Photodynamic Therapy Combined with Antihypoxic Signaling and CpG Adjuvant as an In Situ Tumor Vaccine Based on Metal–Organic Framework Nanoparticles to Boost Cancer Immunotherapy

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Photodynamic therapy (PDT) usually aggravates tumor hypoxia, which promotes the survival and metastasis of residue cancer cells; furthermore, although PDT-induced immunogenic death of cancer cells can induce host antitumor responses, such responses are generally weak and not enough to eliminate the residue cancer cells. Here, metal–organic framework (MOF)-based nanoparticles to combine PDT, antihypoxic signaling, and CpG adjuvant as an in situ tumor vaccine to boost host anticancer responses after PDT are designed. The MOF-based nanoparticles are self-assembled from H$_2$TCP and zirconium ions with hypoxia inducible factor (HIF) signaling inhibitor (ACF) and immunologic adjuvant (CpG) loading, and hyaluronic acid (HA) coating on the surface. The final nanoparticles (PCN-ACF-CpG@HA) can specifically target cancer cells overexpressing CD44 receptor though HA; the aggravated hypoxic survival signaling after PDT can be blocked by ACF to inhibit the HIF-1α induced survival and metastasis. With the help of CpG adjuvant, the tumor associated antigens generated from PDT-based cancer cell destruction can initiate strong antitumor immune responses to eliminate residue cancer cells. Taken together, a novel in situ immunostimulatory strategy is designed to synergistically enhance therapeutic effects of PDT by activating host antitumor immune-responses both in vitro and in vivo, which may have great potential for clinical translation in future.

1. Introduction

Photodynamic therapy (PDT) has been proved as a noninvasive therapeutic modality in the treatment of local cancers.[1] It could generate reactive oxygen species (ROS) to induce hyper-oxidative stresses to ablate tumor cells under laser irradiation. However, due to the continuous O$_2$ consumption and limited O$_2$ supply after the microvasculature destruction by PDT treatment, tumor region always suffers serve hypoxia, which could trigger the activation of hypoxia inducible factor 1 (HIF-1) by stabilizing the subunit HIF-1α in residue cancer cells.[2] Some evidences have shown that activation of HIF-1 pathway could induce survival signaling in cancer cells by regulating downstream gene transcription, such as VEGF, MMP9, and BCL2, to facilitate recurrence and metastasis, which was significantly associated with patients’ poor prognosis.[3]

To alleviate those phenomena, directly blocking HIF-1 mediated survival pathway has emerged as a potential powerful approach for enhancing the therapeutic effect of PDT.[4] Acriflavine (ACF) is known as an antibacterial and anticancer drug with clinic safety that directly inhibits the dimerization of HIF-1α or HIF-2α with HIF-1β to block transcriptional activity.[4c] Recent study showed that ACF could decrease survival and growth of chronic myeloid leukemia and reduce the leukemia stem cell maintenance both in vitro and in vivo by pharmacological inhibiting HIF-1α as well as its downstream targets.[5] Weijer et al. synthesized liposome-delivered zinc phthalocyanine nanoparticle for combining PDT and immunostimulatory effect. However, the combination of PDT and CpG adjuvant is very limited in vivo. Here, we designed a novel in situ immunostimulatory strategy to combine PDT, antihypoxic signaling, and CpG adjuvant to boost host antitumor immune responses after PDT to eliminate residue cancer cells.
and ACF treatment in human perihilar cholangiocarcinoma cells, and the results showed that ACF could enhance PDT efficacy via inhibiting HIF-1α and topoisomerases I and II.[6] Although the combination of PDT and HIF-1α inhibitor could significantly improve the therapeutic efficiency,[7] some invisible tumor microlesions still are very difficult to completely eradicate, which usually would cause short time recurrence.

Cancer immunotherapy by reactivating and maintaining the patient’s own immune system to control and kill cancer cells, has emerged as an effective and promising cancer therapeutic strategy in recent years.[8] It has been reported that PDT treatment could induce the apoptosis or necrosis of cancer cells, then release tumor associated antigens (TAAs) to trigger certain level of antigen-specific immune responses for fighting against tumor.[9] While, such responses generally are not strong enough to eradicate residual tumor cells due to poor antigen presenting efficiency in dendritic cells (DCs) for activating host antitumor immune responses. In addition, the activation of HIF-1α signaling after PDT treatment also would promote the immunosuppression of immune cells in the tumor microenvironment to improve tumor immune evasion.[10] Hence, boosting adaptive immunity after PDT treatment by immuno-adjuvant to enhance antigen-specific responses, then further induce long-term immuno-memory to against residual tumor cells is a potential effective strategy for completely eradicating tumor cells.[11] For instance, Chen et al. reported a poly (lactic-co-glycolic) acid system, that coencapsulated with indocyanine green and toll-like-receptor-7 adjuvant imiquimod, to serve as a cancer vaccine for boosting immune responses after photothermal ablation of tumor.[12] Synthetic oligodeoxynucleotides with unmethylated cytosine-phosphate-guanine (CpG), a Toll-like receptor 9 (TLR9) agonist, is a short DNA segment which could trigger innate and adaptive immunity and induce cytokine secretion by priming immature DC cells. Recently, CpG has been proved as an excellent immunotherapeutic agent and immuno-adjuvant against solid cancer in many clinical trials.[13] However, CpG usually needs multiple injections or relatively high doses for stimulating DC maturation.[14] Thus, it is necessary to develop novel strategy to effectively delivery CpG to synergize with PDT for efficiently activating the immune system to achieve comprehensive therapeutic outcomes.[15]

