Biodegradable Reduce Expenditure Bioreactor For Augmented Sonodynamic Therapy Via Regulating Tumor Hypoxia And Inducing Pro-Death Autophagy

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Research

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Abstract

Backgrounds: Sonodynamic therapy (SDT) as an emerging reactive oxygen species (ROS)-mediated antitumor means is still hampered by the rapid depletion of oxygen, as well as hypoxic tumor microenvironment. Instead of the currently coping strategies through amplifying endogenous O₂ level, herein, a biodegradable O₂ economizer is described as a reduce expenditure bioreactor for augmenting SDT efficacy.

Results: We have successfully fabricated the O₂ economizer (HMME@HMons-3BP-PEG, HHBP) by the conjugation of respiration inhibitor 3-bromopyruvate (3BP) with hollow mesoporous organosilica nanoparticles (HMons), followed by the loading of organic sonosensitizers (HMME) and further surface modification of poly(ethylene glycol) (PEG). The engineered HHBP features controllable pH/GSH/US-sensitive drug release. The exposed 3BP could effectively inhibit cell respiration for restraining the oxygen consumption, which could alleviate the tumor hypoxia. More interestingly, it could exorbitantly elevate the autophagy level, which in turn induce excessive activation of autophagy for promoting the therapeutic efficacy. As a result, accompanied with suppressing O₂-consumption and triggering pro-death autophagy strategy, the HHBP achieves remarkable antitumor activity, which has been systematically validated both in vivo and in vitro assays.

Conclusion: This work not only provides a reduce expenditure strategy for enduring SDT, but also represents an inquisitive strategy for tumor treatments via inducing pro-death autophagy.

Background

As an emerging cancer-treatment modality, sonodynamic therapy (SDT) employs sonosensitizers that are activated by ultrasound (US) in the presence of oxygen, which enables highly toxic reactive oxygen species (ROS) generation in cancer cells, primarily singlet oxygen (¹O₂) [1-3]. Due to the deep tissue penetration of US, SDT affords multiple advantages, such as noninvasiveness and high tissue-penetrating depth compared with photodynamic therapy, thus exerting great potential for deep-seated tumors treatment [4-7]. Although preclinical researches on SDT have shown promising results in various cancer cells, some undesirable limitations severely restricting the clinical translation of SDT. Especially, the efficacy of SDT mainly depends on oxygen content inside the solid tumors, while many solid tumors often feature hypoxia triggered owing to the limited oxygen diffusion and rapid proliferation of cancer cells [8-11]. What makes things worse is that the hypoxia is even further intensified during SDT process, triggering SDT resistance. Thus, overcoming tumor hypoxia is the priority among priorities in SDT process. In principle, the hypoxic tumor microenvironment (TME) can be reshaped through improving exogenous O₂ level or suppressing O₂ consumption [12, 13]. Promoted by the significant advances in nanomedicine, many expectant strategies aimed at modulating the level of oxygen in solid tumor, such as delivering the O₂ to the hypoxic regions by oxygen-carrying nanomaterials and in situ generation of O₂ by nanocatalysts/nanozymes-mediated hydrogen peroxide (H₂O₂) decomposition, have been exploited [11,

14-19]. Nevertheless, the feasibility of these strategies is severely hindered by the leakage of O_2 from the carriers and the insufficient endogenous H_2O_2 **[3, 20-22]**. Compared to the widespread tactics of improving the oxygen content, reducing the oxygen consumption is promising alternative to relieve tumor hypoxia. It has been proved that inhibiting mitochondrial respiration by some oxygen-regulator (e.g., NO **[23]**, atovaquone **[24]** and metformin **[25]**) could effectively oxygenated tumor for improving the therapeutic efficacies. Unfortunately, the design of nanoplatform that can modulate hypoxia by reducing O_2 consumption is still necessary but largely unexploited, especially on oxygen-dependent therapeutic modalities.

In addition to inducing cell apoptosis, the potential benefit of the production of 1O_2 during the SDT process can induce autophagy **[26-28]**. Autophagy is cellular degradation process refers to the degradation of phagocytes cytoplasmic proteins or organelles through the formation of form autophagy lysosomes **[29, 30]**. Autophagy is commonly considered as a self-protection process to achieve the renewal of some organelles and the metabolism and energy of cell itself **[31]**. However, autophagy is a “double-edged sword”. When exceeds a certain threshold, exorbitant and abnormal autophagy can promote cell death under specific circumstances **[32, 33]**. It can be assumed that activation of pro-death autophagy can also be a potential strategy to improve cancer therapy. Due to the autophagy induced by ROS is pro-survival, therefore, the construction of nanosystems with both sonosensitizer activity and capability in regulation of autophagy from self-protection to pro-death is expected to achieve augmented SDT.

