The Strategy of Precise Targeting and In Situ Oxygenating for Enhanced Triple-Negative Breast Cancer Chemophototherapy

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Abstract

The absence of effective therapeutic targets and tumor hypoxia are the main causes of failure in the treatment of triple-negative breast cancer (TNBC). Biomimetic nanotechnology and tumor microenvironment (TME)-responsiveness bring hope and opportunity to address this problem. Here, we develop a core membrane nanoplatfrom (HM/D-I-BL) using hollow mesoporous manganese dioxide (HM) coated with biomimetic cancer cell membrane for enhanced chemotherapy/phototherapy via the strategy of precise drug delivery and hypoxia amelioration. Cancer cell membrane modification endows HM/D-I-BL with excellent homologous targeting and immune escape performance. The cellular uptake and fluorescence imaging studies confirmed that HM/D-I-BL can be accurately delivered to tumor sites. HM/D-I-BL also features efficient in situ O₂ generation in tumor upon laser irradiation, and subsequently enhanced chemotherapy/phototherapy, pointing to its usefulness as a TME-responsive nanozyme to alleviate tumor hypoxia in the presence of H₂O₂. In addition, HM/D-I-BL showed good fluorescence and magnetic resonance imaging performances, which offers a reliable multimodal image-guided combination tumor therapy for precision theranostics in the future. In general, this intelligent biomimetic nanoplatfrom with its homotypic tumor targeting, in situ alleviation of tumor hypoxia and synergetic chemophototherapy would open up a new dimension for the precision treatment of TNBC.

Introduction

According to the latest global cancer data, breast cancer (11.7% of all newly diagnosed cancers) has surpassed lung cancer (11.4%) as the most commonly occurring cancer worldwide and ranks as the first leading cause of cancer mortality in most countries in 2020.¹ Triple-negative breast cancer (TNBC), which is defined by the absence of estrogen receptor (ER), progesterone receptors (PR), and human epidermal growth factor 2 (HER2) is the most malignant subtype of breast cancer and exhibits a poor prognosis.² The lack of effective receptors has greatly limited the successful development of targeted and specific therapy for TNBC, which results in few systemic treatment options besides the use of chemotherapy. Therefore, finding effective targeted and specific therapy is urgently needed to improve the prognosis of TNBC.

Improving drug-targeted delivery into tumor tissues is crucial in traditional chemotherapy and other novel therapeutic modalities. The traditional strategy of targeted drug delivery usually use hydrophilic polymers and various ligands as surface modification agents to extend circulation time and achieve specific targeting.³ However, this method still have some shortcoming, such as cumbersome technology, new immunological response and rapid blood clearance.⁴⁻⁷ Moreover, patients whose specific target molecules have not been found, including TNBC patients, put forward higher requirements and challenges for traditional targeting strategies. In order to develop more effective methods for drug delivery *in vivo*, enormous efforts have been made by researchers. One of the most prominent approaches to NP functionalization is cell membrane coating technology which directly utilizes entire cell membranes as a material for NP coating.^{8,9} When NPs are enclosed in a natural cell membrane, the

membrane-camouflaged NPs have been revealed to offer selective targeting and precise self-recognition, gaining long-term circulation, immune evasion, and targeting capabilities.⁹⁻¹³ Studies have shown that CD47 on the surface of cancer cells can interact with SIRP α on phagocytic cells to realize the immune escape and prolong blood retention time, and the interaction of surface adhesion molecules such as N-cadherin, T antigen-galectin-3 and epithelial cell adhesion molecule 74 (EpCAM74) mediates homologous cell adhesion between malignant tumor cells.¹⁴⁻¹⁷ Inspired by this, the construction of biomimetic liposomes modified by specific tumor membrane proteins can not only load drugs, but also have the homologous targeting and immune escape ability, which is may a promising solution to address the targeting problem for TNBC treatment.