Metal–organic framework (MOF), as a new generation of hybrid porous material with excellent biocompatibility, biodegradability, and suitable size, has been widely applied in nanomedicine including drug delivery and PDT.[16] Owe to the poor solubility, most traditional photosensitizers often need to change pharmaceutical formulations for drug loading into nanoparticles.[17] while such loading efficiency is usually very limited and also suffers from premature leaking behaviors.[18] Interestingly, the MOF nanoparticles also could be directly self-assembled from metal ions and photosensitizers by carefully selecting the composition elements.[19] Comparing with photosensitizers that encapsulated into nanoparticles, the photosensitizer directly formed nanoscale MOFs have extremely high photosensitizer loading efficiency with excellent photoxygenation efficiency and minimal self-quenching of the excitation energy.[20] In additional, the porous inner structure of photosensitizer formed MOFs also could use to load other small molecular drugs for combining PDT with other therapeutic modality.[21]

Herein, to effectively eliminate residue tumor cells after PDT, we have designed photosensitizer directly formed MOF nanoparticles to integrate PDT, antihypoxic signaling and immunologic adjuvant as in situ tumor vaccine to improve PDT efficiency and achieve long-term therapeutic outcomes by boosting the host antitumor immuno-responses. The MOF nanoparticles PCN224 (named as PCN) were self-assembled from H2TCPP (photosensitizer) and Zirconium ions by coordination effects, with HIF signaling inhibitor (ACF) and immunologic adjuvant (CpG) loading as well as the hyaluronic acid (HA) coating on the surface (PCN-ACF-CpG@HA). Here designed MOF nanoparticles could specifically target to tumor cells via HA, effectively block the HIF-1 mediated survival and metastasis signaling after PDT, and efficiently boost the host antitumor immuno-responses to achieve long-term therapeutic effects. Overall, the here reported novel MOF system might provide a promising strategy for synergistically enhancing the therapeutic effects of PDT.

2. Results and Discussion

The preparation procedure and the working principle of PCN-ACF-CpG@HA to integrate PDT, antihypoxic signaling and CpG adjuvant as in situ tumor vaccine were shown in Figure 1. The PCN-ACF-CpG@HA nanoparticles were fabricated through the self-assembly of photosensitizer (H2TPCP) and Zirconium ions (PCN), followed by encapsulating ACF in the pore and subsequently coating with CpG and HA on the out surface. The loading capacities of ACF and CpG in PCN-ACF-CpG@HA were 8.36 wt% and 1.45 wt%, respectively, which were assessed by high performance liquid chromatography (HPLC) and agarose gel electrophoresis (Figure S1, Supporting Information); while the HA content was determined to be 45.35 wt% through calculating the loading amount by subtracting the free HA mass.

As shown in Figure 2a, the morphology of PCN-ACF-CpG@HA examined by transmission electron microscopy (TEM) showed that the obtained nanoparticles had round-shape and well-dispersed in an aqueous solution with an average size around 105.4 nm. In addition, dynamic light scattering was used to determine the hydrodynamic diameter of PCN-ACF-CpG@HA (Figure 2b). The results showed that these nanoparticles exhibited an average size of 117.5 nm, which was similar with the results obtained by TEM. To investigate the surface charge of PCN-ACF-CpG @HA, the zeta potential was determined. Compared with the PCN (2.85 mv) and PCN-ACF (11.93 mv), PCN-ACF-CpG@HA in phosphate buffer saline (PBS) reversed to a negative surface charge (~20.27 mV), indicating the successful ACF encapsulation and CpG/HA coating (Figure 2c).

Considering that the PCN has strong ability to generate ROS under 670 nm laser irradiation, we next evaluated the ROS production ability of PCN-ACF-CpG@HA by ABDA probe in PBS solution. As illustrated in Figure 2d; and Figure S2 (Supporting Information), the absorbance of ABDA in PCN-ACF-CpG@HA gradually decreased, and it was similar with the absorbance change profile of ABDA in PCN under laser irradiation (670 nm, 0.1 W cm−2). These results suggested that the addition of ACF and CpG did not influence the
Figure 1. Schematic illustration of the preparation procedure and the working principle of PCN-ACF-CpG@HA to integrate PDT, antihypoxic signaling, and CpG adjuvant as in situ tumor vaccine.

Figure 2. Preparation and characterization of PCN-ACF-CpG@HA. a) A TEM image of PCN-ACF-CpG@HA nanoparticles (scale bar = 100 nm). b) Hydrodynamic diameters of PCN, PCN-ACF, PCN-ACF-CpG@HA nanoparticles. c) Zeta potential of PCN, PCN-ACF, PCN-ACF-CpG@HA nanoparticles. All data were presented as mean ± SD (n = 3). d) The absorbance change of ABDAB (100 × 10^−6 M) after photodecomposition by ROS generation in PCN-ACF-CpG@HA upon the 670 nm laser (0.1 W cm^−2). All absorbances were normalized at the zero-time point of irradiation. e) In vitro cumulative release of ACF from PCN-ACF-CpG@HA under indicated conditions at the predetermined time points ranging from 0 to 72 h. All data are presented as mean ± SD (n = 3). f) In vitro cumulative release of CpG from PCN-ACF-CpG@HA under indicated conditions at the predetermined time points ranging from 0 to 72 h. All data are presented as mean ± SD (n = 3).
ROS generation ability of PCN-ACF-CpG@HA. In addition, the drug release behaviors of PCN-ACF-CpG@HA with or without laser irradiation (670 nm, 0.1 W cm⁻², 5 min) were also evaluated at the predetermined time points in the presence or absence of HAase. As shown in Figure 2e,f, the final released percentages of ACF and CpG from PCN-ACF-CpG@HA after 72 h were remarkably increased from 21% to 63% and from 12% to 44% in the presence of HAase, respectively. Meanwhile, the release behaviors of ACF and CpG from PCN-ACF-CpG@HA were not significantly affected by laser irradiation. These results indicated that the release of ACF and CpG from PCN-ACF-CpG@HA could be selectively regulated by HAase, which was overexpressed in tumor cells.