Considering the above assumption, we put forward a reduce expenditure bioreactor for augmenting SDT efficacy via regulating tumor hypoxia and inducing pro-death autophagy. This bioreactor was constructed by decorating respiration inhibitor 3-bromopyruvate (3BP) onto the surface of disulfide bonds-hybridized hollow mesoporous organosilica nanoparticles (HMOns) via an amide linker. Organic sonosensitizers (hematoporphyrin monomethyl ether, HMME) were further loaded into the mesopores and hollow interior of HMOns, followed by modification with poly (ethylene glycol) (PEG) (HMME@HMOns-3BP-PEG, designated as HHBP) to enhance their biocompatibility (**Fig. 1**). The engineered HHBP are featured with intrinsic TME-responsive biodegradability, which function as a nanosonosensitizer to produce 1O_2 under the US irradiation. The delivered 3BP reduces significantly reduce intracellular oxygen consumption rate by perturbing the respiration, thus relieving tumor hypoxia for further augmenting the production of 1O_2 . Specifically, 3BP is expected to shut down nutrient supply for inhibiting cellular respiration to rapid depletion of adenosine triphosphate (ATP) and simultaneously induce autophagy **[34, 35]**. The combination of ROS production and ATP supply restriction lead to the changeover of autophagy from self-protection to pro-death, which in turn promotes cell apoptosis. As a result, enhanced SDT therapeutic outcome could be acquired, which was systematically investigated both in vitro at cellular level and in vivo on 4T1 tumor-bearing mice. The findings demonstrate the feasibility of this augmented SDT strategy with the 3BP and HMME co-delivered system. This work provides a reduce expenditure strategy for facilitating ROS production, and simultaneously offers a rationale for the combination of respiration inhibition and pro-death autophagy therapy as a therapeutic strategy against cancer.

Methods

Synthesis of hollow mesoporous organosilica nanoparticles (HMONS) nanoparticles

The HMONS was prepared according to our previous work [36, 37]. In brief, CTAC (10 g) was dissolved in deionized water (100 mL) and TEA (8 g) solution was added. The mixture was heated and stirred in an oil bath to 80 °C and TEOS (0.5 mL) was added dropwise for one hour. Then, a mixture of TEOS (1 mL) and BTDS (0.6 mL) was quickly added to continue the reaction for 4 h to form core/shell structure. The products were washed with a mixture of ethanol (50 mL) and hydrochloric acid (4 mL) in a reflux unit at 80 °C for three times. Then, the products were then dispersed into deionized water (20 mL), followed by adding ammonium hydroxide (3.5 mL) for another 3 h at 98 °C. The final HMION products were obtained by centrifugation, washed and dried in vacuum.

Synthesis of HHBP nanoparticles

HMONS (200 mg) were dispersed in the methanol solution and then functionalized with APTES (1 mL) for 16 h at 120 °C. After that, the precipitate was collected by centrifugation, washed with ethanol for several times and finally dried to obtain HMONS-NH₂ nanoparticles. After that, the 3BP was anchored onto surface of HMONS-NH₂ via amidation reaction. In brief, HMONS-NH₂ (80 mg) nanoparticles were dispersed in DMSO/deionized water mixture solution, containing EDC (12.0 mg) and NHS (7 mg, 0.12 mmol). The reaction system was stirred for 6 h at room temperature and 3BP (10 mg) was slowly added, followed by vigorously stirring for another 12 h. The product (HMONS-3BP) was obtained by centrifuged and purified with ethanol.

For HMME loading, HMONS-3BP was dispersed in the HMME methanol solution at different mass ratios (4:1; 2:1; 1:1; 2:3; 2:4), and stirred at room temperature for 24 h. After centrifugation and purified with methanol for several times, the HMME-loaded formulations (designated as HMME@HMONS-3BP) were obtained. Meanwhile, the collected supernatant was collected to evaluate the HMME loading capacity by UV-vis analysis. Finally, The PEGylated nanoparticles (HHBP) were carried out through adding the mPEG-COOH to the HMME@HMONS-3BP dispersion and stirring for 12 h. The final product (HHBP) was washed and re-dispersed in PBS for further use.

Qualitative and quantitative ¹O₂ detection

The generation of ¹O₂ was measured by electron spin resonance (ESR, Brooke A300, Germany) measurement using the trapping agent TEMP. Typically, HHBP (150 µg/mL) was exposed to US irradiation (1.0 W/cm², 1.0 MHz, 50% duty circle) for 1 min in the mixture containing TEMP (100 mM). Then, ESR spectroscopy was then immediately performed on the mixture. As control groups, PBS with or without US irradiation and HHBP without US irradiation were also tested.

To quantitatively evaluate the production of ¹O₂, HHBP was dispersed in DMF (150 µg/mL, 2.96 ml), followed by adding DPBF (8 mM, 40 µL). Then, the mixture was treated with US irradiation (1.0 W/cm²,

1.0 MHz, 50% duty circle) for 1 min, and then the absorbance intensity at the wavelength of 398 nm was recorded by UV-vis spectrophotometer.

In vitro pH/GSH/US-responsive HMME release

First, the biodegradation behavior of the HHBP nanoparticles in simulated body fluid (SBF) containing GSH at different concentrations (0, 5, and 10 mM) was evaluated by TEM observation. The release behavior of HMME was measured by dialysis method through UV-Vis spectrophotometer. Typically, HHBP dispersion (1 mg/mL HMME equivalent, DMSO 0.5 mL and buffer solution 4.5 mL) was placed in a dialysis bag under different conditions: (1) PBS, pH 7.4; (2) ABS, pH 5.5; (3) PBS, pH 7.4, GSH (10 mM); and (4) ABS, pH 5.5, GSH (10 mM) at 37 °C under stirring. At the given time, 0.5 mL of the releasing solution outside the dialysis bag was taken out for UV-vis analysis at λ 398 nm to determine the amount of released HMME. Meanwhile, the US-triggered release behavior were performed under the same condition of group 3 and 4, and exposed to US irradiation (1.0 W/cm², 1.0 MHz, 50% duty circle) over a period of 1 min at 1, 4, 8 and 16 h incubation.

