigation of intracellular uptake and ROS generation, *in vitro* cytotoxicity of 3BP@PLGA-IR780 enhanced PDT against 4T1 cells was determined by a typical CCK-8 assay. As illustrated in Fig. 4c, a noticeable cell viability drop was observed when treated with 3BP@PLGA-IR780 + Laser and PLGA-IR780 + Laser at equivalent PLGA concentrations of 100 µg/mL and 200 µg/mL. Particularly, the cytotoxicity of 3BP@PLGA-IR780 was higher than that of PLGA-IR780 at equivalent concentrations of IR780, probably indicating the synergistic therapeutic effects of 3BP-induced mitochondrial respiratory depression. It is worth mentioning that 3BP could also perturb tumor metabolism *via* inhibition of HK-II and further induce starvation of ATP [33], which might make tumor cells more vulnerable to PDT damage [35, 36]. According to the *in vitro* assessments, compared to the control group, after 4 hours of incubation with 3BP@PLGA and 3BP@PLGA-IR780, 4T1 cells presented loss of intracellular ATP, and 3BP@PLGA-IR780 led to a more significant ATP decline (**Fig. S3**). These metabolic interruptions subsequently lead to strong cytotoxic activities on tumor cells. As shown in the absence of laser irradiation, 3BP@PLGA-IR780 still displayed more significant cell damage than 3BP@PLGA. Furthermore, flow cytometry analysis presented a similar trend in cellular damage (Fig. 4d). For instance, while assisted by laser irradiation, with equivalent PLGA concentrations at 200 µg/mL, PLGA-IR780 + Laser induced cell viability decrease approximately 60%, whereas, the cell viability remarkably dropped about 80% when treated with 3BP@PLGA-IR780 + Laser. In addition, the cell viability was visualized by CLSM. After various treatments, 4T1 cells were stained with calcein-AM and propidium iodide (PI) to identify the live and dead cells. The CLSM images displayed that almost all cells presented red fluorescence in the 3BP@PLGA-IR780 + Laser group, indicating severe cell apoptosis/necrosis, which exhibited effective targeted cell killing ability (Fig. 4e). The plausible mechanism for cytotoxicity might be explained as follows. On the one hand, 3BP could alkylate succinate dehydrogenase on the complex II in mitochondria respiration chain, causing an obvious decline in O₂ consumption and further alleviating hypoxia [32, 57]. On the other hand, the selectivity of IR780 enables a large number of 3BP@PLGA-IR780 accumulating in mitochondria, which makes ROS more lethal to cells.

3.5 *In Vivo* Distribution (FL Imaging) of 3BP@PLGA-IR780

The efficient accumulation of 3BP@PLGA-IR780 in tumor tissue is a prerequisite for the *in vivo* performance [58]. Fortunately, IR780 can selectively accumulate in tumor cells/tissues and emit fluorescence signals [59]. FL imaging was performed using 3BP@PLGA-IR780 as a contrast agent to

detect its biodistribution *in vivo*. 4T1 tumor models were intravenously administrated with 3BP@PLGA-IR780 suspension. As depicted in Fig. 5a, FL images were collected at pre-injection 1, 2, 4, 6, 24, and 48 h post-injection. It can be seen that the FL signals in the tumor site strengthened over time with a peak at 24 h (Fig. 5b). In addition to *in vivo* FL imaging, pathological examinations of major organs were also performed to further determine the biodistribution of 3BP@PLGA-IR780 *in vivo*. The tumor tissue, livers and spleens were harvested after 24 h post-injection of 3BP@PLGA-IR780. The data revealed a remarkable accumulation of 3BP@PLGA-IR780 in the tumor site, where the fluorescence signals were higher than that of the liver and spleen (Fig. 5c). In comparison, in the 3BP@PLGA treated group, the fluorescence signals extensively accumulated in the liver and spleen due to the phagocytosis effect of the reticuloendothelial system. These results showed that the unique tumor-targeting capability of IR780 endowed 3BP@PLGA-IR780 with efficient accumulation in the tumor sites. It indicates the enormous potential in selectively eradicating tumor cells and circumventing the systemic adverse effects.

