Integration Strategy of ROS Boosting and Antioxidation Inhibiting Initiates Ferroptosis to Enhance Phototherapeutic Effect on Tumor

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Abstract

Conventional phototherapy is often limited by hypoxia, introducing oxygen generators is the common method to relieve it, but the antioxidant path of tumor cell was inevitably initiated. Herein, by integrating oxygen generator (MnO_2) and inhibitor of Nrf2 (brusatol) into one nanoplatform, we strive to relieve hypoxia and inhibit the antioxidation simultaneously. Hypoxia was relieved for the triggered decomposition of MnO_2 by endogenous H_2O_2 and it directly strengthened photodynamic therapy (PDT) through boosting reactive oxygen species (ROS) generation. Moreover, high level ROS greatly enhanced the efficacy of photothermal therapy (PTT) by attacking heat shock proteins(HSP). Antioxidant defense was prevented by brusatol through inhibiting the expression of Nrf2. Importantly, MnO_2 and brusatol collaboratively induced ferroptosis through raising oxidation, remarkably promoting tumor curative effect. Both *in vitro* and *in vivo* experiments demonstrated the strengthened therapeutic effects of synergistic PDT/PTT, highlighting the great promise of the synergistic modulation strategy with a nanomedicine to overcome the drawbacks of phototherapy.

Introduction

Cancer, one of the most severe diseases, seriously endangers human health and is becoming the leading cause of death worldwide. Traditional therapies, including chemotherapy, surgery, and radiotherapy, are the main protocols for the treatment of cancer. Given their inherent flaws and nonideal therapeutic results, including systemic toxicity, multidrug resistance, high risk of recurrence, postoperative complications, and patient suffering, great technical efforts should be made to seek alternative treatments.^[1–3]

Great active research progress has been made to solve the problems. Typically, nanotherapeutics with targeting capability, responsiveness, and controlled release have been designed and applied in clinical practice to avoid systemic toxicity and side effect.^[4] Alternatively, phototherapies, noninvasive tumor ablation approaches that include photothermal therapy (PTT) and photodynamic therapy (PDT), have been investigated extensively for cancer treatment owing to their benefits of low cumulative toxicity, high selectivity, reduced long-term mortality, fewer complications, and high therapeutic selectivity.^[5, 6] Despite great efforts, it is still difficult to find a single treatment method that can circumvent all of the inherent shortcomings.^[7]

The combination of PTT and PDT offers a more promising strategy to eliminate tumors, which not only achieves synergy but also maintains a minimum number of side effects.^[8, 9] However, their inherent flaws, such as off-target photosensitizers, insufficient reactive oxygen species (ROS) levels, interference by heat shock protein (HSP), limited tissue penetration depth, and activated antioxidant defense systems, have not yet been resolved. Thus, the maximum treatment efficacy is largely restricted.^[10, 11] In particular, ROS in the tumor microenvironment (TME) play a crucial role in either PTT or PDT. PTT adopts photothermal conversion agents (PTAs) to generate on-site hyperthermia upon laser irradiation to combat solid tumor.^[12] However, under hyperthermal stimulation, cancer cells will produce HSPs to resist heat damage,

leading to the survival of deep tumors.^[13] It has been reported that ROS can damage HSPs due to their oxidation properties.^[14] Thus, improving ROS production is beneficial to enhance the efficacy of PTT by attacking HSPs.

In addition, PDT employs photosensitizers, such as chlorine e6 (Ce6), to generate cytotoxic ROS under light irradiation to combat tumors.^[15, 16] A large number of nanosystems have been synthesized for PDT, but their therapeutic effects are still not satisfactory. It is largely due to the low ROS yield and its easy scavenging in the TME.^[17] Hypoxia in the TME is the main factor that contributes to low ROS yield. Numerous strategies have been developed to relieve hypoxia, such as transmitting oxygen to the tumor site and degrading glucose to produce oxygen.^[18, 19] Among these strategies, in situ catalytic oxygen generation by nanoparticles (NPs), such as platinum NPs,^[20] Prussian blue NPs^[21] and MnO₂ NPs,^[22] has attracted great interest owing to its simple technical operation. MnO₂ acted as the oxygen generator has been extensively investigated to relieve hypoxia for the excellent characteristics, including its low toxicity, high drug loading capacity, and sensitivity to H₂O₂/H⁺.^[23, 24] Additionally, it reported that high concentrations of MnO₂ NPs have considerable photothermal conversion properties.^[25]

It needs to be emphasized is that high level ROS usually triggers the activation of Nrf2, which dissociates from the Nrf2/Keap1 complex in cells. The activated Nrf2 regulates the expression of its downstream target genes, such as HO-1, GPX4, and FTH, which contribute to the antioxidant capabilities of tumor cells and thus inhibit the effect of phototherapy.^[26, 27] To avoid antioxidant protection of tumor cells, it is essential to reduce the expression of Nrf2 as well as its downstream target genes. Brusatol was reported to be an efficient inhibitor of Nrf2. Thus, the presence of brusatol can reduce the antioxidant capacity of tumor cells and maintain an ideal level of ROS, efficiently alleviating the resistance of cancer cells to phototherapy.^[28, 29]

Based on the above facts, it is extremely attractive to combine the expanded production of ROS and weakened antioxidation of tumor cells for enhanced synergistic phototherapy. To the best of our knowledge, this strategy based on dual-mechanism regulation of synergistic phototherapy has rarely been reported. In this work, polyethylene glycol-folic acid (PEG-FA)-modified polydopamine (PDA)-coated hybrid NPs (composed of a silica core with brusatol incorporation and a MnO₂ layer with Ce6 adsorption), denoted as brusatol/silica@MnO₂/Ce6@PDA-PEG-FA (BSMCPF) NPs, were rationally designed and synthesized to achieve this prospective therapy.