For TNBC population, the other reasons for the treatment failure are therapy resistance caused by single treatment and the hypoxia tumor microenvironment (TME).^{18,19} Consequently, only improving drug delivery is still insufficient. Developing methods to improve the anti-tumor efficacy by overcoming the therapy resistance of TNBC is also an urgent clinical problem for researchers. To acquire the therapeutic benefit, collaborative therapy using various modalities has become a vigorous trend, typically the synergism of phototherapy and chemotherapy. However, increasing evidence shows that the hypoxia TME has greatly limited the therapeutic efficacy of phototherapy and chemotherapy.^{20,21} For example, cancer cells in a hypoxia environment exhibited increased drugs efflux and reduced ROS induced DNA damage, while selectively eliminate the hypoxia cells can enhance antitumor response.²²⁻²⁴ Consequently, substantial research efforts have been focused on the development of effective ameliorating hypoxia strategies based on nanotheranostics to amplify therapeutic effect for TNBC treatment. In recent years, enzymes and catalytic nanomaterials (nanozymes) have been widely used as oxygen-replenishing agents through reaction with TME for in situ O₂ production with appreciable anti-tumor efficiency.²⁵⁻²⁷ Compared to natural enzymes, nanozymes possess the features of low cost, good stability, high catalytic capacity, and easy scalability, and have attracted extensive attention of researches.^{28,29} As an ideal TME responsive nanozyme, manganese dioxide (MnO₂) can continuously supply sufficient O₂ to alleviate tumor hypoxia by degradation in acidic and hydrogen peroxide (H₂O₂) rich conditions of tumor.³⁰ In addition, compared with normal cells, the glutathione (GSH) concentration in cancer cells is about 1000 times higher, and GSH can protect cells against on a variety of oxidizing species weakening ROS dependent therapy, such as photodynamic therapy (PDT) and chemodynamic therapy (CDT).^{31,32} MnO₂ can be reduced into Mn²⁺ by the GSH in cancer cells, resulting in GSH depletion and Fenton-like reaction for enhanced cancer PDT and CDT.^{33,34} Moreover, as the reduction product Mn²⁺ has T1-weighted magnetic resonance imaging (MRI) ability, redox-active MnO₂ can serve as a molecular switch for tumor MRI. In general, MnO₂ is a multifunctional theranostic agent for improving TNBC therapy.

Here, we designed an intelligent multifunctional TME-responsive theranostic platform based on biomimetic cancer cell membrane and hollow mesoporous MnO₂ (HM) for the delivery of tumor-targeted phototherapeutic and chemotherapeutic agents. This nuclear membrane nanocomposites (HM/D-I-BL) utilized 4T1 cell membrane proteins camouflaged indocyanine green (ICG)-loaded liposomes as outer

biomimetic cancer cell membrane for active target, and utilized DOX loaded HM as core for TEM responsive mediated passive target and in situ tumor hypoxia alleviation (Scheme 1). *In vitro* studies revealed that when the HM/D-I-BL is exposed to high level of H_2O_2 , GSH, and laser irradiation, the increased permeability of outer membrane would lead to O_2 production and release of DOX, simultaneously resulting in significantly enhanced T1-weighted MRI signal, suggesting excellent TME-responsive ability of HM/D-I-BL. Meanwhile, owing to excellent active targeting and O_2 generation ability, HM/D-I-BL exhibited remarkably enhanced chemotherapeutic and phototherapeutic efficacy after laser irradiation. *In vivo*, using a TNBC mouse model, the active targeting ability of HM/D-I-BL was further verified by near-infrared (NIR) fluorescence imaging and MRI. It is also worthy of note that phototherapy combined with chemotherapy provided better inhibiting effect of TNBC with the proposed HM/D-I-BL. Therefore, this intelligent multifunctional nuclear membrane nanocomposites with the ability to active tumor targeting and tumor hypoxia amelioration could provide a promising protocol for enhanced chemotherapy and phototherapy against TNBC.