3.6 Deep Penetration of 3BP@PLGA-IR780

Nanomedicine is of great significance in tumor regression on account of its biocompatibility and reduced toxicity. However, manufacturing nanomedicine that allows tumor accumulation and deep diffusion inside solid tumor tissues still needs continuous optimization [60, 61]. Taken into account the complicity of the tumor microenvironment, 3D tumor spheroid was employed to mimic the *in vivo* solid tumor as it could offer near-identical tumor microenvironmental characteristics (including pH, heterogeneous tumor perfusion, *etc.*) [62, 63]. As shown in Fig. 6a, red fluorescence originating from DiI-labeled 3BP@PLGA-IR780 was found to diffuse throughout the tumor spheroid, while 3BP@PLGA was found only distributing around the peripheral areas, suggesting that IR780 endowed the nanoplateforms desirable capability to penetrate deep inside the 4T1 spheroids more efficiently. In addition, we further explored the distribution of the nanoplateforms around the tumor vessels. The vasculature was stained by CD31 antibody (green fluorescence), and 3BP@PLGA-IR780 was labeled by DiI (Fig. 6b). It was found that substantial 3BP@PLGA-IR780 distributed farther away from the blood vessels and spread over the tumor tissue, while 3BP@PLGA mainly gathered inside or only adjacent to tumor vessels. The solid tumor tissue was then sliced for further observation. As shown in Fig. 6c, the red fluorescence of 3BP@PLGA only accumulated around the surface of the tumor tissue, while 3BP@PLGA-IR780 could widely distribute deep inside the tumor. It again confirmed the effective penetration of IR780.

3.7 *In Vitro* and *In Vivo* PA Imaging of 3BP@PLGA-IR780

Probes with imaging guidance ability have great practical benefits for enhancing the accuracy of anticancer therapy [64]. On account of the unique absorbance in NIR region, IR780 was considered to be an eligible PA contrast agent. PA imaging capability of 3BP@PLGA-IR780 nanoplateforms was tested both *in vitro* and *in vivo*. For PA laser excitation wavelength from 680 nm to 970 nm (interval = 5 nm), 3BP@PLGA-IR780 showed an optimal wavelength at 785 nm (**Fig. S4**). Meanwhile, the PA signals increased linearly with elevated concentrations of IR780 from 10 to 50 µg/mL (Fig. 7a). For *in vivo* evaluation, mice with 4T1-xenograft tumors were intravenously injected with 3BP@PLGA-IR780 suspension. As shown in Fig. 7b, an obvious PA signal highlighted the tumor region, strengthened over

time, and reached a maximum value at 24 h post-injection (Fig. 7c). The results confirmed the strong imaging capability of 3BP@PLGA-IR780 to differentiate the targeted regions from normal tissue.

3.8 Alleviation of Tumor Hypoxia Assisted by 3BP@PLGA-IR780

It has been reported that 3BP is capable of overcoming tumor hypoxia as it is able to suppress oxygen consumption of tumors *via* abolishing the excessive metabolism of tumor cells and simultaneously interfering with mitochondria-associated OXPHOS [55]. To verify this hypothesis, immunofluorescence of hypoxia-inducible factor (HIF-1 α), the corresponding western blot assay and the quantifying oxygenated hemoglobin with PA imaging system were conducted after various treatments. As shown in Fig. 8a and 8b, the expression of HIF-1 α was relatively high in the control group, implying that the tumor was in a hypoxia state. Comparatively, tumor hypoxia was greatly relieved after the treatment of 3BP@PLGA-IR780, as indicated by the weaker fluorescence in the tumor. Moreover, the results of western blot were consistent with immunofluorescence staining, demonstrating that 3BP@PLGA-IR780 induced a significant decrease in the expression of HIF-1 α (Fig. 8c and 8d). In particular, with the addition of IR780, the expression of HIF-1 α in tumor cells treated with 3BP@PLGA-IR780 was drastically less than that in cells treated with 3BP@PLGA only, indicating that the tumor-targeted properties of IR780 endowed 3BP@PLGA-IR780 with more effective accumulation in tumor cells.

Additionally, a comparison of oxygenated hemoglobin amount within tumor tissue further confirmed the function of 3BP in relieving hypoxia (Fig. 8e and 8f). Oxyhemoglobin signal significantly increased and diffused around the tumor region after injection of 3BP@PLGA-IR780, while in 3BP@PLGA group, the signal intensities showed only negligible increase compared to the control group. These results revealed that 3BP loaded in 3BP@PLGA had a noticeable yet limited capability of alleviating hypoxia in the tumor area. Only when in combination with IR780, 3BP@PLGA-IR780 was able to achieve more effective alleviation of tumor hypoxia, which could also be expected to ensure the efficiency of PDT.