As shown in Scheme 1, BSMCPF NPs can efficiently flow into tumor tissue due to their prolonged blood circulation conferred by PEG and enhanced permeation and retention (EPR) effects and then be phagocytosed by tumor cells via FA-mediated active targeting. The PDA coating, the main PAT in PTT, not only prevents premature release of the loaded molecule but also facilitates postmodification of PEG and FA. In the acidic TME, NPs collapse due to the responsive degradation of MnO₂ to H⁺, H₂O₂ and GSH, triggering the release of guest molecules (Ce6 and brusatol) and the generation of O₂. Thus, abundant O₂

contributes to hypoxia relief, which elevates $^1\text{O}_2$ production during PDT. Notably, the enhanced ROS production can not only attack tumor cells directly through PDT but also damage HSPs to strengthen PTT. Importantly, the incorporated brusatol could inhibit the expression of Nrf2 and downstream targeted genes, thus decreasing the antioxidation and antihyperthermia capacity of the tumors and prompting ferroptosis, greatly enhancing the synergistic phototherapy effects.

Results And Discussion

Synthesis and characterization of BSMCPF NPs

The procedure for the synthesis of BSMCPF is illustrated in Fig. 1A. Due to its excellent biocompatibility, simple synthetic process and porous properties, silica was used as the carrier of brusatol. However, brusatol has poor solubility in aqueous solution and cannot be adsorbed onto the hydrophilic surface of silica by the conventional method. Therefore, brusatol is incorporated into a network of porous silica by a modified coassembly strategy rather than a postadsorption method (**Figure S1**).^[30] Specifically, the amphiphilic silane coupling agent hexyltriethoxysilane (HTES) is prehydrolyzed into a surfactant molecule with a nonpolar hexyl group and polar silanol groups. Once brusatol was added, the nonpolar hexyl tail interacts with the lipid-soluble brusatol via hydrophobic interactions to form a thermodynamically stable system similar to micelles. The polar silanol group then crosslinks with the silicic acid precursors hydrolyzed from tetraethyl orthosilicate (TEOS) by condensation under alkali catalysis. After undergoing nucleation and growth processes similar to the Stöber method, brusatol/silica (BS) hybrid NPs are obtained. The morphology and size of these NPs were characterized by transmission electron microscopy (TEM). As shown in Fig. 1B and **Figure S2A**, compared to pure silica NPs, BS hybrid NPs displays a lower contrast or higher porosity due to the incorporation of brusatol.

By measuring the absorbance of supernatant after the BS NPs were separated by centrifugation, the loading content of brusatol was determined to be 9.8%. As shown in **Figure S3A**, the absorption peak at approximately 285 nm during the subsequent release process of brusatol indicates the successful loading of brusatol into the silica. The diameter of the BS NPs was measured by TEM and dynamic light scattering (DLS) to be 82 nm (Fig. 1B and **Figure S4A**) and 89 nm (Fig. 1G and **Figure S5A**), respectively. Considering the influence of the hydration layer in the DLS measurement, the two values are consistent with one other.

MnO_2 was coated onto the surface of as-prepared BS NPs by mixing with KMnO_4 , which can be reduced by the residual organosilica on the surface of BS NPs. From the TEM image of Fig. 1C, it can be seen that a thin island-like shell formed on the surface of the BS NPs, and the solution color changed from light blue to orange, indicating the formation of brusatol/silica@ MnO_2 (BSM). Moreover, the aqueous solution of BSM washed three times with deionized H_2O exhibits broad absorption at ~ 335 nm (Fig. 1H), the characteristic peak of MnO_2 .^[31] Furthermore, BSM NPs have relatively obvious UV-vis absorption in the near-infrared region (NIR), whereas bare BS NPs are nearly optically transparent in this region (**Figure S6**).

Finally, compared to the BS NPs, the diameters of the BSM NPs increased to 94 nm (Fig. 1C and **Figure S4B**) or 101 nm (Fig. 1G and **Figure S5B**). The BS core was removed in Na_2CO_3 aqueous solution, leaving the hollow MnO_2 structure (Fig. 1D). This evidence highlights the successful synthesis of BSM.^[25]

The loading of Ce6 onto BSM was executed through the conventional adsorption-desorption process in solution.^[32] Due to the electrostatic interaction and Mn-N coordination between Ce6 and MnO_2 , Ce6 can be efficiently adsorbed onto the porous MnO_2 layer yielding brusatol/silica@ MnO_2 /Ce6 (BSMC).^[33] After Ce6 loading, the sample was washed three times with H_2O and displayed the characteristic peaks at 410 nm and 660 nm belonging to Ce6 (Fig. 1H), which is the proof of Ce6 loading. The absorbance of the solution containing Ce6 was measured before and after loading, and then the loading content and loading efficiency were calculated to be 14% and 56%, respectively.

Due to its excellent photothermal effects and biocompatibility, PDA is often used in nanomedicines. By self-polymerization of dopamine under alkaline conditions, PDA is easily coated onto the surface of the BSMC NPs to act as the PAT, yielding brusatol/silica@ MnO_2 /Ce6@PDA (BSMCP) NPs. As observed in the TEM image in Fig. 1E, a thin uniform shell formed on the surface of the BSMC NPs, and the color of the sample solution ranged from red-brown. In addition, the diameters of the BSMCP NPs measured by TEM and DLS were 103 nm (Fig. 1E and **Figure S4C**) and 106 nm (Fig. 1G and **Figure S5D**), respectively, showing an obvious increase in contrast to the BSM NPs. Compared with the BSM NPs, the UV-vis absorption of the BSMCP NPs in the NIR region increases due to the excellent absorption properties of PDA in this region (**Figure S6**), offering excellent photothermal conversion abilities to the nanoplatform. Finally, the BSMC core was successively removed in H_2O_2 solution and Na_2CO_3 aqueous solution, leaving a hollow PDA shell (Fig. 1F), which is direct evidence of PDA encapsulation.