In vitro ROS production at cellular level

DCFH-DA was served as a probe to detect intracellular ROS generation. Briefly, 4T1 cells were incubated into 12-well plates at a density of 1×10^5 cells overnight. Subsequently, the cell medium was replaced and the cells were treated with different formulations including PBS, HMME@HMONs-PEG, HHBP for another 4 h. Then, the cells were further exposed to US irradiation (1.0 W/cm², 1.0 MHz, 50% duty circle, 1 min). Then, the cells were incubated with DCFH-DA (1:1000 dilution) for 30 min according to the provided protocols. The cells were washed three times with PBS and then imaged by fluorescence microscope. Finally, cells were collected and the quantitative analysis of fluorescence intensity was analyzed by flow cytometry.

In vitro nanotherapeutic performance of HHBP

4T1 cells were seeded into 96-well plates at a density of 1×10^4 cells and treated with PBS, HMME@HMONs-PEG or HHBP for 4 h. Then, part of the cells were further exposed to US irradiation (1.0 W/cm², 1.0 MHz, 50% duty circle, 1 min). After incubated for a total time of 12 h, the cells were washed with PBS for three times and the cell viability was measured by the CCK-8 assay. Correspondingly, all the treated cells were digested with trypsin and stained by Calcein-AM/PI Cell Double Dyeing Kit according to the instructions. After incubation for 15 min, the cells were visualized under a fluorescence microscope. Next, cell apoptosis apoptosis detection assay was also carried out to further investigate the cell death mechanism. 4T1 cells were seeded in 6-well plates for 24 h and then treated as the same procedure in the CCK-8 assay. Then, the cells were washed with PBS and collected by centrifugation, and then treated with Annexin V-FITC apoptosis detection kit, and finally quantitated by flow cytometer (FACS-Calibur, BD Biosciences).

Detection of HIF-1 α and HK-II by western blot assay

The expression of HIF-1 α and HIF-2 in cells was evaluated by Western blot assay. The BCA protein concentration determination kit was used to measure the protein concentration, and the specific method was referred to the kit instructions. Briefly, the 4T1 cells were inoculated into 6-well plates (2×10^5 cells per well) and incubated with PBS, HMME@HMNs-PEG (100 μ g/mL) or HHBP (100 μ g/mL) for 4 h. Then, part of the cells were further exposed to US irradiation (1.0 W/cm², 1.0 MHz, 50% duty circle, 1 min). After incubated for a total time of 6 h, cell lysates with protease inhibitor are added to extract the proteins. The proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (10%) and then transferred to polyvinylidene fluoride membrane (Millipore, IPVH00010). The membrane was decolorized and sealed with 5% skim milk (0.5% TBST) on a shaker for 1 h. The primary antibodies (HIF-1 α (1:2000 dilution, Abcam, ab16066) and HK-2 (1:5000 dilution, Abcam, ab227198)) was diluted and incubated overnight at 4 °C, and then the membranes were washed with TBST for three times and hybridized with relevant secondary antibody for 1 h. Finally, the film was observed by chemiluminescence. The protein expression and the band strength was quantified using Image Pro Plus 6.0 software. β -actin is served as a loading control. Each treatment had three parallel samples, and the data were expressed as mean $\pm$ standard deviation (n = 3).

Animals and tumor model

Healthy female BALB/c nude mice (4-6 weeks) were provided by the Animal Center of Shanghai Sixth People's Hospital. All animal experiments were conducted under the protocols approved by the Institutional Animal Care and Use Committee (IACUC) of Shanghai Jiao Tong University Affiliated Sixth People's Hospital (Animal Welfare Ethics acceptance number No: DWLL2020-0582). The 4T1 cell subcutaneous tumor model was established by subcutaneously injecting 100 μ L of 2×10^6 cells suspension into female nude mice. The body weight of the mice was monitored and the tumor volume (V) was calculated as $V = 0.5 \times L \times W^2$, where L and W represented the length and width of the tumor respectively.

In vivo antitumor assays

When the tumor size reached 150 mm³, the mice were randomly divided into five groups (n = 6): I) PBS, II) HMME@HMNs-PEG, III) HHBP, IV) HMME@HMNs-PEG + US, V) HHBP + US. Mice were intravenously injected with 100 μ L HMME@HMNs-PEG (HMME: 8 mg/kg) or HHBP (HMME: 8 mg/kg, 3BP: 3.4 mg/kg), respectively. The parameters of US irradiation are as follows: 1.5 W/cm², 1.0 MHz, 50% duty circle for 5 min. Mice were administrated drugs and irradiated at day 1 and 4. After 12 h of treatments, tumor blocks of mice were taken out and frozen at -20 °C for DHE staining to detect ROS, HIF-1 α staining to measure the extent of tumor hypoxia, and LC3 staining to detect autophagy level. After treatments, the tumor-bearing nude mice were sacrificed and the tumor tissues were collected for H&E, Ki67, and TUNEL staining. Additionally, the major tissues were also dissected to make paraffin sections for further H&E staining.