Experimental

Materials

Tetraethyl orthosilicate (TEOS) and 1,3-diphenyl-isobenzofuran (DPBF) were purchased from Sigma-Aldrich (China). 4,6-diamidino-2-phenylindole (DAPI), Cell Counting Kit-8, and Membrane Protein Extraction Kit were obtained from Beyotime Institute of Biotechnology (Jiangsu, China). Potassium permanganate ($KMnO_4$), ammonium hydroxide ($NH_3 \cdot H_2O$), and sodium carbonate (Na_2CO_3) were obtained from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China). Hydrogen peroxide (H_2O_2) 30 wt% solution, Doxorubicin (DOX), Lecithin, and Cholesterol were purchased from Aladdin (China). ICG was purchased from Dalian Meilun Biotechnology Co., Ltd. (Dalian, China). 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene-glycol)-2000] (DSPE-PEG2000) was obtained from lipid Co. (Ludwigshafen, Germany). The chemicals and solvents were of analytical grade and used as received.

Preparation of DOX-loaded HM (HM/D)

Solid silica NPs ($sSiO_2$) were synthesized following the reported method.³⁵ Then, an aqueous solution of $KMnO_4$ (300 mg) was added dropwise into the suspension of $sSiO_2$ (40 mg) under ultrasonication. After 6 h, the precipitate was obtained by centrifugation at $10,000 \times g$. The as-prepared mesoporous MnO_2 -coated $sSiO_2$ was dissolved in 2 M Na_2CO_3 aqueous solution at $60^\circ C$ for 12 h. The obtained hollow mesoporous MnO_2 (HM) was centrifuged and washed with water several times. For DOX loading, the HM (1 mg/mL) was mixed with different concentrations of DOX for 12 h, yielding HM/D that was used for further experiments.

Preparation of cancer cell membrane proteins

4T1 cell membrane proteins were obtained first by membrane protein extraction kit. Briefly, the collected 5×10^7 4T1 cells (a mouse TNBC line) were dispersed in 1 mL of membrane protein extraction buffer solution A pre-added 1 mM phenylmethanesulfonyl fluoride (PMSF) and placed in an ice bath for 10–15 min. The resulting 4T1 cell suspension was frozen at -80°C and then thawed at room temperature. This freezing–thawing cycle was repeated for 3 times, followed by centrifugation at $700 \times g$ for 10 min at 4°C . The obtained supernatant was further centrifuged at $14,000 \times g$ for 30 min at 4°C to remove cytoplasmic proteins in the supernatant. Then, membrane protein extraction buffer solution B 200 μL was added to the obtained precipitate, followed by violent vortex for 5 seconds and ice bath 5–10 min in turn. The previous steps were repeated for 2 times to fully extract membrane protein. After centrifugation at $14,000 \times g$ for 5 min at 4°C , the supernatant was collected as cell membrane protein solution. The cell membrane protein concentration was determined by BCA assay. Finally, the supernatant was preserved and stored at -80°C for future use.

Preparation of ICG-loaded liposomes

First, ICG-loaded liposomes were synthesized by thin layer evaporation method. Briefly, appropriate amounts of ICG-octadecylamine, lecithin, cholesterol, and DSPE-PEG2000 were dissolved in chloroform at a specific ratio and then rotary-evaporated at 45°C until a lipid film was formed. Subsequently, the obtained lipid film was dried for 3 h under vacuum. The thin-dried phospholipid blends were hydrated at 65°C in PBS to produce ICG-loaded liposomes.

Preparation of biomimetic cancer cell membrane-coated HM/D (HM/D-I-BL)

The HM/D-I-BL was constructed by co-extrusion method using Avanti mini extruder. Briefly, ICG-loaded liposomes and the HM/D were dissolved in water at a concentration of 2 mg/mL, respectively. Then, 0.5 mL of ICG-loaded liposomes (2 mg/mL) and 4T1 cell membrane proteins were extruded at least 10 times by Avanti mini extruder using polycarbonate porous membrane (200 nm). Next, 0.5 mL of HM/D (2 mg/mL) was added into the pretreated ICG-loaded liposomes, and the mixture was extruded at least 10 times using polycarbonate porous membrane (200 nm). Finally, the mixture was centrifuged at $6000 \times g$ for 10 min at 4°C and washed with water for 3 times.