The PDA shell not only prevents premature drug leakage during circulation in the blood but also facilitates subsequent modification of PEG and FA through the abundant quinone, amino, and phenolic hydroxyl groups on its surface.^[34] The PEG- and FA-modified product (brusatol/silica@ MnO_2 /Ce6@PDA-PEG-FA, denoted as BSMCPF) possesses an extended half-life and active targeted delivery. The successful modification of PEG-FA is confirmed from the characteristic absorption peaks of HS-PEG-FA at approximately 280 nm and 360 nm.^[22] In the step-by-step synthesis process, the stepwise altered diameters and zeta potentials indicate the successful preparation of the BS, BSM, BSMC, BSMCP and BSMCPF NPs (Fig. 1G and 1I).

Photothermal performance, controlled release and in vitro $^1\text{O}_2$ generation of NPs

To examine the photothermal conversion effects of MnO_2 and PDA, the temperature changes of H_2O , BS, BSM, BSMP and BSMCP were monitored with an infrared (IR) thermal camera under exposure to an 808 nm laser (1.5 W cm^{-2}) for 10 min (Fig. 2A). The temperatures of the BSMP and BSMCP NPs was rapidly increase to approximately 58.6°C and 57.9°C, respectively, from ambient temperature in a time-dependent

manner. These two NPs reached almost the same distinct cumulative photothermal effects of BSMCP NPs is potentially attributed to the combination of the MnO_2 layer and PDA film. In addition, the temperature of the BSMCP NPs increased rapidly in a power density-dependent and concentration-dependent manner (Fig. 2B and 2C). Moreover, as shown in Fig. 2D, the BSMCP NPs exhibited excellent photothermal stability after five on-off cycles of the laser, showing no obvious temperature decay. Collectively, the as-synthesized BSMCP NPs showed high photothermal conversion performance, superior photothermal stability and outstanding thermal cumulative effects.

Owing to the high cytotoxicity of brusatol, its controlled release is necessary to avoid damage to normal cells or tissues. In the BSMCPF delivery system, brusatol is embedded in the network of silica that allows the sustained release of brusatol (black curve in **Figure S3B**). In addition, the MnO_2 layer and PDA film can prevent its premature release or leakage. As a result, brusatol can be released only in a controlled manner (red curve in **Figure S3B**). In addition, the release of Ce6 from the BSMCPF NPs was evaluated under different physical conditions (Fig. 2E). In the absence of H_2O_2 , at pH 7.4, only approximately 18.4% of Ce6 was released from the BSMCPF NPs after 50 h, which was largely attributed to the diffusion driven by the concentration gradient. The amount of Ce6 released rapidly increased to approximately 48.3% at pH 5.5, illustrating acid-triggered release. In the simulated TME (pH 5.5, H_2O_2 concentration of 100 μM), this release amount increased to 66.1%. This result may originate from the subsequent reaction between the exposed MnO_2 layer and $\text{H}_2\text{O}_2/\text{H}^+$, which leads to the collapse of the MnO_2 layer and accelerates the release of Ce6. In comparison, without PDA encapsulation, approximately 71.7% of Ce6 was released from the BSMC NPs, which was higher than that of the BSMCPF NPs, illustrating the blocking effect of the PDA shell. These results demonstrated that MnO_2 functioned as a pH/ H_2O_2 -sensitive gatekeeper to prevent the premature release of Ce6 during the circulation period, facilitating drug release at tumor site in the acidic and H_2O_2 -rich TME. Hypoxia, a typical characteristic of the TME, contributes to PDT resistance. In the PDT procedure, $^1\text{O}_2$ is an essential requirement, and its production highly depends on oxygen. MnO_2 can act as a catalyst to trigger the decomposition of H_2O_2 into H_2O and O_2 or can be decomposed by H^+ to produce O_2 . Herein, we tested dissolved O_2 under various conditions (with or without H_2O_2 ; at pH 5.5 and pH 7.4) after the BSMCPF NPs were added (Fig. 2F). As expected, the dissolved O_2 concentration increased dramatically in the presence of H_2O_2 at pH 5.5, whereas it increased slowly without the addition of H_2O_2 . In contrast, there was almost no change in dissolved O_2 at pH 7.4. In addition, there was no obvious change in the dissolved O_2 content at pH 5.5 in the absence of the BSMCPF NPs. The above phenomena demonstrate that only the acidic TME containing H_2O_2 can largely trigger BSMCPF NPs to generate oxygen. Moreover, O_2 production was highly dependent on the concentrations of MnO_2 (Fig. 2G) and H_2O_2 (**Figure S7**).

To validate the association between $^1\text{O}_2$ generation and O_2 level, the $^1\text{O}_2$ produced from various samples in the presence of H_2O_2 was measured with a $^1\text{O}_2$ probe, 9,10-anthracenediyl-bis(methylene)dimalonic acid (ABDA), whose absorbance at 380 nm can be decreased by $^1\text{O}_2$. As observed in **Figure S8** and

Fig. 2H, the absorbance of ABDA at 380 nm decreased drastically in BSMCPF solution in the presence of H_2O_2 , indicating the production of a large amount of $^1\text{O}_2$. However, the absorbance dropped slightly without the addition of H_2O_2 . As a comparison, the $^1\text{O}_2$ generation ability of free Ce6 has been investigated, and the decrease in absorbance was notably lower than that of BSMCPF, highlighting that the improved O_2 provided by MnO_2 boosts the generation of $^1\text{O}_2$. In the absence of H_2O_2 , the $^1\text{O}_2$ yield of BSMCPF was lower than that of free Ce6, which is due to the quenching effect of Ce6 by MnO_2 . As a control, there was almost no production of $^1\text{O}_2$ in the BS and H_2O sample. The above results coincide well with the basic mechanism depicted in Fig. 2I, demonstrating the great potential of BSMCPF NPs to relieve hypoxia in the TME.^[35]