Furthermore, the ROS generation ability and oxygen consumption of PCN-ACF-CpG@HA in H22 cells were also studied by using DCFH-DA and [Ru(dpp)₃]Cl₂ as the fluorescence indicator, respectively. As the presence of ACF may interfere the observation of DCFH-DA fluorescence, the PCN-CpG@HA was used to conduct relative experiments instead of the PCN-ACF-CpG@HA. As shown in Figure 3a, the PCN-CpG@HA exhibited excellent ROS generation ability with strong DCFH-DA fluorescence and simultaneously consumed significant amount of oxygen with strong [Ru(dpp)₃]Cl₂ fluorescence, indicating the ROS generation of PCN-CpG@HA would aggravate the hypoxia of microenvironment. It has been reported that severe hypoxic microenvironment in tumor region could trigger the activation of HIF-1α signaling and subsequently promote tumor cell survival and metastasis. Therefore, we assessed the expression of HIF-1α in H22 cells treated with PCN-ACF-CpG@HA under 670 nm laser irradiation. As illuminated in Figure 3b, HIF-1α was significantly upregulated in H22 cells treated with PCN-CpG@HA or PCN-ACF-CpG@HA with laser irradiation comparing with the cells treated with PBS or ACF alone, due to PDT-induced hypoxic signaling. Since ACF mainly inhibits antihypoxic signaling by blocking the dimerization of HIF-1α with HIF-1β rather than by affecting the expression of HIF-1α, we further detected the expression of HIF-1α regulated and survival/metastasis-associated crucial genes (VEGF, BCL-2, and MMP9) in H22 cells with indicated treatments under laser irradiation. Significantly, all of those three genes were downregulated in H22 cells treated with PCN-ACF-CpG@HA under laser irradiation when compared with the cells treated with PCN-CpG@HA under laser irradiation (Figure 3c), demonstrating that PCN-ACF-CpG@HA could significantly inhibit the HIF-1α mediated cell survival and metastasis signaling after PDT therapy. We also could clearly see the down regulation of these 3 genes only with ACF treatment under laser irradiation (Figure 3c); this is due to certain degree of HIF-1α expression on H22 cells even under normoxia, therefore the ACF also could down-regulate the HIF-1α signaling in such conditions.

HA receptor CD44 was frequently overexpressed in many solid tumor cells and known as potential cancer therapeutic target, which could be specifically targeted by HA.[22] H22 cells also showed overexpression of CD44 receptor on membrane, while no obvious expression of CD44 receptor on normal cells, such as NIH3T3 cells was observed (Figure S3a, Supporting Information). To further investigate whether the HA-coating of
PCN-ACF-CpG@HA could facilitate the specific targeting and enhance the cellular uptake. The H22 cells were incubated with PCN-ACF-CpG or PCN-ACF-CpG@HA for 2 and 4 h, respectively. After that, the fluorescence of Cy3 labeled CpG, ACF, and PCN were imaged by confocal microscopy, indicated by yellow, green, and red signals, respectively. As shown in Figure 4a; and Figure S3b (Supporting Information), the H22 cells treated with PCN-ACF-CpG@HA exhibited stronger fluorescence signals of all three components than the H22 cells treated with PCN-ACF-CpG. Meanwhile, as expected, the cellular uptake efficacy of PCN-ACF-CpG@HA was remarkably decreased in the H22 cells with HA preblocking or CD44 antibody preblocking, and the NIH3T3 cells with negative CD44 receptor expression (Figure 4a; and Figure S3c, Supporting Information). Taken together, these results demonstrated excellent HA-mediated cellular uptake behavior in cancer cells.

Considering that low toxicity or nontoxicity is a prerequisite for biomedical applications of nanomaterials, we first investigated the dark toxicity of our drug carrier PCN@HA in H22 cells by cell viability assay (CCK8). As shown in Figure 4b, the PCN@HA showed good biocompatibility and almost no influence on the cell survival of H22 cells with viable cells above 95% at a relatively high dose of 32 µg mL\(^{-1}\) after 24 h incubation. Next, we further analyzed the synergistic anti-tumor effect of PCN-ACF-CpG@HA in vitro (Figure 4c). The results showed that the PCN-ACF-CpG@HA exhibited dose-dependent cytotoxicity and PDT effects under laser irradiation. Furthermore, the H22 cells treated with PCN-ACF@HA or PCN-ACF-CpG@HA under laser irradiation at 32 µg mL\(^{-1}\) both showed significantly improved synergistical effects of combining PDT with antihypoxic signaling on killing cancer cells, resulting in significantly lower cell viability (16% and 11%) comparing to H22 cells treated with PCN@HA and laser irradiation (PDT alone, 47%), PCN-ACF@HA without laser (ACF alone, 69%), as well as PCN-ACF-CpG@HA without laser (ACF alone, 75%). Similar results were also observed in fluorescence images of propidium iodide (PI) stained H22 cells with similar treatments as mentioned above (Figure 4d). These results clearly demonstrated the remarkably enhanced antitumor effects of PCN-ACF-CpG@HA through inhibiting HIF-1α survival signaling by ACF after PDT treatment, which could be beneficial for its in vivo applications.