Cell uptake

4T1 cells and antiphagocytosis against macrophage (RAW264.7) cells were seeded in cell culture dishes, and incubated with HM/D-I-L and HM/D-I-BL for 2 h, respectively. The cellular uptake was measured via confocal laser scanning microscopy (CLSM) and flow cytometry. HM/D-I-L and HM/D-I-BL were incubated with different cell lines, including 4T1, bEnd.3, HeLa, MCF-7, and MDA-MB-468 cells for 2 h. Then, cells were washed with PBS and collected for flow cytometry.

O_2 and $^1\text{O}_2$ detection *in vitro*

HM/D-I-BL was dispersed in PBS at different pH (6.5 and 7.4) with GSH (10 mM). Then, certain volume of H_2O_2 was added to the previous solution in turn under vigorous stirring making the final concentration of H_2O_2 to be 10 mM, and the O_2 concentration of solution was monitored by a portable dissolved oxygen meter in real time.

The $^1\text{O}_2$ generation under laser irradiation in the absence or presence of 10 mM H_2O_2 solution by HM/D-I-BL was measured with a 1,3-diphenylisobenzofuran (DPBF) probe. In brief, DPBF solution (20 μL , 10 mM in dimethyl sulphoxide) was added to the sample solution (20 $\mu\text{g}/\text{mL}$, 2 mL) under irradiation (808 nm, 1 W/cm^2) for 5 min. Finally, the absorbance change of DPBF at 420 nm was recorded at appointed times.

Intracellular ROS generation

ROS generation of NPs was detected by DCFH-DA probe. 4T1 cells were seeded in cell culture dishes at a density of 10^5 per well. The cells were treated with ICG, DOX, HM, HM/D-I-L or HM/D-I-BL for 4 h. Then, the cells were irradiated for 5 min (1 W/cm^2) and washed 3 times with PBS. After incubating with DCFDA (10 $\mu\text{mol}/\text{L}$) for 30 min, the cells were further stained with DAPI and finally imaged using Leica DMI3000 inverted microscope.

Mn^{2+} -mediated fenton-like reaction

The $\cdot\text{OH}$ -generation was measured by methylene blue (MB). In detail, HM/D-I-BL was mixed with GSH (1 mmol/L) and received laser irradiation for 5 min before MB solution (final containing 25 mmol/L HCO_3^- , 8 mmol/L H_2O_2 and 4 $\mu\text{g}/\text{mL}$ MB) were added. Then, the mixture was incubated at 37 $^\circ\text{C}$ for 30 min, and the MB degradation was monitored by the UV–Vis absorbance intensity at 665 nm.

Cell cytotoxicity assay

The chemical and photo cytotoxicity of HM/D-I-BL were investigated by CCK-8 as follows: 4T1 cells were seeded at 1×10^4 cells/well in a 96-well plate using 1640 cell culture medium containing 10% FBS. After 24 h incubation, the medium was replaced with 100 μL fresh medium containing different concentrations of free ICG, free DOX or HM/D-I-BL. The HM/D-I-BL+Laser group was exposed to laser irradiation to assess therapeutic effect. After 24 h, CCK-8 assay was used to evaluate cell viability.

Animal models

Female Balb/c mice (6–8 weeks old) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China), and received care in accordance with protocols approved by Ningbo University Laboratory Animal Center (China). 4T1 cells (5×10^6) suspended in 50 μL of PBS were subcutaneously injected into the back of mouse. The mice bearing 4T1 tumors were treated when the volume of tumor reached $\sim 100 \text{ mm}^3$.

Dual-modal imaging

For NIR fluorescence imaging, HM/D-I-BL was administered via tail vein injection when the tumor size was $\sim 100 \text{ mm}^3$. For comparison, HM/D-I-L was administered via tail vein injection in 4T1 tumor-bearing mice. Before and at 2, 4, 8, 12, and 24 h after injection, images were obtained by an *in vivo* imaging system (IVIS Lumina, USA). Then, mice were sacrificed and the major organs (heart, liver, spleen, lung, kidney, and tumor) were collected for *ex vivo* imaging and semi-quantitative biodistribution analysis by using the imaging system. Emission spectra of ICG (710–900 nm) were obtained at a fixed excitation wavelength of 675 nm. Dye accumulation and retention in organs was evaluated by using the NIR analysis software Living Image 4.5.