Biocompatibility and targeting of nanocarrier NPs

Inspired by the excellent photothermal properties and ROS generation ability of the nanocomposites, it was then necessary to evaluate the synergistic effects of the phototherapeutics. First, the delivery efficiency and *in vitro/in vivo* biodistribution of the targeted nanocarrier (silica/Ce6@PDA-PEG-FA, denoted as SCPF) or nontargeting nanocarrier (silica/Ce6@PDA-PEG, denoted as SCP) were investigated (concentration of Ce6 = $3.25 \mu\text{g mL}^{-1}$). The biocompatibility of the SCPF NPs was tested with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as presented in Fig. 3A. The negligible cytotoxicity observed indicates the biosafety of the carrier. Of paramount importance for therapy is the efficient cellular uptake of the nanotherapeutic. Due to the red fluorescence of Ce6, Ce6-loaded NPs could be tracked to determine whether they entered cancer cells. Initially, to evaluate the cellular uptake behaviors of the nanocarriers, Mia-PaCa-2 cells were incubated with free Ce6, SCP or SCPF. Next, the fluorescence of Ce6 in the cells was monitored by flow cytometry analysis and confocal laser scanning microscopy (CLSM). As shown in Fig. 3B-D, after 4 h, the Mia-PaCa-2 cells that received SCP had an obviously higher fluorescence signal than the cells that received the free Ce6 molecule, illustrating that the cellular uptake efficiency of the Ce6 molecule can be greatly enhanced by loading it into a nanocarrier. In particular, a much higher fluorescence signal was observed in the cells incubated with SCPF than the cells mentioned above, indicating that FA enhanced the endocytosis behavior by active targeting. Moreover, the SCPF NPs located in the cell can also be observed by TEM, which further demonstrated that FA enhanced the delivery efficiency (**Figure S9**).

The *in vivo* distribution of the nanocarriers was evaluated through fluorescence imaging after intravenous injection of free Ce6 and SCPF NPs (dose of Ce6 = 3.25 mg kg^{-1}). Ce6 fluorescence signals gradually accumulated in the tumor region 8 h after injection of SCPF, whereas there was almost no fluorescence signal in tumor tissue injected with free Ce6 (Fig. 3E). Semiquantitative biodistribution was determined from the *ex vivo* imaging of the major organs and tumors collected 24 h postinjection to evaluate the biodistribution of the SCPF NPs. The most intense fluorescence signal was detected in the tumor tissue, confirming the high tumor accumulation of SCPF NPs (Fig. 3F and 3G). The above results illustrated that SCPF NPs could efficiently accumulate at the tumor site and be endocytosed into the cells, which was

mostly attributed to the EPR effect from nanodelivery,^[36] the improved blood circulation capacity from PEG and the enhanced internalization efficiency from FA.^[37]

Strengthened PDT and PTT by MnO₂ and activated Nrf2 expression

As discussed above, the incorporated MnO₂ not only facilitates hypoxia relief but also contributes to photothermal conversion. To investigate the enhanced effects of MnO₂ on phototherapy, *in vitro* experiments were conducted. Specifically, Mia-PaCa-2 cells were treated with different combinations of medium and irradiation, including PBS, irradiation at 660 nm + 808 nm, SCPF + irradiation at 808 nm, SCPF + irradiation at 660 nm, SCPF + irradiation at 660 nm + 808 nm, and SMCPF (MnO₂ = 10 µg mL⁻¹, optimal concentration obtained by screening, **Figure S10**) + irradiation at 660 nm + 808 nm. Next, cell survival was assessed by MTT assay. As shown in Fig. 4A, cell survival of the above groups decreased sequentially 4 h posttreatment. Among the groups, the presence of MnO₂ (SMCPF) gave the highest cell death rate. In addition, the ROS yield detected by flow cytometry analysis and CLSM in the SMCPF group was also the highest (Fig. 4B-D). These results show that SCPF NPs have a synergistic PDT and PTT effect under the combination laser irradiation of 660 nm and 808 nm, displaying better antitumor efficiency than PDT or PTT alone. Notably, the introduction of MnO₂ further enhances synergistic phototherapy by its oxygen generation capacity and photothermal conversion performance.

However, the elevated ROS yield inevitably activates the Nrf2 signaling pathway, which regulates the expression of a series of cytoprotective genes that could resist the harmful effects of oxidative stress, leading to the survival of tumor cells. As shown in Fig. 4E, in contrast to the PBS group, the SMCPF-treated cells had notably higher expression and nuclear translocation of Nrf2. Moreover, the related genes, including HO-1 and GPX4, displayed high expression (Fig. 4F and 4G). This evidence confirms the activation of the Nrf2 signaling pathway.

Antioxidation inhibited synergistic phototherapy

Brusatol, an inhibitor of Nrf2, was introduced into the nanoformulation (BSMCPF) to inhibit the expression of Nrf2 and its downstream genes, thus avoiding the elimination of ROS and activation of the antioxidant defense system. First, *in vitro* experiments were carried out to test double-enhanced synergistic therapy with MnO₂ and brusatol. Specifically, Mia-PaCa-2 cells underwent four different treatments, PBS, BSCPF, SMCPF and BSMCPF (brusatol = 0.1 µg mL⁻¹) and were irradiated by lasers at 660 nm and 808 nm. Then, the intracellular ROS levels and cell viabilities were measured. As shown in Fig. 5A and **Figure S11**, the produced ROS in the BSMCPF group was significantly higher than those in the other groups and this level was maintained for more than 12 h. Consistently, the viability of the cells treated with BSMCPF under irradiation dramatically decreased (Fig. 5B). The improved ROS yield and death rate of the tumor cells are probably attributed to the inhibitory effects of antioxidation by brusatol.