Statistical analysis

Data were expressed as mean or mean $\pm$ standard deviation. The statistical significance was determined using a Student's *t* test: ns: $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. All the statistical analyses were conducted using the SPSS software.

Results And Discussion

Design, fabrication, and characterization of HHBP nanosonosensitizers

The procedure for fabrication of HHBP nanosonosensitizer and the corresponding therapeutic mechanism are illustrated in Fig. 1. To endow the nanosonosensitizer with TME-responsive biodegradability, we introduced disulfide bond components into the framework of mesoporous silica to prepare organic-inorganic hybrid HMONS for the delivery of both 3BP and HMME. Typically, HMONS were prepared by employing an “ammonia-assisted selective etching” approach reported in the literature [37–39]. Initially, the monodispersed SiO_2 @MONs nanoparticles were synthesized through the co-hydrolysis of tetraethoxysilane and bis(3-triethoxysilylpropyl) disulfide by employing cetyltrimethylammonium chloride as the structural-directing agents [40, 41]. As visualized by transmission electron microscopy (TEM) image, the as-synthesized SiO_2 @MONs exhibits a core/shell structure (Fig. S1). After etching in ammonia solution, the SiO_2 core was removed and obtained the hollow HMONS of around 85 nm in size (Fig. 2a). Elemental mapping images show the presence of sulfur with the coexistence of silicon, carbon, and oxygen in HMONS (Fig. 2b–f), thus suggesting the formation of disulfide bond hybrid silsesquioxane framework within HMONS, as further proved by energy-dispersive spectrometry (EDS) analysis (Fig. S2). Meanwhile, the characteristic C signals in ^{13}C cross-polarization solid-state NMR spectra and silicon resonances in the ^{29}Si magic-angle spinning confirms the successful hybridization of disulfide bond into the framework of HMONS (Fig. S3). The amino-functionalized HMONS (HMONS- NH_2) were prepared and then covalently modified with 3BP through the amide reaction between carboxyl groups of 3BP and amino groups of HMONS- NH_2 (HMONS-3BP). The presence of peaks of amide group at 1623 (C=O stretching vibration) and 3278/cm (N-H stretching vibration) in Fourier-transform infrared (FT-IR) spectrum demonstrate the success formation of HMONS-3BP (Fig. S4). According to the N_2 absorption-desorption isotherms analysis, the BET surface area of HMONS decreased from 481 m^2/g to 301 m^2/g after the incorporation of 3BP (Fig. S5a), and the pore size decreased to about 3.8 nm (Fig. S5b). Even so, this HMONS-3BP was still highly suitable for encapsulation of hydrophobic molecules. Thus, organic sonosensitizers HMME was next loaded into the hollow cavity of the HMONS-3BP (designated as HMME@HMONS-3BP). Meanwhile, this nanosonosensitizer was further PEGylated through noncovalent interactions for improving the stability (yielding HHBP). It can be found from TEM images that the surface engineering and drug loading exhibited negligible change of morphology (Fig. 2g), while the hydrodynamic diameter of HHBP was increased to 142 nm (Fig. 2h), slightly larger than that of HMONS-3BP. After loading of HMME and PEGylation, the BET surface area was decreased to 206 m^2/g and the mesopore size was also dramatically reduced to about 2.4 nm (Fig. S5). Also, the serial change of zeta potential further reconfirms the desirable synthesis in each step (Fig. S6). Furthermore, UV-vis spectra of HHBP show the characteristic absorption peaks at 398 nm (Fig. 2i), which originates from the HMME

molecules. The loading capacity of HMME was found to be around 38% (HMME: HMONS-3BP, w/w) when the HMONS-3BP/ HMME feeding ratios is 0.5 (Fig. S7). These results suggested that HMONS could be efficiently loaded with HMME and modified with 3BP and be coated with PEG on the surface.

As the disulfide-bridged silsesquioxane framework of HMONS can be cleaved in the reductive TME [42, 43], the biodegradation behavior of HHBP was evaluated in simulated body fluids (SBF) mimicking intracellular redox conditions (GSH, 5 mM or 10 mM) in TME. From the TEM images, HHBP in SBF without GSH shows a certain extent but unobvious biodegradation during one week immersion in SBF. In contrast, HHBP are inclined to be gradually degraded in SBF solution containing 5 mM GSH and shows a time-dependent biodegradable behavior (Fig. S8). Especially, the biodegradation rate was significantly quicken and the nanoparticles were found to be entirely biodegraded in SBF solution containing GSH (10 mM) for 7 d. These results indicated the GSH-responsive biodegradability of this HHBP nanosized sonosensitizers.

In vitro HMME release and SDT effect of HHBP

Based on the unique GSH-sensitive biodegradation behavior of HHBP, we further evaluated their releasing performance and behavior under different GSH concentrations and pH values (Fig. 2j). As expected, the HMME release behavior from HHBP is highly dependent on its pH values as well as GSH concentrations. The percentage of HMME released after 24 h was less than 10% at pH 7.4 in absence of GSH, indicating the high stability of HHBP under physiological conditions. In contrast, the amount of released HMME dramatically increased to 26.2% at pH 5.5. Notably, the drug-releasing percentage sharply increased to $\approx 33.7\%$ and $\approx 60.5\%$ under GSH concentrations of 10 mM at pH 7.4 and 5.0, respectively. This finding can be attributed to the gradual biodegradation of the HMONS framework induced by GSH-induced cleavage of the disulfide bond. Excitingly, the release rates are further enhanced and approximately 76.5% HMME release was observed after 24 h upon exposure to US irradiation, which may be due to the dissociation of HMME from HMONS caused by the mechanical/cavitation effects of US [2, 44, 45]. Taking into account the tumor environment is distinguished by mild acidic and high GSH concentration, the pH/GSH/US tri-stimuli-responsive HHBP are expected to deliver hydrophobic sonosensitizers for substantially enhanced SDT efficacy.