HM/D-I-BL at different Mn concentrations (0, 0.05, 0.1, 0.2, 0.4, 0.6, and 0.8 mM) in PBS were incubated at different pH (6.5 and 7.4) with or without GSH under laser irradiation before MRI. Images were acquired on a 3.0 T clinical MRI scanner (GE healthcare, USA). The longitudinal (T1) phantom images and relaxation times (T1) were recorded. For *in vivo* MRI, a special coil was used for small animal imaging.

Photothermal properties evaluation *in vivo*

Another important property of HM/D-I-BL was their adjustable photothermal effects. The temperature changes in deionized water, ICG, HM/D-I-BL aqueous solutions under 808 nm laser irradiation at 1.0 W/cm^2 for 5 min were initially investigated.

Therapeutic efficacy evaluation *in vivo*

4T1 tumor-bearing mice were intravenously injected with 200 μL of saline, I-BL, HM/D or HM/D-I-BL (dose of ICG = 0.5 mg/kg, and DOX = 3 mg/kg), respectively. The 808 nm light irradiation was conducted at 2 h post injection (1 W/cm^2 , 5 min). Tumor sizes and body weights were monitored every 3 days for 2 weeks. The tumor volume was calculated following the formula: $\text{volume} = \text{width}^2 \times \text{length} / 2$. The tissue and tumor slices were stained by hematoxylin and eosin (H&E) following the standard protocol.

Results And Discussion

Preparation and characterization of HM/D-I-BL

The procedure for the synthesis of HM/D-I-BL is illustrated in Fig. S1. First, HM-SiO₂ was obtained *via* the reduction of KMnO₄ with organosilica existing on freshly prepared silica NPs. Then, Na₂CO₃ solution was incubated with HM-SiO₂ NPs to dissolve silica for achieving hollow mesoporous MnO₂ (HM).³⁶ Transmission electron microscope (TEM) images of HM clearly revealed the spherical morphology and the hollow structure of HM (Fig. S1). The hollow structure of HM was further confirmed by high-angle annular dark-field scanning TEM (HHAADF-STEM)-based elemental mapping (Fig. S1). Fig. S2 showed Mn 2p and O 1s XPS spectrum of HM. XRD results demonstrated that HM is amorphous (Fig. S3). Next, the HM were mixed with certain concentration of DOX to form H-M/D. To achieve the effective encapsulation of ICG in the lipid bilayer of liposomes, a hydrophobic modification was first performed by conjugating the amino groups on octadecylamine (ODA) with the sulfonic groups on ICG. Then the