It has been reported that PDT could trigger the initial generation of antitumor T cells by producing tumor associated antigens from apoptotic tumor cells.[23] As well known, DCs served as the major antigen presenting cells for T cell priming to induce cytotoxic T lymphocytes in tumor microenvironment.[24] DCs could undergo maturation after acquiring exogenous and endogenous substances, such as TAAs and then present antigens on major histocompatibility complex (MHC) at the cell membrane. After that, MHC-antigen complexes on DC membrane surface could be specific recognized by T cell receptors for priming T cells, and subsequently triggering T cell activation to kill tumor cells.[25] Thus, we wonder whether PCN-ACF-CpG@HA could also serve as an immune adjuvant to trigger and enhance the antitumor immune responses after PDT therapy at tumor site. We first investigated the DC stimulation effects of our nanoparticles in vitro by Trans-well system. As shown in Figure 5a, the H22 cells were incubated in the upper chamber and then treated with various conditions as

Figure 4. The cellular uptake and synergistically combined PDT and antihypoxic signaling effects of PCN-ACF-CpG@HA in vitro. a) Confocal images of H22 cells incubated with PBS, PCN-ACF-CpG, PCN-ACF-CpG@HA, PCN-ACF-CpG@HA with HA preblocking and with CD44 antibody preblocking for 4 h (scale bar = 50 µm). b) Cell viability of H22 cells treated with different concentration of PCN@HA without laser irradiation. Data are presented as mean ± SD (n = 3). c) Cell viability of H22 cells treated with different concentrations of PCN@HA, PCN-ACF@HA, as well as PCN-ACF-CpG@HA with or without laser irradiation (670 nm, 0.1 W cm\(^{-2}\), 5 min). Data are presented as mean ± SD (n = 3, ***P < 0.001). d) Fluorescence images of H22 cells stained by PI, after indicated treatment as follows: PBS, PCN@HA, PCN-ACF@HA, PCN-ACF-CpG@HA with or without laser irradiation (670 nm, 0.1 W cm\(^{-2}\), 5 min).
indicated; after that, the immature DCs were coincubated in the lower chamber for 48 h. Then the maturation of DCs was determined by flow cytometry after staining CD11c, CD80, and CD86 and then analyzed by flow cytometry. All data are presented as mean ± SD (n = 3). c–e) The statistical analysis of the percentages of CD11c+/CD86+/CD80+, CD11c+/MHCII+, and CD11c+/CD317+ cells in DC population after indicated treatments. Data are presented as mean ± SD (n = 3, **P < 0.01, ***P < 0.001). f–h) Cytokine levels of TNF-α, IFN-γ, and IL-12p70 in medium on day 3 after indicated treatments with or without laser irradiation (670 nm, 0.1 W cm⁻², 5 min). Data are presented as mean ± SD (n = 3, **P < 0.01, ***P < 0.001).
in the PCN-ACF-CpG@HA treated group with laser irradiation when compared with other groups (Figure 5d,e; and Figure S4a,b, Supporting Information).

Moreover, the secreted levels of immune-related pro-inflammatory cytokines by DCs, such as IL-12p70, IFN-γ and TNF-α in the medium were also analyzed by Enzyme linked immunosorbent assay (ELISA). As shown in Figure 5f–h, the levels of these 3 cytokines were obviously higher in the PCN-ACF-CpG@HA treated group with laser irradiation than other groups. Taken together, our results suggested that PDT could induce the release of TAAs from tumor site and combining with CpG-coating nanoparticles as adjuvant could trigger and effectively enhance DC maturation.

Inspired by the high therapeutic efficiency and the antitumor immune responses in vitro, we next evaluated the synergistic antitumor efficiency of PCN-ACF-CpG@HA in H22 cell bearing Balb/c mice (Figure 6a). The overexpression of CD44 receptor in H22 tumors were first confirmed by immunohistochemistry (Figure S5, Supporting Information). Then, the in vivo distribution of these nanoparticles in major organs and tumors were observed at indicated time points after intravenous injection by the IVIS Series Preclinical In Vivo Imaging System. As shown in Figure 6b, the strong fluorescence signal of ACF from PCN-ACF-CpG@HA could be clearly observed at the tumor site after 4 h of injection, and there was no obvious increase at 8 h or 12 h postinjection. Therefore, the H22 bearing mice were randomly divided into eight groups and received various treatments as indicated by intravenous injection, and then the tumors were irradiated by 670 nm laser after 4 h of injection. As shown in Figure 6c,d, according to the tumor volume change profiles, the tumor volumes in PBS-treated group with or without laser irradiation both showed a rapidly increase over 16 d, while the PCN@HA treated mice with laser irradiation and the PCN-ACF@HA treated mice without laser irradiation exhibited certain tumor growth delay due to PDT or ACF mediated antihypoxic signaling alone. Furthermore, the PCN-ACF@HA treated mice with laser irradiation displayed obvious tumor inhibition, due to the combination of PDT effects and ACF mediated antihypoxic signaling. Most significantly, the tumor growth of PCN-ACF-CpG@HA treated mice with 670 nm laser irradiation showed most dramatic tumor suppress comparing with any other groups, most likely owing to the synergize of PDT/ACF and in situ tumor vaccine mediated antitumor immunotherapy. Furthermore, the weight of treated mice showed no significant change during the period of treatment, indicating the excellent biocompatibility and minimal toxicity (Figure 6d).