Subsequently, the SDT performance of HHBP was evaluated based on the presence of HMME, by using 1,3-diphenylisobenzofuran (DPBF) as a $^1\text{O}_2$ probe to measure the ROS production under US irradiation (Fig. 2k). After irradiation with the US, the production of $^1\text{O}_2$ was further improved, as evidenced by the attenuated UV absorption peak of DPBF at 398 nm when the US irradiation time increased. Additionally, electron spin resonance (ESR) with the spin traps of 2,2,6,6-tetramethylpiperidine (TEMP) were also acquired. According to ESR spectra (Fig. 2l), the strong $^1\text{O}_2$ signal (1:1:1) was detected in the HHBP + US group, while no obvious ESR signal could be observed in the HHBP alone group and PBS group, even upon irradiation with US (1.0 W/cm², 1.0 MHz, 50% duty circle, 1 min). The above results indicate that HHBP could be an ideal nanosonosensitizer for US-triggered ROS generation.

Intracellular uptake and hypoxia alleviation of HHBP

Inspired by the afore-mentioned results, we next evaluated the cellular uptake and O₂-consumption reduction ability of HHBP in 4T1 cells. Initially, the in vitro cytotoxicity of HMONS-PEG was determined by a cell counting kit-8 (CCK-8) assay. HMONS-PEG shows no significant cytotoxicity to various cancer cells in vitro (Fig. 3a), including HUVEC, 4T1, A375, and A549 cells, even at high concentrations of 400 µg/mL, demonstrating the relatively good biocompatibility of HMONS-PEG. In order to evaluate the intracellular endocytosis behavior, the cellular uptake efficiency of HHBP in cells was investigated. Confocal laser scanning microscopy (CLSM) images notices a time-dependent endocytosis process, as evidenced by increased FITC signals at extended co-incubation durations (1, 2, 4, and 8 h) of HHBP with 4T1 cancer cells (Fig. 3b). More excitingly, after further irradiated with US, a stronger green fluorescence was found in 4T1 cells in the presence of HHBP. In addition, flow cytometry analysis was also performed to measure the fluorescence intensity of FITC (Fig. S9). The cells treated with HHBP exhibit high uptake efficiency as the incubation time prolonged and the US stimulation could enhance the cellular uptake of nanoparticles, which are consistent with the CLSM observation.

As an excellent respiration inhibitor, 3BP is expected to be a hypoxia ameliorator for reducing O₂ consumption, which is the essential condition for amplifying SDT.[46] To verify it, the expression of and hexokinase (HK)-II, which involves in the first stage of cellular respiration, were evaluated in the cells after treated with different formulations by western blotting analysis (Fig. 3c). Notably, negligible change of HK-II and HIF-1α levels was detected in 4T1 cell after incubated with HMME@HMONS-PEG nanoparticles, while HHBP with or without US irradiation could markedly reduce the protein expression levels (Fig. S10). Arguably, the introduction of 3BP played a vital role in the down-regulation of HK-II and HIF-1α. These results proved that the inhibition of HK-II used by such HHBP exposure could inhibit cellular respiration, which would cause changes on cellular oxygen consumption. Furthermore, the effect of HHBP under US irradiation to the mitochondrial dysfunction was evaluated by measuring the variation of mitochondrial membrane potential, with commercial JC-1 dye. We found that the 4T1 cells treated with HHBP with or without US irradiation displays strong green fluorescence (Fig. 3d), in marked contrast to the prominent red fluorescence after treated with PBS or HHP, indicating respiration is further suppressed.

In vitro augmented SDT efficacy of engineered HHBP nanosonosensitizer at cellular level

Inspired by the excellent O₂-economization properties of HHBP nanosonosensitizer, the intracellular ROS generation and in vitro augmented SDT efficacy were further assessed. The intracellular ROS levels was visualized by a of 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) probe, which could be oxidized into green fluorescent, 2',7'-dichlorofluorescein (DCF) in the presence of ROS [47, 48]. As revealed in Fig. 3e, negligible fluorescence was detected in PBS, HMME@HMONS-PEG, and HHBP group, while the cells treated with the latter two under the US irradiation display conspicuous green fluorescence, indicating a strong ROS generation. Further quantitative flow cytometric results summarized in Fig. 3f demonstrated that the cellular fluorescence intensity of HHBP + US treatment was significantly higher than that of other treatments. As a result, HHBP is able to induce plentiful ROS production under US irradiation, shows great