3.9 *In Vivo* Synergistic Tumor Therapy

After confirmation of the mitochondria-targeting capability, deep penetration, and hypoxia relieve of 3BP@PLGA-IR780, the *in vivo* synergistic tumor therapy was further assessed. Bearing these properties in mind, 4T1 tumor-bearing mice were randomly divided into six treatment groups (n = 5): (i) Control (Saline); (ii) Laser only; (iii) 3BP@PLGA; (iv) 3BP@PLGA-IR780; (v) PLGA-IR780 + Laser; (vi) 3BP@PLGA-IR780 + Laser. When the tumor volume reached a size of 50–70 mm³, mice were intravenously injected with 3BP@PLGA, PLGA-IR780 or 3BP@PLGA-IR780 suspensions, followed by NIR laser irradiation (1.0 W/cm²) 24 h after injection. To exclude the photothermal effect, the laser irradiation was implemented for 40 s on and off to room temperature to keep the tumor temperature under 42 °C. The above irradiation was repeated for 15 cycles (Fig. S5). The tumor volumes of mice were monitored every other day during the therapeutic period (Fig. 9a), and the tumor tissues were excised and weighted at the end of treatment (Fig. 9b and 9c).

As a result, the weights of tumors resected from the mice in each group presented a similar trend as the tumor volumes. The tumors in the control group and laser only group grew steadily. In contrast, the tumor growth of mice treated with 3BP@PLGA was slightly suppressed, which might be due to the therapeutic effects induced by 3BP, such as metabolic disorder. Whereas in the PLGA-IR780 group, the tumor inhibition rate was less than 20% (Fig. 9d). Comparatively, obvious tumor growth inhibitions were observed both in the PLGA-IR780 + Laser group and 3BP@PLGA-IR780 + Laser group, implicating the outstanding therapeutic efficacy of PDT treatment. Importantly, the IR780 + Laser group induced about 53.67% inhibition rate, while the mice receiving 3BP@PLGA-IR780 + Laser therapy exhibited the highest tumor suppression ($80.83 \pm 10.29\%$), which might be resulted from the therapeutic synergy.

Furthermore, H&E, TdT-mediated dUTP Nick-End Labeling (TUNEL), and proliferating cell nuclear antigen (PCNA) staining on tumor sections were conducted to confirm the synergistic and amplified PDT effect (Fig. 10). As shown in H&E and TUNEL-stained tumor tissues, almost all cells suffered from severe apoptosis/necrosis in the 3BP@PLGA-IR780 group. The PCNA staining of tumor tissue followed a similar tendency and presented a significantly lower proliferation index in the 3BP@PLGA-IR780 + Laser group. Therefore, it is evident that combining the PS and metabolic poison could maximize the therapeutic efficacy of PDT.

3.10 Biosafety assessment of 3BP@PLGA-IR780

The relative weight of mice was also measured during the treatment period, which showed negligible changes (Fig. S6). H&E staining of the main organs was performed at the end of various treatments, and no obvious histopathological lesion was observed (Fig. S7). The above results indicated the satisfactory biosafety of 3BP@PLGA-IR780-mediated therapy. To further determine the *in vivo* biocompatibility of 3BP@PLGA-IR780, blood cell analysis and biochemical examination of blood were conducted in healthy mice after intravenous administration with 3BP@PLGA-IR780. The results of blood cell analysis and biochemical examination showed no obvious abnormality in the indicators of hepatotoxicity, nephrotoxicity, and blood cell count (Fig. 11a). In addition, the main organs (heart, liver, spleen, lung, and kidney) of the mice were harvested at different time points (7, 15, 30 d). There was no histopathological lesion found in these organs (Fig. 11b), showing rare systemic toxicity to mice at the treatment dose and confirming the high biocompatibility of 3BP@PLGA-IR780.

Conclusion

In conclusion, multifunctional 3BP@PLGA-IR780 nanoplatform was synthesized for highly efficient PDT and a series of synergy to strengthen the therapeutic effects. The nanoplatforms have been demonstrated to alleviate tumor hypoxia by decreasing physiological O_2 consumption. Besides, 3BP@PLGA-IR780 could interrupt the energy metabolism of tumor cells. Moreover, these nanoplatforms showed tumor-targeting, mitochondria-targeting and deep tumor penetration capabilities, which were favorable to therapy delivery. Moreover, PA/FL dual-modal imaging capabilities allows guidance and improves precision for PDT. In addition, the desirable biosafety of 3BP@PLGA-IR780 nanoplatforms

ensured the feasibility towards clinical transformation. This study provides an ideal strategy for cancer therapy by concurrent oxygen consumption reduction, oxygen-augmented PDT, energy supply reduction, mitochondria-targeted/deep-penetrated nanoplateforms and PA/FL dual-modal imaging guidance/monitoring.

Declarations

Consent for publication

Not applicable.

Availability of data and materials

All data analyzed during this study are included in this published article.

Acknowledgements

Not applicable.

Authors' contributions

JXW, YL, HG conceived and carried out the experiments. LZ, DW and PL deigned the research. JXW, YL, HG, XW and JH analyzed the data and drafted the manuscript. TTS, DZ, DW, ZGW and ZXW supervised the research. All authors read and approved the final manuscript.