Furthermore, the histopathology and immunochemistry examinations were performed to confirm the therapeutic effects. The representative tumor tissues collected from above treated groups on day 3 after treatment were analyzed. Histochemical staining showed that the mice with PCN-ACF-CpG@HA treatment and laser irradiation displayed the most obvious cell destruction with lowest cell density in tumor tissues than any other groups, and these results were well consistent with the Ki67 (a marker of cell proliferation) staining analysis and the tumor volume results, confirming that the tumor growth has been effectively inhibited in the PCN-ACF-CpG@HA treated group with laser irradiation (Figure 6e). As ACF encapsulated inside our nanoparticles could significantly inhibit hypoxia-induced cell survival and metastasis signaling after PDT treatment in vitro, we also further confirmed such inhibition effects in vivo by detecting the expression of VEGF, MMP9, and BCL-2 in tumor tissues. Compared with the expression of these 3 genes in the PBS-treated groups with or without laser irradiation, the tumors treated with PCN@HA with laser irradiation showed stronger expression due to PDT-induced hypoxic signaling activation, whereas their expression were significantly inhibited in tumor regions in the presence of ACF in our nanoparticles (such as PCN-ACF@HA and PCN-ACF-CpG@HA). These results imply that the PCN-ACF-CpG@HA exhibited excellent therapeutic effect against tumor by combination of PDT, antihypoxic signaling, and immunotherapy.

After the PDT and antihypoxic signaling treatment as mentioned above, the tumor cells could be lysed into large amount of tumor debris to release TAAs. With the help of CpG adjuvant in our nanoparticles as the in situ tumor vaccine, the released TAAs could trigger strong antitumor immune responses to further eliminate residue unskilled tumor cells. Therefore, we also carefully investigated the downstream triggered antitumor immune responses by our nanoparticles, including DC mature in tumors and tumor draining lymph nodes (TDLNs) and the cytokine levels at tumor site after 3 d of treatment. As shown in Figure 7a,b, a slight increase of DC maturation in TDLNs could be observed in the PCN@HA with 670 laser irradiation (44.05%) and the PCN-ACF@HA without laser group (44.37%), when compared with the PBS group (36.76%), PBS with laser group (34.30%), and PCN@HA without laser (37.33%), because of the released TAAs by PDT or ACF treatment could simulate DC maturation at certain level. Significantly, such efficacy was further improved in the PCN-ACF@HA with laser irradiation group (51.40%) and the PCN-ACF-CpG@HA without laser irradiation group (51.45%), due to the combined immuno-stimulate mechanism of PDT plus ACF or ACF plus CpG, respectively. Most significantly, the PCN-ACF-CpG@HA with 670 nm laser irradiation could induce highest percentage (61.21%) of DC maturation, owing to the synergistic immuno-stimulate effects of PDT, ACF, and CpG.

Furthermore, we also harvested the tumor tissue homogenates after different treatments and measured the IL-12p70, IFN-γ and TNF-α levels by ELISA assay. The results showed that the secretion levels of these 3 cytokines in the PCN-ACF-CpG@HA treated mice with laser irradiation were the highest at the tumor site comparing with any other groups (Figure 7c–e), which were well consistent with the data in DC maturation in TDLNs. CD8+ T cells and CD4+ T cells, which could be primed and activated by mature DC, were major effector cells for antitumor immune responses to kill tumor cells. Thus, we further assessed the number of infiltrating T cells at tumor site after 16 d of treatment. As expected, more infiltrating CD8+ T cells and CD4+ T Cells at the tumor site were identified in the PCN-ACF-CpG@HA treatment group with laser irradiation comparing with any other treatment groups (Figure 7f). Taking together, these results suggested that our PCN-ACF-CpG@HA could act as in situ tumor vaccine to generate strong antitumor immune responses in vivo, due to the effective tumor-associate antigens release, immune adjuvant function of CpG and the inhibition of HIF-1 signaling pathway.
The in vivo distribution results of the PCN-ACF-CpG@HA demonstrated that our nanoparticles were mainly enriched in the tumor, although some accumulation in the heart and liver also could be observed (Figure 6b). To further evaluate the potential systemic toxicity of our PCN-ACF-CpG@HA, biochemical tests were performed at the day 16 after injection.

Figure 6. The synergistically combined PDT, antihypoxic signaling and antitumor immunotherapy effects of PCN-ACF-CpG@HA in vivo. a) Schematic illustration of in vivo tumor therapy. b) The biodistribution of PCN-ACF-CpG@HA in excised major organs and tumors that were observed at the indicated time points after intravenous injection by the IVIS Series Preclinical In Vivo Imaging System (\(E_s = 488\) nm, \(E_m = 506\) nm). c) Tumor volumes of Balb/c mice after indicated treatments. All data are presented as mean ± SD (\(n = 4\), \(\ast\ast\ast\)P < 0.001). d) Mean body weights (±SD) of Balb/c mice in different groups after treatment (\(n = 4\)). e) The representative H&E staining, as well as Ki67 immunohistochemical staining images of tumors after 3 d of treatments as indicated (scale bar = 50 µm). f) The expression of VEGF, BCL2, and MMP9 in tumor tissues by QPCR analysis after receiving indicated treatments. Data are presented as mean ± SD (\(n = 3\), \(\ast\ast\ast\)P < 0.001).
The results showed that no significant differences of these tested biochemical indexes (including ALB, MTP, UREA, CREA, GLU, TG, CHOL, ALT, AST, and CK) were noticed among the normal mice, the H22 tumor bearing mice treated with PBS and the H22 tumor bearing mice treated with PCN-ACF-CpG@HA (Figure S6, Supporting Information). These results demonstrated the biosafety of our PCN-ACF-CpG@HA. Furthermore, in vivo toxicity in major organs after various treatments was also assessed by hematoxylin and eosin (H&E) staining. As shown in Figure S7 (Supporting Information), no obvious organ damages were observed in all treated groups, further proving the lower toxicity and biosafety of our PCN-ACF-CpG@HA. While the long-term toxicity to liver and heart during any clinical translation should be further considered in the future since our nanoparticles were also partially accumulated in these organs. Taken together, these results clearly demonstrated that our designed PCN-ACF-CpG@HA nanoparticles could integrate PDT and antihypoxic signaling to act as an in situ tumor vaccine, which could display excellent synergistic antitumor effects with minimized side effects both in vitro and vivo.