potential for SDT against tumors. Encouraged by the prominent ROS generation performance of HHBP, in vitro augmented SDT effect was assessed on 4T1 cell by CCK-8 assay. As expected, the viabilities of the cells after treatment with “HMME@HMONS-PEG + US” and “HHBP” decreased to 62.5% and 44.9% at a HMME concentration of 38 $\mu\text{g/mL}$, respectively (Fig. 4a). In contrast, much lower cell viabilities were detected after treated with HHBP at corresponding concentrations under US irradiation, suggesting the excellent synergistic effect of SDT and 3BP. Similar results were also revealed by calcein AM/PI stain assay (Fig. 4b). Moreover, the treatment of HHBP under US irradiation could also achieved effective inhibition effect on diverse tumor cells including A375 and A549 cells with a strong concentration-dependent cytotoxicity (Fig. S11). Significantly, almost no green fluorescence could be observed when the cells were treated with HHBP and US irradiation. In addition, the apoptosis effect induced by the synergistic effect was evaluated by Annexin V-FITC/PI based flow cytometer assay (Fig. 4c). The apoptosis/necrosis rate of cells treated with “HHBP + US” was 69.7%, which was much higher compared with other treatments. Taken together, the HHBP nanosonosensitizer could reduce O_2 consumption for enhanced SDT, promising in improving the therapeutic outcomes cancer treatment.

Pro-death autophagy induced by HHBP promoted cancer cell apoptosis

Here, it should be noted from the vitro therapeutic results that HHBP treatment without US irradiation can also acquire moderate inhibitory effects. Therefore, 3BP implicated an essential role in inhibiting cell viabilities. In previous studies, 3BP can suppress both mitochondrial respiration and glycolysis to lead to starvation-induced autophagy [46]. To further verify the hypothesis that 3BP can induced pro-death autophagy, we evaluated the expression of autophagy-related protein (LC3 and p62) in 4T1 cells receiving different treatments by Western blot assay. Notably, highest cellular autophagy levels were detected when treated with HHBP plus US irradiation (Fig. 4d), which was proved by the p62 inhibition as well as the LC3-II/LC3-I ratio elevation. Quantification results shown on Fig. S12 further indicated the LC3-II/LC3-I ratio increased by 98-fold after the HHBP + US treatment comparing with control cells, while the expression of p62 reduced to 1.5%. These phenomena demonstrate that the synergistic effect of 3BP and SDT could effectively induce autophagy in 4T1 cells. Furthermore, immunofluorescence staining was performed to characterize the autophagy level by visualized monitoring of the LC3 punctate dots (Fig. 4e). Both HHBP alone and HMME@HMONS-PEG + US treatments exhibit green significant fluorescence signals, which are attributed to the autophagy induced by 3BP and ROS, respectively. When combined with HHBP and extra US irradiation, the green fluorescence was significantly enhanced. The formation of acidic vesicular autophagosomes during autophagy process was examined by utilizing the monodansylcadaverine (MDC) staining (Fig. 4e). It can be observed that the treatment of HHBP incubation plus US exposure resulted in most strong green fluorescence of acidic autophagosomes compared to the cells treated with HHBP and HMME@HMONS-PEG + US. To observe the autophagosomes more intuitively, the 4T1 cells receiving different treatments were observed by TEM (Fig. 4f). Unequal in quantity of autophagosomes could be detected in all treatments except the control group, which indicated that 3BP or SDT-induced ROS could trigger cell autophagy. Meanwhile, HHBP incubated 4T1 cells exposed by US represented more autophagic vesicles than that in other treatments. These

results suggested that the synergistic effect can activate the excessively autophagy, which could also promote cell death. Concomitantly, the ATP level in 4T1 cells was investigated. As shown in Fig. S13, HMME@HMONS-PEG treatment had no obvious influence intracellular ATP level as compared to the control group. However, both 3BP and HHBP dramatically reduced the ATP to 17.8% and 20.2%, respectively, suggesting the depletion of ATP induced by 3BP could significantly elevated the autophagy level. These results agreed well with the therapeutic evaluations described above and offered reliable evidence that the improved anticancer activity of HHBP-mediated augmented SDT could be ascribed to the synergistic effect of apoptosis and autophagy.

In vivo biodistribution of the HHBP nanosonosensitizer

The in vitro outstanding properties of the nanosonosensitizer encouraged us to further assess the therapeutic performance on 4T1 breast tumor xenografts in nude mice. For tracking the in vivo distribution and tumor accumulation behavior of HHBP, a near infrared dye, indocyanine green (ICG) was loaded to HHBP through a simple mixing method. From the in vivo fluorescence images (Fig. 5a), the fluorescence signals in the tumor site were increased upon prolonging time, with the maximum signal at 12 h post-injection, which is also maintained at 16 h, and then declined due to the degradation of ICG. Correspondingly, the tumor and main organs of mice were excised at 24 h for ex vivo imaging. Obviously, with ICG-HHBP-treated mice showed strong ICG fluorescence in the tumor (Fig. S14). Also, strong fluorescence signal was detected in liver, which may be attributed to the specific uptake of mononuclear phagocyte system [49]. These results suggested the favourable tumor accumulation performance of HHBP.

In vivo alleviation of hypoxia

On the basis of the above in vitro findings, HHBP is expected to be a hypoxia modulator for reducing O₂ consumption, which is beneficial to overcome tumor hypoxia and improving SDT efficiency. Before testing the antitumor efficacy, the ability of HHBP to ameliorate tumor hypoxia was evaluated by hypoxia inducible factor-1 α (HIF-1 α) immunofluorescence staining assay. As observed under the fluorescence microscopy (Fig. 5b), extensive green fluorescence was observed in PBS and HMME@HMONS-PEG group, confirming the overexpression of HIF-1 α under hypoxic conditions. In contrast, the indicators of HIF-1 α were obviously downregulated after treatment with HHBP with or without US irradiation, which proved that 3BP could effectively relieve tumor hypoxia.