Funding

This work was supported by the National Natural Science Foundation of China (Grant No. 81801711 and 81801717), Key Project of Chongqing Natural Science Foundation (cstc2019jcyj-zdxmX0019), Key Project of Application Development Plan of Chongqing (cstc2019jscx-dxwtBX0004) and Joint Project of Chongqing Municipal Science and Technology Bureau Health Committee (2021MSXM259).

Competing interests

The authors declare no conflicts of interest.

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Figures

Figure 1

Characterizations of 3BP@PLGA-IR780. (a) SEM image of 3BP@PLGA-IR780. (b) TEM image of 3BP@PLGA-IR780. (c) Hydrodynamic diameter of 3BP@PLGA-IR780. (d) Surface zeta potential of 3BP@PLGA-IR780. (e) Time-dependent ROS generation of 3BP@PLGA-IR780 detected by SOSG. (f) Concentration-dependent ROS generation of 3BP@PLGA-IR780 detected by SOSG.

Figure 2

(a) CLSM images of intracellular uptake of 3BP@PLGA-IR780 (the red represents nanoplateforms and the blue represents cell nuclei). (b) Flow cytometry analysis of phagocytosis rate of 3BP@PLGA or 3BP@PLGA-IR780.

Figure 3

(a) CLSM images of 3BP@PLGA-IR780 colocalization with mitochondria (the red represents nanoplateforms and the green represents mitochondria). (b) Western blot results of HK-II expression in 4T1 cells after various treatments. (c) CLSM images of JC-1 stained-4T1 cells after co-incubating with 3BP@PLGA and 3BP@PLGA-IR780 (red signal indicates normal membrane potential, green signal indicates declined membrane potential). (d) Schematic illustration of the measurement of dissolved O_2

with an oxygen electrode. (e) The changes of dissolved O₂ of cell culture medium after incubation with 3BP@PLGA or 3BP@PLGA-IR780 for 4 h. (f) The changes of apoptotic proteins in 4T1 cells after various treatments.

Figure 4

(a) Intracellular ROS generation of 3BP@PLGA-IR780 visualized by CLSM. (b) Flow cytometry analysis of ROS generation after different treatments. (c) Cell viability of 4T1 cells after various treatments. (d) Flow

cytometry analysis of apoptosis rates after receiving different treatments. (e) CLSM images of CAM/PI co-stained 4T1 cells after receiving various treatments, live and dead cells are stained green and red, respectively.

Figure 5

(a) In vivo FL imaging of 4T1 tumor-bearing mice after intravenous injection of 3BP@PLGA-IR780. (b) The corresponding FL intensity within tumor region with prolonged circulation time. (c) The biodistribution of 3BP@PLGA-IR780 in tumor region, liver and spleen (scale bar is 100 μ m).

Figure 6

(a) Intertumoral diffusion of 3BP@PLGA or 3BP@PLGA-IR780 in 3D tumor models (scale bar is 20 μm). (b) The distribution of 3BP@PLGA or 3BP@PLGA-IR780 around tumor vessels (scale bar is 50 μm). (c) distribution of nanoplatforms in different tumor sections (the interval is 100 μm).

Figure 7

(a) Concentration-dependent PA intensity of 3BP@PLGA-IR780. (b) In vivo PA images of 4T1 tumor tissue with prolonged injection time (0, 1, 2, 4, 6, 24, and 48 h). (c) The PA intensity data of tumor tissues at different time points.

Figure 8

(a) Immunofluorescent images of 4T1 tumor slices stained by HIF-1 α (scale bar is 50 μ m) and (b) quantitative analysis of tumor hypoxia. (c) Western blot results of HIF-1 α expression in tumors after treatment of 3BP@PLGA or 3BP@PLGA-IR780. (d) The corresponding quantitative analysis of HIF-1 α after different treatments. (e) Oxyhemoglobin saturation monitoring after injection of 3BP@PLGA or 3BP@PLGA-IR780. (f) Quantification analysis of sO₂ within tumor regions.

Figure 9

(a) Tumor growth curves of mice after various treatments. (b) Digital photographs of tumor nodes dissected from mice in six groups after kinds of treatments. (c) Weight of tumor tissues in each group at the end of treatment. (d) Tumor inhibition rate of mice after different treatments.

Figure 10

H&E, TUNEL and PCNA staining of tumors after different treatments.

Figure 11

(a) Hematological and blood biochemistry analysis of healthy mice after intravenous injection of 3BP@PLGA-IR780. (b) H&E staining of major organs (heart, liver, spleen, lung and kidney) of mice post injection of 3BP@PLGA-IR780.

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