3. Conclusion

In summary, we have constructed a multifunctional MOF system (PCN-ACF-CpG@HA nanoparticle) to synergistically combine PDT, antihypoxic signaling, and in situ immunostimulant for cancer therapy. The PCN in PCN-ACF-CpG@HA nanoparticles exhibited excellent PDT efficiency to kill tumor cells; interestingly, the ACF could further inhibit the HIF-1α mediated survival and metastasis signaling after PDT; most importantly, the immunologic adjuvant CpG combined with TAAs generated from tumor site after PDT could serve as in situ tumor vaccine...
to further boost strong host anticancer immune responses to eliminate residue cancer cells. By integrating these strategies, our designed novel MOF system could significantly improve the cancer therapeutic efficiency both in vitro and in vivo. Taken together, our here presented novel strategy is enable to serve as a promising way for synergistically and immunologically enhancing the therapeutic responses of PDT.

4. Experimental Section

Materials: Tetakis (4-carboxyphenyl) porphyrin (H$_2$TCP) was purchased from Frontier Scientific (USA). CpG-ODNs (5'-TCCATGACGTTCCTGACGTT-3') was purchased from BioSunya (Fuzhou, China). Zirconium oxychloride octahydrate (ZrOCl$_2$·8H$_2$O), benzoic acid (BA), ACF, HA, Hyaluronidase from bovine testes (HAase), BBI Life Sciences (Shanghai, China). Molecular Technologies (Tokyo, Japan). Paraffin oil was purchased from Sigma-Aldrich (Fuzhou, China). Zirconium oxychloride octahydrate (ZrOCl$_2$·8H$_2$O), benzoic acid (BA), ACF, HA, Hyaluronidase from bovine testes (HAase), BBI Life Sciences (Shanghai, China). Molecular Technologies (Tokyo, Japan). Paraffin oil was purchased from Sigma-Aldrich (Fuzhou, China).

Preparation of PCN-ACF-CpG@HA: PCN was synthesized according to the previous study.[20] Briefly ZrOCl$_2$·2H$_2$O (300 mg, 0.93 mmol), H$_2$TCP (100 mg, 0.13 mmol), and BA (2.8 g, 23 mmol) were dissolved in 100 mL of anhydrous N,N-Dimethylformamide (DMF) and subsequently stirred for 5 h at 90 °C under the dark environment. Then, the product was collected and washed with anhydrous DMF for 3 times at room temperature. After that, ACF (10 mg) and PCN (10 mg) were dissolved into 10 mL deionized water and further stirred for 12 h at room temperature under the dark environment. Next, the PCN-ACF nanoparticles were collected by centrifugation and washed with deionized water for 3 times.

To optimize the mass ratio of CpG and PCN-ACF, the same amounts of CpG (0.6 μg) were added into different amounts of PCN-ACF, and then sonicated for 5 min to obtain PCN-ACF-CpG with various wt/wt ratios (1:0, 1:10, 1:20, 1:30, 1:40, 1:50, 1:60, 1:70) at the equal volume. Then the loading mass ratios of PCN/ACF and CpG were evaluated by agarose gel electrophoresis and photographed using a Gel Doc XR imaging system (Bio-Rad, Lab, Hercules, CA). Following the optimized mass ratio (1:40, Figure S8, Supporting Information), the CpG was loaded to PCN-ACF under sonication; then a deionized water solution of HA (20 mg, 1 mL) was immediately added to PCN-ACF-CpG (10 mg, 4 mL) drop-wisely, and further stirred for 4 h at room temperature in the dark. Afterward, the final nanoparticles PCN-ACF-CpG@HA was collected by centrifugation and washed with deionized water for 3 times to remove uncoated HA. The PCN@HA and PCN-ACF@HA nanoparticles were synthesized with similar method as the control.

Then the supernatant from centrifugation and washing effluent was collected and dried in a vacuum oven to get the residual HA mass. The loading efficiency of HA was calculated as follows: (Mt−Ms)/Mt × 100, where Mt represented the total HA mass and Ms represented the residual HA mass in the supernatant. The encapsulation capacity of ACF and CpG in PCN-ACF-CpG@HA nanoparticles were assessed by HPLC and agarose gel electrophoresis analysis, respectively. Briefly, 200 μL heparin sodium (15 mg mL$^{-1}$) was added into 200 μL PCN-ACF-CpG@HA nanoparticle solution (4 mg mL$^{-1}$) to completely release ACF and Cy3-CpG. Then, the ACF amounts were analyzed by HPLC on an Agilent 1260 Infinity LC (Agilent Technologies, Inc. Germany) with a ZORBAX eclipse plus C18 column (4.6 × 100 mm, 3.5 μm, USA). The mobile phase was consisted by a mixture of acetonitrile and phosphate buffer saline (45:55, v/v), and the injection volume was 50 μL and the flow rate was 1 mL min$^{-1}$ with the UV detector wavelength at 442 nm. Finally, the loading amounts of ACF were calculated through the standard calibration curve. While, the loading amounts of Cy3-CpG were evaluated by agarose gel electrophoresis through the standard calibration curve.