Next, the *in vivo* combined phototherapeutic efficacy of the BSMCPF NPs was evaluated in a Mia-PaCa-2 mouse tumor model. Mice bearing Mia-PaCa-2 tumors were divided into four groups (the same as those described above, with doses of 10 mg kg⁻¹ MnO₂, 3.25 mg kg⁻¹ Ce6, and 100 µg kg⁻¹ brusatol). The nanoformulations were administered every four days over the 12 days, and the *in vivo* synergistic phototherapy against the tumor was executed by exposing the tumor to irradiation with a laser at 660 nm + 808 nm for 10 min at 12 h postinjection. For *in vivo* thermal imaging, the temperatures of mice exposed to laser irradiation were recorded using an IR thermal camera (**Figure S12**). The temperature in the tumor regions of the SMCPF NP group increased from 31°C to 52°C within 10 min, and the IR images gradually displayed a clear tumor outline, indicating excellent targeted delivery efficiency and photothermal performance. In contrast, the PBS group had only a slight temperature increase to 37°C.

In addition, the tumor sizes were measured every two days (Fig. 5C), and the tumors of all mice were collected and weighed at the end of therapy (Fig. 5D and 5E). Mice treated with PBS showed no appreciable effect on tumor growth. However, a moderate inhibitory effect was observed on the growth in the BSCPF group and SMCPF group, owing to the synergistic phototherapeutic effects enhanced by brusatol or MnO₂ alone, respectively. Notably, the BSMCPF-treated mice showed significant inhibition of tumor growth compared to the other groups, illustrating that the introduction of brusatol further enhanced the synergistic phototherapy. Importantly, mouse survival in the BSMCPF groups was obviously longer than that of the other groups (Fig. 5F).

Furthermore, EDU assays (Fig. 5G and **Figure S13**; red: EDU, blue: nucleus) and Transwell assays (Fig. 5H and **Figure S14**) were used to evaluate cell proliferation and invasion, respectively. The most obvious decreases in proliferation and invasion were observed in Mia-PaCa-2 cells treated with BSMCPF compared with the other groups, indicating the prominent potential of enhanced synergistic phototherapy with MnO₂ and brusatol for tumor suppression. Importantly, hematoxylin and eosin (H&E) and Ki67 staining examinations of the tumor tissues also indicated that BSMCPF could effectively induce tumor cell apoptosis, cause tumor tissue necrosis and inhibit tumor proliferation (Fig. 5I, 5J and **Figure S15**).

Finally, H&E staining was also used to evaluate the biosafety of the nanotherapeutics on the major organs, including the heart, liver, spleen, lung and kidney, and the results showed that the major organs had no appreciable tissue damage in any of the groups (**Figure S16**). Moreover, immunofluorescence staining of Nrf2 was performed on these organs, and the expression of Nrf2 among all groups was not significantly different, indicating that the nanotherapy had no influence on the expression of Nrf2 in other organs (**Figure S17**). The of H&E staining results and Nrf2 immunofluorescence staining results are consistent with each other, demonstrating the efficient targeted capacity and safety of the nanotherapeutics.

Mechanistic studies of synergistically enhanced phototherapy

To understand the fundamental mechanism of the brusatol and MnO_2 synergistically strengthened phototherapeutic process, TEM characterization of tumor cell sections and content detection of malondialdehyde (MDA, serving as a lipoxidation end product) and free Fe^{2+} (an essential substance of ferroptosis) were conducted. As displayed in Fig. 6A, the TEM images showed that the mitochondria in the BSMCPF group became smaller, the membrane density increased, and the mitochondrial ridge disappeared, which is a typical characteristic of ferroptosis.^[38, 39] In contrast, the morphology of mitochondria in the PBS group remained normal. Moreover, compared with the PBS group and the SMCPF group, the contents of free Fe^{2+} and MDA in the BSMCPF group increased significantly. Similarly, in animal tumor tissue cells, these indicators had almost the same trends as the cell groups (Fig. 6B). This evidence shows that the death mechanism of the tumor cells is likely ferroptosis. To confirm this, deferoxamine (DFO, 100 μM), an Fe^{2+} chelator, was introduced to cotreat the tumor cells with the BSMCPF NPs. The results showed that compared with BSMCPF, BSMCPF + DFO had a significantly reduced tumor suppression effect, illustrating that DFO inhibits ferroptosis by decreasing free Fe^{2+} (**Figure S18**).^[40] In addition, the proliferative (**Figure S19**) and migratory abilities (**Figure S20**) of the tumor cells were significantly reduced, which further supports the occurrence of ferroptosis in the BSMCPF group.

Furthermore, Western blot (WB) and confocal microscopy analyses were used to detect the expression levels of Nrf2 and its downstream genes, including FTH1, GPX4, HSP and SLC7A11, all of which play an important role in antioxidant function or inducing ferroptosis.^[41, 42] WB staining of the cell experiments showed that the expression of these genes stabilized 12 h after laser irradiation; thus, subsequent tests were based on this time point (**Figure S21**). As shown in Fig. 6C, compared to the SMCPF group, these genes in Mia-PaCa-2 cells treated with BSMCPF NPs were downregulated by inhibiting the expression of Nrf2 with brusatol, which further promoted synergistic phototherapy by destroying the defense system of the tumor cells. Most importantly, the downregulation of GPX4 can evoke ferroptosis by compromising the cellular antioxidant defense.^[43] Likewise, WB analysis from the animal experiments showed similar results (Fig. 6D). Moreover, HSP downregulation could assist PTT by weakening the defense capacity of tumor cells against hyperthermia. Finally, from both the cell experiment and animal experiment, CLSM analysis showed that the expression and nuclear translocation of Nrf2 in the BSMCPF group was significantly reduced (Fig. 6E and 6F), demonstrating that the antioxidant defense function of the tumor cells was efficiently inhibited.