In vivo antitumor efficacy enabled by HHBP nanosonosensitizer

To investigate whether the HHBP-mediated augmented SDT strategy was applicative to in vivo experiments, the therapeutic performance of HHBP against 4T1 tumor-bearing mice model was performed. Mice were randomly divided to six groups (n = 5): (I) PBS, (II) HMME@HMONS-PEG, (III) HMME@HMONS-PEG + US, (IV) HHBP, and (V) HHBP + US. The injected dosage was set as HMME 8 mg/kg and 3BP 3.4 mg/kg (100 μ l). The tumor site of mice in Group I and II were exposed to US (1.5 W/cm², 1.0 MHz, 50% duty circle) for 5 min and twice as much US exposure on days 1 and 4, respectively. To

examine the ROS level at tumor sites, the tumors after different treatments were collected after US irradiation for dihydroethidium staining (Fig. 5c). It was uncovered that both HMME@HMons-PEG and HHBP under US exposure could improve ROS levels, both of which were obviously higher than other treatments. More specifically, higher ROS level could be achieved by HHBP + US treatment, which validated that 3BP could relieve tumor hypoxia for cause tremendous ROS generation via the amplification of HMME-initiated sonodynamic effect. Following the treatments, tumor volumes (Fig. 6a) and body weights (Fig. 6b) of the mice monitored within two weeks. Rapid tumor growth displayed in PBS and HMME@HMons-PEG treated group, while HHBP and HMME@HMons-PEG plus US exposure led to moderate growth of tumors (inhibition rate: 40% and 28.5%, respectively). More excitingly, the tumors on the mice after treated with HHBP and US exposure were the most effectively suppressed (inhibition rate: 89.1%). The corresponding images of representative tumors on day 14 in different treatments further confirmed that the HHBP + US group realized an intelligent antitumor effect (Fig. 6c). In addition, there were negligible body-weight loss of mice in any of the groups during the treatment (Fig. 6b), demonstrating the low adverse effects of all formulations in vivo. The above desirable therapeutic efficacy might be benefited from the excellent synergistic therapeutic effect, including the 3BP-mediated hypoxia modulation, HMME-based augmented SDT, as well as 3BP-induced excessive activation of autophagy.

To further reveal the performance mechanism of HHBP, the apoptosis and autophagy level of the collected tumors from all groups were evaluated. Compared to other treatments, most significant large-area histological damage regions from H&E staining, most significant inhibition of cell proliferation from Ki-67 assay, as well as the highest evident apoptosis of tumor cells from TUNEL staining were observed in the “HHBP + US” treatment group (Fig. 6d). Such results are consistent with the foregoing results of in vivo anticancer experiment. Subsequently, we also checked the autophagy levels in tumor sections after different treatments by immunofluorescence imaging staining of LC3 (Fig. 6e). It was found that both HMME@HMons-PEG + US and HHBP alone could improve autophagy levels, according to the green fluorescence of LC3 puncta showed in immunofluorescence images. Consistent with the in vitro data, the HHBP group showed the strongest green fluorescence after US irradiation, which represented an excessive activation of autophagy by 3BP and SDT.

Last, but not the least, the safety assessment of HHBP on mice was performed. H&E sections of the collected major organs did not observe obvious inflammation after injection with HHBP for 3, 7, and 14 days (Fig. S15), indicating the favorable biocompatibility of HHBP. Concomitantly, the blood sample was collected from healthy female Balb/c mice at the different times post-injection of HHBP for blood biochemistry and blood routine examination. No obvious changes detected in relation to blood routine indexes and liver/kidney function associated biomarker induced by HHBP compared to the control group (Fig. S16), partly indicating ignorable systemic toxicity. These results indicated that the fabricated HHBP may augment SDT efficacy with causing relatively lower systematic toxicity in vivo.

Conclusion

In summary, we proposed an O₂-economizer strategy to reverse the tumor hypoxia for improving the therapeutic efficacy of SDT, instead of the currently mainstream hypoxia regulation precept of elevate intratumoral O₂ level. To prove the concept, a biodegradable reduced expenditure bioreactor (HHBP) was constructed by the integration of 3BP and HMME into biodegradable HMONs, which is respond to TME and subsequently augmenting SDT efficacy. The 3BP inhibit cell respiration through down-regulation of HKII expression, which decreasing the O₂ consumption and sparing more O₂, facilitating the SDT by HMME against hypoxic tumors. Importantly, the enhanced ROS generation and 3BP can transform the actor of autophagy from self-protection to pro-death, which was proved by the increase of the LC3 level and the inhibition of p62 expression. Consequently, HHBP nanosonosensitizers offered more efficient antitumor performance than the traditional SDT sonosensitizer, which has been systematically verified both in vitro and in vivo. This O₂-economizer concept not only offers a general strategy to alleviate the tumor hypoxia in sonodynamic therapy but also paves a new way for manipulating autophagic processes to enhance the efficacy of ROS-mediated cancer therapy.

Declarations

Supporting Information

Supporting Information accompanies this paper at: <https://doi.org/xx.xxxx/xxxxx xxxxxxxxxx>.