Nanoparticle Characterization: The morphology of PCN-ACF-CpG@HA was characterized by TEM (FEI Company, Hillsboro, OR). The size and zeta potential of PCN-ACF-CpG@HA were detected by a Zetasizer NanoZS system (Malvern Instruments, Southborough, MA).

Assessment of the ROS Generation Ability and In Vitro ACF and CpG Release: The ROS generation ability of PCN-ACF-CpG@HA was detected by a ROS indicator (ABDA). Briefly, the PCN-ACF-CpG@HA mixture with 100 × 10$^{-6}$ m ABDA was added into a 96-well plate, using deionized water as the blank control. The absorbance of ABDA was recorded by a Spectra Max M5 microplate reader (Molecular Devices, USA) after 670 nm laser irradiation (0.1 W cm$^{-2}$) at the predetermined time. The experiments were independently repeated three times.

For ACF release assay, 250 μL PCN-ACF-CpG@HA (4 mg mL$^{-1}$) with or without laser irradiation, together with 100 μL HAase (5 mg mL$^{-1}$), were added into 650 μL PBS in the dialysis bag (3500 Da cutoff), respectively. The system was dialyzed against 9 mL PBS with a shaking speed of 220 rpm at 37 °C. At predetermined time intervals, 1 mL of medium was collected while another 5 mL of fresh PBS was added to the dialysis medium. The cumulative released amounts of ACF from PCN-ACF-CpG@HA were determined according to above-mentioned ACF quantitative method. The experiments were independently repeated three times.

For the CpG release kinetics study, 250 μL PCN-ACF-CpG@HA (4 mg mL$^{-1}$) with or without laser irradiation, together with 100 μL HAase (5 mg mL$^{-1}$), were added into 650 μL PBS, respectively. Then the mixture was added in to a dialysis bag (8000–14 000 Da cutoff) and was dialyzed against 2 mL PBS with a shaking speed of 220 rpm at 37 °C; at the predetermined time intervals, 1 mL of dialysis buffer was collected, while another 1 mL of fresh PBS was added into the dialysis medium. The cumulative released amounts of Cy3-CpG from PCN-ACF-CpG@HA were determined by agarose gel electrophoresis through the standard calibration curve.

Cell Culture: The mouse hepatocellular carcinoma H22 cells (ATCC, Manassas, VA) and mouse embryonic fibroblast NIH3T3 cells were cultured in Dulbecco’s Modified Eagle Media (DMEM) medium supplemented with 10% fetal bovine serum at 37 °C in a humidified incubator containing 5% CO$_2$.

Murine bone-marrow derived DCs were isolated from Balb/C mice according to the previously reported method. After bone marrow collection, red blood cell lysis buffer was added to lysis the red blood cells. The remaining cells were washed twice and cultured in RPMI 1640 medium containing recombinant murine GM-CSF (10 ng mL$^{-1}$) and 1% antibiotics (penicillin-streptomycin). After 48 h incubation, adherent cells were collected. After incubation for another 72 h, nonadherent and loosely adherent cells were harvested as immature DCs for downstream experiments.

The ROS Generation and Oxygen Consumption Assay of PCN-ACF-CpG@HA In Vitro: As the fluorescence of ACF may interfere the observation of DCFH-DA, PCN-CpG@HA was used to conduct the related experiments to instead the PCN-ACF-CpG@HA. Briefly, the H22 cells were seeded in a 96-well plate (4 × 10$^4$ cells per well) and further incubated for 24 h. Then, 50 μg mL$^{-1}$ PCN-CpG@HA was added into corresponding wells and cultured for another 4 h. Then the culture medium was substituted for fresh culture medium containing 40 × 10$^{-6}$ m DCFH-DA or 20 × 10$^{-6}$ s [Ru(dpp)$_3$]Cl$_2$, and further incubated for another 30 min. After washing with PBS for 2 times, the cells were reincubated in fresh medium and irradiated under 670 nm laser (0.1 W cm$^{-2}$, 5 min). Then, the cells were visualized at fluorescence microscope immediately (Zeiss Axio Vert.A1, USA). PBS and PCN-CpG@HA without laser were used as control. The experiments were independently repeated three times.