extractive membrane proteins and the ICG-loaded liposomes were mixed by successive extrusion to form a stable biomimetic liposome nanomedicine (I-BL). In the process of surface functionalization, the I-BL were mixed with H-M/D by coextrusion method to produce biomimetic cell membrane-coated NPs (HM/D-I-BL). To characterize the specific nanostructure of HM/D-I-BL, TEM imaging was performed. The TEM imaging of HM (Fig. 1A) and HM/D-I-BL (Fig. 1B) demonstrated that the prepared biomimetic NPs are core/membrane nanostructures, when compared to HM, a bright film was formed outside the core. Dynamic light scattering (DLS) analysis showed that the mean size of HM and HM/D-I-BL were 121.67 ± 0.98 nm and 174.64 ± 1.37 nm, respectively (Fig. 1C). The zeta potentials of HM, I-BL, H-M/D, and HM/D-I-BL were -21.3 ± 1.5 mV, -27.1 ± 1.6 mV and -24.8 ± 1.0 mV, respectively (Fig. 1D), indicating HM/D-I-BL is generally spherical in shape with good mono-dispersity and suitable for its potential application *in vivo*. The surface area and average pore diameter of HM were determined to be $121.529 \text{ m}^2/\text{g}$ and 3.932 nm, respectively, by Brunauer–Emmett–Teller (BET) measurement (Fig. 1E). The formation of HM/D-I-BL and H-M/D, as well as I-BL were investigated by measuring their UV–visible absorption. Fig. 1F showed that HM/D-I-BL has an absorption band peak around 780 nm indicating that ICG was successfully loaded. In comparison, I-BL, H-M/D, and HM/D-I-BL showed a UV–visible absorption band around 500 nm, which could be attributed to DOX. Next, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to verify whether the extracted 4T1 tumor membrane proteins were efficiently embedded in the phospholipid bilayer on the surface of NPs. As shown in Fig. 1G, the protein profile of HM/D-I-BL was consistent with that of the 4T1 cell membrane, indicating that 4T1 cell membrane proteins were successfully decorated on the surface of HM/D-I-BL. Moreover, the DOX encapsulation efficiency (EE) of HM/D-I-BL increased from 25.15% to 64.79% when the weight ratio of DOX:HM increased from 0.5 to 4 (Fig. 1H). Further, the DOX release behavior under laser irradiation was investigated when NPs were exposed to GSH solution with different PH. It was found that HM/D-I-BL+GSH showed little DOX release in the absence of laser irradiation. However, HM/D-I-BL+GSH exhibited a remarkably enhanced DOX release after laser irradiation, which was closely related to the intensity and time of irradiation (Fig. 1I, S4A, S4B). Besides, the amount of DOX release in different PH solutions showed no significant difference indicating the effect of reductive environment on the drug release profile is important than pH values. In general, the drug release mechanism might be the disorder and fusion of lipid bilayer caused by high temperature after laser irradiation.

Targeting capability characterization

It has been proven that NPs modified with cancer cell membrane could inherit homologous targeting and immune-evading characteristics from their source cancer cells.^{37,38} To investigate the specific homotypic targeting ability of HM/D-I-BL to homologous cancer cells, the uptake of DOX and ICG of HM/D-I-BL in 4T1 cells was evaluated by confocal laser scanning microscope (CLSM) imaging and flow cytometry. After incubation at 37°C for 2 h with HM/D-I-L and HM/D-I-BL, a higher amount of HM/D-I-BL penetrated the 4T1 cells, which exhibited a stronger fluorescence signal when compared to the HM/D-I-L group (Fig. 2A, 2B), indicating that HM/D-I-BL had homotypic targeting ability. In order to further verify the specific cellular uptake, HM/D-I-BL was evaluated by incubating the NPs with several different cell lines, including

4T1, bEnd.3, HeLa, MCF-7 and MDA-MB-468 cells. The results revealed that the fluorescence intensity of DOX in the 4T1 cell group was higher compared to that of other cell lines (Fig. 2C). The higher uptake efficiency of 4T1 cells confirmed the excellent active targeting ability of HM/D-I-BL, which benefit from cellular adhesion molecules including EpCAM, N-cadherin and galectin-3 on cancer cell membranes mediated specific adhesion junctions between homologous cells.^{16,17} In addition, HM/D-I-L and HM/D-I-BL uptake by RAW264.7 cells were further investigated by CLSM imaging and flow cytometry. As shown in Fig. 2D, only a very small amount of HM/D-I-BL was internalized by RAW264.7 macrophages. Quantitative analysis by flow cytometry showed 0.51-fold fluorescence as compared to that for HM/D-I-L treated cells (Fig. 2E), suggesting the good immune escape ability of HM/D-I-BL. The excellent homologous cellular uptake and immune escape ability of HM/D-I-BL may make it a promising delivery system for TNBC precision nanomedicine.³⁹