Conclusion

We creatively designed a synergistic nanomedicine platform that greatly enhanced tumor therapy efficacy by boosting ROS yield while simultaneously inhibiting antioxidation of tumor cells. By integrating MnO_2 and brusatol into the nanoplatform, the obstacles affecting conventional phototherapy were eliminated in this system, including the hypoxic characteristics of the TME and the defense functions of the tumor cells. In addition, PEG conjugation prolongs the circulation period of nanomedicines, allowing their substantial accumulation in tumor tissues, and FA modification enhances cellular uptake through the

affinity of FA with receptors on the surface of tumor cells. Notably, extensive *in vitro* and *in vivo* evaluations have demonstrated that the synergistic effects of MnO₂-boosted ROS and brusatol-inhibited Nrf2 expression induced ferroptosis, thereby efficiently combating cancer cells and suppressing tumor growth. Finally, the high biocompatibility of this multifunctional nanoformulation *in vivo* was also demonstrated, guaranteeing its biosafety. We believe that this enhanced synergistic therapeutic strategy could compensate for the shortcomings of single phototherapy, either PDT or PTT, and lay a promising foundation for synergistic therapies to battle cancer.

Experimental Section

Synthesis of brusatol/silica (BS) NPs and silica NPs.

First, 100 mg of hexyltriethoxysilane (HTES) was added to 150 mL of a mixed solution of ethanol and DI H₂O at a volume ratio of 2:1, followed by stirring for 30 min for hydrolysis. Then, 5 mL of brusatol (1 mg mL⁻¹ in DMSO) was added to the above solution with stirring. Thirty minutes later, 5 mL of ammonia solution (25%-28%) and 0.5 mL of TEOS were added. The mixture stirred at room temperature for 14 h, and then brusatol/silica NPs (BS NPs) were separated by centrifugation at 12000 rpm for 10 min, followed by washing three times with DI H₂O. The preparation of silica NPs is similar to that of BS NPs, except for the process of adding brusatol.

Synthesis of brusatol/silica@MnO₂ (BSM) NPs and silica@MnO₂ (BM).

Fifty milligrams of KMnO₄ dissolved in 1 mL of DI H₂O was added dropwise into a 50 mL aqueous dispersion of BS NPs under stirring. The mixture stirred at room temperature for 4 h and was then washed with DI H₂O three times to remove the residual KMnO₄, obtaining brusatol/silica@MnO₂ NPs (BSM NPs). The preparation of SM NPs is the same as that of BSM NPs, the TEM is shown in Figure S2B.

Loading of Ce6.

For Ce6 loading, BSM NPs were dispersed in 50 mL of phosphate-buffered saline (PBS; pH 7.4) containing Ce6 (10 mg) and stirred for 24 h in darkness. Then, the solution was centrifuged at 10000 rpm for 10 min to remove the free Ce6, obtaining brusatol/silica@MnO₂/Ce6 NPs (BSMC NPs). The loading of Ce6 on silica@MnO₂ (SM) NPs is the same as that of BSMC NPs. As for silica/Ce6 (SC) NPs preparation, Ce6 was loaded into silica NPs with mesopores by physical adsorption, the loading procedure is similar to that of BSMC NPs.

PDA coating.

The above BSMC NPs were dispersed in a 30 mL mixed solution of ethanol and DI H₂O at a volume ratio of 2:3. Then, 10 mg of dopamine hydrochloride and 0.2 mL of ammonia solution were added, followed by stirring for 4 h at room temperature to obtain PDA-coated brusatol/silica@MnO₂/Ce 6 NPs. The final product, denoted brusatol/silica@MnO₂/Ce6@PDA NPs (BSMCP NPs), was centrifuged at 10000 rpm for 10 min and washed three times with DI H₂O. The preparation of silica/Ce6@PDA (SCP) NPs and silica@MnO₂/Ce6@PDA (SMCP) NPs is the same as that of BSMCP NPs, the TEM were shown in Figure S2C and S2D.

Modification with PEG and FA (BSMCPF NPs).

For this experiment, the brusatol/silica@MnO₂/Ce6@PDA NPs and HS-PEG-FA were well dispersed in Tris-HCl buffer (pH 8.5) and mixed for 6 h at room temperature in the dark. Then, the mixture was centrifuged and washed several times with DI H₂O to remove the remaining HS-PEG-FA. The final product, brusatol/silica@MnO₂/Ce6@PDA-PEG-FA NPs (BSMCPF NPs), were dispersed in DI H₂O.

Photothermal effect.

To investigate the impacts of MnO₂, PDA and Ce6 on the photothermal performance, different samples (H₂O, BS NPs, BSM NPs, BSMC NPs, and BSMCPF NPs) were irradiated with an 808 nm laser for 10 min at a power density of 1.5 W cm⁻² and the temperatures were recorded. To examine the concentration dependency of the photothermal effect, various concentrations of BSMCP NPs in solution (50 µg mL⁻¹, 100 µg mL⁻¹ and 200 µg mL⁻¹) were irradiated for 10 min. 0.5 W cm⁻², 1.0 W cm⁻² and 1.5 W cm⁻² were employed to investigate the influence of power density on photothermal performance. In the study of the photothermal stability of the BSMCP NPs, a BSMCP solution (200 µg mL⁻¹) was irradiated with five on-off cycles with an 808 nm laser at a power density of 1.5 W cm⁻². An IR thermal camera (FOTRIC 225s) was used to monitor the changes of temperature during irradiation.

Oxygen detection.

BSMCP NPs were dispersed in phosphate-buffered saline (PBS) at pH 7.4 or pH 5.5, and in the presence or absence of H₂O₂. The amount of produced oxygen was measured with a JPB-608 portable dissolved oxygen meter.

Singlet oxygen detection.

According to a previously published report, the ¹O₂ yield can be measured by using the probe 9,10-anthracenediyl-bis(methylene)dimalonic acid (ABDA). The UV-vis absorption of ABDA at 380 nm

gradually decreases with increasing $^1\text{O}_2$ concentration. Specifically, 100 μL of ABDA solution (1.5 mg mL^{-1} in DMSO) was mixed well with different formulations ($\text{Ce6} = 3.25 \mu\text{g mL}^{-1}$) in PBS (pH 7.4) in the absence or presence of 100 μM H_2O_2 . Then, the mixture was irradiated with a 660 nm laser at a power density of 500 mW/cm^2 . The amount of generated $^1\text{O}_2$ was determined by measuring the absorbance change of ABDA at 380 nm at different time points.