Assessment of HIF-1α Expression In Vitro by Immunofluorescence Staining: H22 cells were cultured in 96-well plate at a density of 4 × 10$^4$ cells per well for 12 h and then treated as follows for 4 h: 1) PBS, 2) ACF only, 3) PCN-CpG@HA, and 4) PCN-ACF-CpG@HA. All wells were sealed with paraffin oil before light irradiation (670 nm, 0.1 W cm$^{-2}$, 5 min). After another 12 h incubation, the cells were fixed with 4% polyformaldehyde for immunofluorescence staining by HIF-1α antibody (Santa Cruz, USA) and Alexa Fluor 546 secondary antibody (Molecular Probes, USA). Then the cells were imaged by confocal laser scanning microscopy (CLSM) (Zeiss LSM780, USA). The experiments were independently repeated three times.
Assessment of the Expression of VEGF, MMP9, and BCL-2 In Vitro and In Vivo by Real-Time Quantitative PCR: Total RNA was isolated from H22 cells or tumor tissues using TRIzol reagent (Invitrogen, USA). Then reverse transcription were performed by using Transcriptor first strand cDNA synthesis kit (Invitrogen, USA) according to the manufacturer’s instructions. The primers for VEGF, BCL-2, MMP9, and internal control gene (GAPDH) were designed as follows: VEGF-F: GCACATAGAAGATGACCTTCC, VEGF-R:RCCGGTGCTCAGAAAGGCT; BCL-2-F:GCACATAGAAGATGACCTTCC, BCL-2-R:GAACATGAAAGGTCAG; MMP9-F: CTGCAGAGAGATGGAGCTTCC, MMP9-R:RCCGGTGCTCAGAAAGGCT; GAPDH-F: GCCACATAGAGAGAATGAGCTTCC, VEGF-R:CTCCGGTGCTCAGAAAGGCT; the H22 cells preincubated 3 times to remove free nanoparticles, and then exposed to 670 nm laser ACF-CpG@HA. Afterward, the H22 cells were washed with PBS for upper chamber were received indicated treatments for 4 h as follows: 1) PBS, 2) PBS with light irradiation, 3) PCN@HA, 4) PCN@HA with light irradiation, 5) PCN-ACF-CpG@HA, 6) PCN-ACF@HA with light irradiation, 7) PCN-ACF-CpG@HA, and 8) PCN-ACF-CpG@HA with light irradiation. The irradiation (670 nm, 0.25 W cm\(^{-2}\)) was conducted after 4 h of injection. After 12 h of treatment, the representative tumor in each group was further excised and embedded by paraffin for immunohistochemistry evaluation of HIF-1\(\alpha\). After treatment for 3 d, the representative tumor in each group was further excised and embedded by paraffin for H&E staining and immunohistochemistry evaluation of Ki67. During the treatment, the relative body weight and tumor volume of mice were recorded every 2 d. The tumor volume was calculated by the following equation: tumor volume (mm\(^3\)) = width\(^2\) × length/2. The experiment was end at the 16 d. All mice were sacrificed and major organs (including heart, liver, spleen, lung, and kidney) were collected from the mouse orbit and diluted for analysis. The cytokines that are important for the antitumor responses including tumor necrosis factor (TNF-\(\alpha\)), interferon gamma (IFN-\(\gamma\)), and IL-12p70 were detected by using ELISA kits (Boster Biological Technology, China) according to the manufacturer’s instructions. The experiments were independently repeated three times.

Tumor Xenograft Establishment and In Vivo Antitumor Effect of PCN-ACF-CpG@HA: The male Balb/C mice of 4–6 weeks old were purchased from Beijing Vital River Laboratories Animal Technology Co., Ltd (Beijing, China). All experiments were performed in compliance with the Animal Ethics Committee of Mengchao Hepatobiliary Hospital of Fujian Medical University. 100 µL solution of H22 cells in PBS including 5 × 10\(^5\) cells were subcutaneously injected into the right axillary of each Balb/C mouse for establishing the tumor xenograft model. For ex vivo fluorescence imaging and biodistribution of PCN-ACF-CpG@HA, the H22 bearing mice were injected with 10 mg kg\(^{-1}\) PCN-ACF-CpG@HA by tail vein injection and sacrificed at 0, 4, 8, and 12 h. Tumor and major organs (including heart, liver, spleen, lung, and kidney) were taken out and imaged to analyze the enrichment and biodistribution of PCN-ACF-CpG@HA throughout the body by observing AC fluorescence.

When the volume of tumor reached around 100 mm\(^3\), the H22 bearing mice were divided into eight groups (n = 4) randomly and treated as follows at the first day: 1) PBS, 2) PBS with light irradiation, 3) PCN@HA, 4) PCN@HA with light irradiation, 5) PCN-ACF-CpG@HA, 6) PCN-ACF@HA with light irradiation, 7) PCN-ACF-CpG@HA, and 8) PCN-ACF-CpG@HA with light irradiation. The irradiation (670 nm, 0.25 W cm\(^{-2}\), 10 min) was conducted after 4 h of injection. After 12 h of treatment, the representative tumor in each group was further excised and embedded by paraffin for H&E staining and immunohistochemistry evaluation of Ki67. During the treatment, the relative body weight and tumor volume of mice were recorded every 2 d. The tumor volume was calculated by the following equation: tumor volume (mm\(^3\)) = width\(^2\) × length/2. The experiment was end at the 16 d. All mice were sacrificed and the tumor were excised and imaged. Furthermore, the representative tumor in each group was further excised and embedded by paraffin for immunohistochemistry evaluation of CD4 and CD8 T cell infiltration; major organs (including heart, liver, spleen, lung, and kidney) were also obtained and analyzed by H&E.

In Vivo Immune Responses: After treatment for 3 d, the blood samples were collected from the mouse orbit and diluted for analysis. Then, TDLNs were also isolated to examine the antitumor immune responses. The amount of cytokines including IL-12p70, TNF-\(\alpha\), and IFN-\(\gamma\) inside tumor tissues were examined by ELISA kits according to the manufacturer’s instructions. Subsequently, the matured dendritic cells (CD11c\(^{+}\), CD80\(^{+}\), and CD86\(^{+}\)) in TDLNs were analyzed by flow cytometry according to above-mentioned methods. The experiments were independently repeated three times.

Statistical Analysis: The quantitative data and sample size of all results were expressed as mean ± standard deviation (SD) and numbers (n), respectively. For in vitro test, 3–4 independent replicates in each group were applied to calculate the mean ± SD; for animal experiments, finally 4 mice were used in each group to calculate the mean ± SD of data representing therapeutic efficiency. The statistical significance among different groups was evaluated by using one-way analysis of variance (ANOVA) conducted on the software GraphPad Prism 6 (GraphPad Software, Inc., USA). Results with \(*p < 0.05\), \(*\*p < 0.01\), and \(*\*\*p < 0.001\) were considered as statistically significant.

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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