O₂ and ROS generation evaluation *in vitro*

Next, the TME-responsive capability of HM/D-I-BL as a multifunctional nanoplatfrom at the *in vitro* level was investigated (Fig 3A). The capability of generating O₂ *in vitro* by HM and HM/D-I-BL upon reaction with H₂O₂ (10 mM) and GSH (10 mM) at endogenous levels was first evaluated. Fig.3B showed that HM could rapidly trigger O₂ generation by a HM concentration-dependent manner. The O₂ generation in the presence of HM/D-I-BL was measured to clarify whether laser irradiation would promote the process. As shown in Fig. 3B-D, the O₂ generation triggered by HM/D-I-BL with laser irradiation was the same with HM, but no obvious O₂ generation was observed from HM/D-I-BL without laser irradiation. O₂ bubbles produced by HM/D-I-BL were visible by eye observation after different treatments (Fig. S5). These results suggested that biomimetic membrane modification can block the reaction between the MnO₂ core and external environment, reducing the production of O₂. Whereas, laser irradiation inducing the outer membrane phase transition took place at a certain temperature, which resulted in an increase on O₂ production.

In addition, ¹O₂ generation from H₂O₂ solution was tested in the absence or presence of laser irradiation (808 nm laser, 1 W/cm², 5 min). The results showed the absorbance of solution in the presence of HM/D-I-BL+DPBF without significant change, while obvious decrease was observed upon laser irradiation, indicating ¹O₂ production ability was remarkably enhanced due to more O₂ production from H₂O₂ and that laser irradiation is an essential element in the process of ¹O₂ production (Fig. 3E). Next, the intracellular ROS generation were observed by using 2',7'-dichlorofluorescein diacetate (DCFDA) probe⁴⁰. There is no obvious fluorescence in control group just exposed to DCFDA, as well as DOX and HM group with laser irradiation showed weak fluorescence. Among the other three groups, the intensity of fluorescence in ICG, HM/D-I-L and HM/D-I-BL suffered from laser irradiation was increased in turn, which were highly consistent with the targeting capability and O₂ generation, further verifying the advantages of HM/D-I-BL.

Finally, after exposed to GSH MnO_2 could be reduced to Mn^{2+} with excellent Fenton-like activity to generate highly toxic hydroxyl radical ($\cdot\text{OH}$) from H_2O_2 in the presence of HCO_3^{3-} for chemodynamic cancer therapy. To investigate the Fenton-like reaction, we utilized the methylene blue (MB) as an $\cdot\text{OH}$ generation probe. Compared with MB solution, the color and characteristic absorbance of $\text{MB}+\text{Mn}^{2+}$ solution was almost vanished, revealing the Fenton-like reaction (Fig. S6). As shown in Fig 3G, the HM/D-I-BL without laser pretreatment and GSH failed to induce Fenton reaction, while an obvious Fenton-like MB degradation was observed in the presence of laser pretreatment and GSH. These results indicated that HM/D-I-BL only with laser pretreatment could release Mn^{2+} in response to GSH, which subsequently generates $\cdot\text{OH}$ via Fentonlike reaction. In general, the O_2 and ROS production, as well as $\cdot\text{OH}$ generation mechanism might be because the HM core was exposed to the outer environment caused by phase transition of lipid bilayer, associated with an increase of the outer membrane permeability after ICG laser treatment induced hyperthermia.⁴¹

Enhanced chemotherapy and phototherapy efficacy *in vitro*

Increasing evidence showed that hypoxic TME is responsible for the limited chemotherapy and PDT efficacy for treatment of solid tumors. Considering that HM/D-I-BL can successfully catalyze H_2O_2 into O_2 , the cytotoxicity of HM/D-I-BL on 4T1 cells was investigated. As shown in Fig. 3H, low dose DOX and HM/D-I-BL showed no obvious cytotoxicity to 4T1 cells for 24 h, whereas HM/D-I-BL plus laser irradiation significantly induced cell death, especially at increased concentration. These results confirmed that 4T1 cells were resistant to low dose DOX, while O_2 and ROS generation after laser irradiation could synergize with DOX and eliminate hypoxia-induced DOX resistance, which is consistent with previous studies.⁴²⁻⁴⁴ The *in vitro* phototherapy efficiency of the NPs was further tested by cell viability assay. The cell viability in ICG (20 $\mu\text{g}/\text{mL}$) group was $103.25\pm8.58\%$, whereas in the ICG+Laser and HM/D-I-BL+Laser group, the viability was $56.39\pm6.37\%$ and $20.63\pm4.04\%$, respectively (Fig. 3I), indicating that HM/D-I-BL plus laser irradiation exhibited excellent phototherapeutic effects on 4T1 cells. These *in vitro* results implied potential advantages for *in vivo* applications.