Biocompatibility of nanocarriers (MTT assay).

A MTT assay was used to quantify cell viability. In brief, Mia-PaCa-2 cells were seeded into a 96-well plate at a density of 5×10^3 cells per well and cultured for 24 h to allow cell attachment. Then, the medium was replaced with fresh medium containing SCP NPs at a concentration of 0, 20, 30, 50, 100, 200 or 400 mg mL^{-1} . The supernatant was discarded after coculturing for another 24 h at 37°C , and the cells were washed twice with PBS. Then, fresh medium (100 mL) containing MTT (0.5 mg mL^{-1}) was added. After incubation for another 4 h, the absorbance at 570 nm was measured using a microplate reader (Thermo Fisher) to analyze cell viability.

Measurement of intracellular ROS.

ROS levels in Mia-PaCa-2 cells were evaluated by confocal laser scanning microscopy (CLSM) and flow cytometry using 2',7'-dichlorofluorescein diacetate (DCFH-DA; 10 μM) as a ROS detection probe. First, the Mia-PaCa-2 cells were seeded on a 24-well plate at 5×10^4 cells per well and cultured overnight. After removal of the medium, the cells were treated with different samples for 4 h, and then DCFH-DA was loaded into the cells followed by incubation for another 1 h. Subsequently, the cells were irradiated with a 660 nm laser for 10 min (500 mW cm^{-2}). Then, before measuring the fluorescence of DCFH by CLSM and flow cytometry, the medium was removed, and the cells were washed with PBS twice.

Synergistic therapy *in vitro*.

The phototherapeutic effects of the different NPs were investigated by using MTT as follows. Mia-PaCa-2 cells were seeded into a 96-well plate at a density of 5×10^3 cells per well and cultured for 12 h. Then, the medium was replaced with fresh medium containing different samples. After 6 h of incubation, the cells were washed with PBS, fresh medium was added, and the cells were then irradiated with a laser for 10 min and further incubated for 24 h. Cell viability was measured by using the standard MTT method.

MDA assay.

To monitor lipid peroxidation, a TBARS assay kit was used to detect the MDA content in the cells or tumor tissues treated with either PBS, SMCPF NPs or BSMCPF NPs. Briefly, 50 mg of tumor tissue was

homogenized with 500 µL of RIPA buffer containing protease inhibitors, followed by centrifugation at 1500 g for 5 min, and the supernatant was used for MDA analysis. In addition, the protein concentration of the supernatant was measured using a BCA protein assay kit for normalization of the MDA content.

Western blot.

Cellular proteins were evaluated by Western blot (WB). First, the protein concentrations in the samples were quantified by a BCA protein assay kit, and then the proteins were isolated by SDS-PAGE. The obtained proteins were transferred onto polyvinylidene difluoride membranes in Tris-glycine buffer, blocked with 5% fat-free skim milk at room temperature for 1 h, and then incubated with primary antibodies overnight at 4 °C. Subsequently, the membranes were rinsed and incubated with secondary HRP-conjugated antibody at room temperature for 1 h. The membranes were washed twice with TBST solution and imaged with a Gel Logic system (2200 Pro, Crestream, USA) in the dark. Primary antibodies against Nrf2, GPX4, FTH1, HO-1, SLC7A11 and HSP90 were used. Lamin B and GAPDH were used as loading controls.

Immunofluorescence assay.

Mia-PaCa-2 cells after treatment on different glass slides were washed, fixed, and permeabilized. Then, the cells were labeled with specific primary antibodies (Nrf2 at a 1:200 ratio) at 4 °C overnight. The obtained cells were incubated with the corresponding fluorescently conjugated secondary antibody together with DAPI nuclear counterstain and phalloidin and then used for CLSM imaging after rinsing.

Invasion assay.

Invasion was performed using transwell inserts for a 24-well plate that contained 8 µm pores (Millipore).

5-Ethynyl-2'-deoxyuridine (EDU).

To assess cell proliferation, Mia-PaCa-2 cells displaying logarithmic growth were inoculated into 96-well plates at a density of 4×10^3 to 1×10^5 cells per well and cultured until they reached the normal cell development stage. Then, the cells were incubated with 100 µL of culture medium containing EDU (50 µM) for 2 h and fixed with 50 µL of cell PBS solution containing 4% paraformaldehyde for 30 min. Subsequently, 50 µL (2 mg mL^{-1}) of glycine was added to incubate the cells in a shaker for 10 min. After discarding the glycine solution, the cells were incubated with 100 µL of infiltrator (PBS containing 0.5% Triton X-100) for 10 min. Next, 100 µL of Apollo staining reaction liquid was added to the cells for incubation for 30 min, then washed with infiltrator three times. Finally, the cells were incubated with Hoechst for 30 min in the dark.

Animals.

Male nude mice (6 weeks old), purchased from ChangZhou Cavens Laboratory Animal Center, were used for animal experiments in accordance with the regulations of the Animal Care and Use Committee of Nanjing University of Chinese Medicine (Nanjing, China). The Mia-PaCa-2 tumor model was generated by subcutaneous injection of 1×10^7 Mia-PaCa-2 cells in 200 μ L of PBS into the armpits of male nude mice. When the tumor volume reached approximately 100 mm³, the mice were injected with various formulations.

Enhanced PTT/PDT synergistic therapy *in vivo*.

In the *in vivo* synergistic therapy experiments, the mice were randomly divided into four groups (n = 6): group 1: PBS; group 2: BSCPF NPs+808 nm+660 nm laser irradiation; group 3: SMCPF NPs+808 nm+660 nm laser irradiation; group 4: BSMCPF NPs+808 nm+660 nm laser irradiation (dose of brusatol = 100 μ g kg⁻¹; dose of MnO₂ = 10 mg kg⁻¹; dose of Ce6 = 3.25 mg kg⁻¹). Dose administration occurred every three days for a total of 12 days. Laser irradiation was carried out for 10 min 12 h postinjection, and the laser power density was 500 mW cm⁻² at 660 nm and 1.5 W cm⁻² at 808 nm. Finally, the mice were sacrificed after therapy. Tumor sections and the major organs, including the heart, liver, spleen, lung and kidney, were collected and examined by hematoxylin and eosin (H&E) staining to evaluate the biosafety of the nanoformulations.