Authors' contributions

WZ, JH and JW conceived the study and participated in the experiment design. WZ performed the experiment, and XC provided experimental technical guidance. WZ and JW completed figures construction and manuscript writing. ZW, BH and YZ checked and revised the manuscript. All authors discussed the results and approved the final version.

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Availability of data and materials

The datasets and materials used in the study are available from the corresponding author.

Ethics approval and consent to participate

All the animal procedures were performed under the protocol approved by the Institutional Animal Care and Use Committee of Shanghai Jiao Tong University Medical College. All the animal experimental

operations were in compliance with the National Guidelines for Animal Protection.

Consent for publication

All authors have approved the manuscript be submitted.

Competing interests

The authors declare no competing interests.

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Figures

Figure 1

Schematic illustrations of the construction of reduce expenditure bioreactor based on HHBP nanosonosensitizer and the therapeutic mechanism of the HHBP to enhance the SDT efficacy via regulating tumor hypoxia and inducing pro-death autophagy.

Figure 2

Characterization and in vitro performance of HHBP. a TEM image and (b-f) the elemental mappings of C, O, Si, and S of HMONS nanoparticles. g TEM image of HHBP. h Hydrodynamic diameter of HMONS, HMONS-NH₂, HMONS-3BP, and HHBP. i UV-vis absorption spectra of 3BP, HMME, HMONS, HMONS-3BP, HMME@HMONS-PEG, and HHBP. j Release profiles of HMME from HHBP at different pHs and GSH concentration with or without US irradiation (1.0 W/cm², 1.0 MHz, 50% duty circle, 1 min, n = 3). k Time-dependent oxidation of DPBF indicating 10² generation by HHBP under US irradiation. l ESR spectra demonstrating 10² generation for different groups.

Figure 3

Intracellular uptake, hypoxia alleviation and SDT efficacy of HHBP. **a** Relative viabilities of HUVEC, 4T1, A375, and A549 cells after incubation with HMONS-PEG nanoparticles (concentrations of 25, 50, 100, 200, and 250 µg/mL) for 24 h. **b** CLSM images of 4T1 cells incubated with FITC-conjugated HHBP for 1, 2, 4, and 8 h without or with US irradiation. Scale bar: 25 µm. **c** Western blot assay results of HIF-1α and HK-II expression in 4T1 cells after different treatments. Fluorescence images of 4T1 cells after different treatments and stained with **d** JC-1 dye and **e** an intracellular ROS probe, DCFH-DA. Scale bar: 50 µm and 100 µm. **f** Flow cytometry data to show intracellular total ROS after different treatments. In panels **d**, **e**, **f** treatment groups are denoted as: (I) PBS, (II) HMME@HMONS-PEG, (III) HHBP, (IV) PBS + US, (V) HMME@HMONS-PEG + US and (VI) HHBP + US.

Figure 4

Pro-death autophagy induced by HHBP promoted cancer cell apoptosis. **a** Relative viabilities of 4T1 cells after different treatments, quantified by CCK-8 assay ($n = 6$). *** $P < 0.001$. **b** Fluorescence images of 4T1 cells stained by calcein AM (green) and PI (red) after treated with PBS, HMME@HMONS or HHBP without or with US irradiation. Scale bar: 200 μm . **c** Flow cytometry analysis of annexin V-FITC/PI-stained 4T1 cells after treated with PBS, HMME@HMONS or HHBP without or with US irradiation. **d** Western blot assay of p62, LC3-I, and LC3-II levels in 4T1 cells after different treatments. (□) PBS, (□) HHBP, (□) HMME@HMONS, (□) HHBP + US, and (□) HMME@HMONS + US. **e** Representative immunofluorescence staining of LC3 punctate dots and MDC-stained fluorescent images after 4T1 cells were treated with HMME@HMONS or HHBP for 4 h with or without US irradiation. Scale bars: 100 μm . **f** TEM images showing the formation of autophagosomes after 4T1 cells were treated with HMME@HMONS or HHBP for 4 h with or without US irradiation. Scale bars: 2 μm . The yellow arrows in the magnification of TEM images represents the typical structures of autophagosomes. Scale bars: 1 μm .

Figure 5

In vivo biodistribution of the HHBP and alleviation of hypoxia. **a** In vivo fluorescence images of 4T1 tumor-bearing mice at different time points after intravenous injection of ICG-loaded HHBP. The red arrows indicate the tumor sites. **b** HIF-1 α staining tumor tissues harvested from 4T1 tumor-bearing mice after different treatments. **c** Fluorescence images of tumor slices collected at 24 h post different treatments and stained with the ROS probe, dihydroethidium (DHE, red).

Figure 6

In vivo antitumor efficacy enabled by HHBP. a Tumor growth curves following different treatments (n = 3). ***P < 0.001. b Body weight of 4T1 tumor-bearing mice during different treatments (n = 3). c Representative images of the excised tumors after different treatments. (□) PBS, (□) HMME@HMONS-PEG, (□) HHBP, (□) HMME@HMONS-PEG + US, and (□) HHBP + US. d Histological and immunohistochemical analysis of H&E, Ki67 and TUNEL assays for the excised tumor tissues after various treatments. Scale bar: 200 μ m. e Representative immunofluorescence images of LC3 in tumor tissues of mice after different treatments. Scale bar: 100 μ m.

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