Statistical analysis.

Data are presented as means $\pm$ SD and analyzed using Student's *t* test or one-way ANOVA to determine significance with Statistical Product and Service Solutions (SPSS) 19.0. Statistical significance was considered at $P < 0.05$. Differences of $*P < 0.05$ and $**P < 0.01$ were considered statistically significant.

Declarations

Authors contributions

J. D. and M. Z. conceived the project. W. T. and D.S. designed and supervised the experiments. N. W. performed the material experiments. J. R. performed the biological experiments. D. S., P. Z. and C. L. took part in analyzed data. L. F., Y. H. C.W. and C. C. took part in discussions. W. T. and N. W. wrote the first paper draft. All authors discussed the results and commented on the manuscript.

Declaration of competing interest

The authors declare no competing financial interest.

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Figures

Figure 1

Synthetic procedure and characterization of the BSMCPF NPs. (A) Schematics illustrating the step-by-step synthesis of the BSMCPF NPs. (B) TEM image and sample solution picture of the BS NPs. (C) and (D) TEM image and sample solution picture of the BSM NPs and TEM image of the corresponding hollow MnO_2 nanostructure obtained by removing the BS core. (E) and (F) TEM image and sample solution picture of the BSMCP NPs and TEM image of the corresponding hollow PDA nanostructure obtained by removing the BSMC core. (G), (H) and (I) The size distribution, UV-vis absorption, and zeta potentials of the BS, BSM, BSMC, BSMCP and BSMCPF NPs. Scale bars, 100 nm.

Figure 2

Photothermal performance and oxygenation property evaluations of the nanoformulations. (A) Temperature increase versus irradiation duration curves of H₂O, BS, BSM, BSMP, and BSMCP. (B) and (C) The concentration- and power density-dependent increases in the temperature of the BSMCP solution under laser irradiation. (D) Heating/cooling profiles of the BSMCP group after five on-off laser irradiation cycles (808 nm, 1.5 W cm⁻²). (E) Triggered release of Ce6 from BSMC or BSMCPF in the different environment. (F) and (G) Oxygen production performance under different physical conditions and different concentrations of BSMCPF NPs. (H) ROS production of different samples (H₂O₂, BS NPs, Ce6, Ce6+H₂O₂, BSMCPF NPs and BSMCPF NPs+H₂O₂) under laser irradiation (660 nm, 500 mW cm⁻²) measured by UV-vis with ABDA as a ROS probe. (I) Schematics illustrating the oxygen supply and ¹O₂ boosting mechanism of the BSMCPF NPs in acidic aqueous solution in the presence of H₂O₂.

Figure 3

The biocompatibility and targeting ability of the nanocarrier (SCPF). (A) Viability of Mia-PaCa-2 cells incubated with different concentrations of SCPF NPs. (B), (C) and (D) Flow cytometry data, quantitative analysis and confocal fluorescence images of ROS generation in Mia-PaCa-2 cells after incubation with PBS and SCPF NPs for 4 h. (E). In vivo fluorescence images of mice after injection of free Ce6 and SCPF NPs at different time points. (F) Ex vivo fluorescence images of the tumor and major organs collected 24

h postinjection. (G) Semiquantitative biodistribution of the SCPF NPs in mice measured based on the average fluorescence intensity of the tumors and major organs. *P < 0.05, **P < 0.01.

Figure 4

MnO2 boosted ROS levels and activated Nrf2 expression. (A) Survival of Mia-PaCa-2 cells treated with various formulations at a constant concentration of Ce6 (10 μ M) after laser irradiation at 660 nm (500 mW cm⁻², 10 min), 808 nm (1.5 W cm⁻², 10 min), or 660 nm (500 mW cm⁻², 10 min) + 808 nm (1.5 W cm⁻², 10 min). (B), (C) and (D) Flow cytometry data, quantitative analysis and fluorescence images of ROS generation. (E) Expression of Nrf2 by CLSM observation. (F) and (G) WB assay and quantitative analysis of the expression of Nrf2 and its downstream proteins in Mia-PaCa-2 cells treated with PBS or SMCPF NPs. *P < 0.05, **P < 0.01.

Figure 5

Antioxidation inhibited synergistic phototherapy by brusatol. (A) and (B) ROS generation and viability of Mia-PaCa-2 cells treated with PBS, BSCPF NPs, SMCPF NPs and BSMCPF NPs upon laser irradiation at 660 nm (500 mW cm⁻², 10 min) + 808 nm (1.5 W cm⁻², 10 min). (C) Tumor growth curves of the mice during the 12 days of treatment. (D) and (E) Photographs and weight quantification of the tumors harvested from mice after 12 days of treatment. (F) Survival rates of the mice in different treatment

groups. (G) CLSM observation of cell proliferation by EDU assay. (H) Images of cell invasion by Transwell assay. (I) and (J) H&E and Ki67 staining of the tumor tissue from different groups. *P < 0.05, **P < 0.01.

Figure 6

Mechanistic studies of ferroptosis and inhibited antioxidation. (A) and (B) TEM observations of the morphology of the mitochondria and the content of free Fe²⁺/MDA corresponding to the in vitro and in vivo studies. (C) and (D) WB assay and quantitative analysis of the expression of Nrf2 and the

downstream proteins corresponding to the in vitro and in vivo studies. (E) and (F) The expression and nuclear translocation of Nrf2 by CLSM observation corresponding to the in vitro and in vivo studies. *P < 0.05, **P < 0.01

Figure 7

Scheme 1. Schematics illustrating the targeted accumulation of the nanoformulations and strengthened synergistic phototherapy by amplifying ROS and inhibiting antioxidation.

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