Dual-modal imaging

MnO_2 is known to be stable under neutral and basic pH, but can be decomposed into Mn^{2+} which has been widely utilizing as a contrast agent for MRI under reductive environment.⁴⁵⁻⁴⁷ Interestingly, the obtained T1-weighted signals of HM/D-I-BL demonstrated a significant concentration-dependent contrast enhancement effect (Fig. 4A). The T1-weighted signal of HM/D-I-BL NPs has not significant difference between pH 6.0 and 7.4 under reductive environment (GSH concentration: 10 mM), and the longitudinal relaxivity (r_1) value was calculated to be 2.344 and 2.114 $\text{mM}^{-1}\text{s}^{-1}$, respectively (Fig. 4B, S7). The remarkably increased relaxivity of the NPs in GSH was attributed to the decomposition of MnO_2 into paramagnetic Mn^{2+} indicating the switch of T1-weighted MR imaging from “OFF” to “ON” states. The T1-weighted MR images of TNBC were examined after intravenous injection of HM/D-I-L or HM/D-I-BL into mice with TNBC at 0, 2, 4, 8, 12, and 24 h (Fig. 4C). TNBC tissue and normal tissue showed no

difference in T1-weighted signal intensity before injection. However, the intensity was significantly increased in the TNBC area from 4 h ($RSI=1.46\pm0.18$) to 8 h ($RSI=2.34\pm0.31$) after injection with HM/D-I-BL but not with HM/D-I-L (Fig. 4D). Moreover, the enhanced T1-weighted signal intensity in TNBC areas lasted for 24 h after injection with HM/D-I-BL ($RSI=1.81\pm0.24$). These results further confirmed the accumulation of HM/D-I-BL in 4T1 tumor tissue and responsive imaging in TEM, which may have great potential for MRI diagnosis of TNBC.

After the establishment of the TNBC model in Balb/c mice, the distribution of HM/D-I-L and HM/D-I-BL in mice was evaluated by NIR imaging. The fluorescence of ICG distributed widely throughout the mouse body after 2 h injection of the NPs (Fig. 4E, 4F). Subsequently, HM/D-I-BL showed remarkable accumulation in the tumor area in mice with TNBC from 2 to 12 h. In contrast, the mice with HM/D-I-L did not show obvious accumulation in the tumor area, which indicated excellent *in vivo* tumor targeting of the NPs. To further prove the *in vivo* uptake of the NPs, *ex vivo* imaging of excised major organs and tumors at 24 h post-injection were investigated (Figure 4G, 4H), and the results were consistent with the previous fluorescence imaging results.

Photothermal properties evaluation *in vivo*

Another important property of HM/D-I-BL is its adjustable photothermal effect. Temperature changes in deionized water, ICG, and HM/D-I-BL aqueous solutions under 808 nm laser irradiation at 1.0 W/cm^2 were initially investigated (Fig. S8). The initial temperature of deionized water, ICG, and HM/D-I-BL solution was 27.9°C , 28.6°C , and 27.7°C , respectively. The solution temperature increased precisely by 22.0°C and 18.8°C (ICG: $20\text{ }\mu\text{g/mL}$). On the contrary, the temperature of pure water exhibited no obvious change. Given the ideal *in vitro* results, the photothermal effect of NPs were further examined *in vivo*. TNBC tumor-bearing mice were randomly divided into three groups and treated with saline+Laser, HM/D-I-L+Laser, and HM/D-I-BL+Laser, respectively. Among the three groups, the temperature of tumor site in HM/D-I-BL+Laser group reached 58.2°C , which was higher than those of other groups, indicating efficient conversion of light to heat by the HM/D-I-BL NPs to induce elimination of cancer cells (Fig. 5A-B).

Therapeutic efficacy evaluation *in vivo*

Mice with TNBC were randomly divided into seven groups: control, I-BL, HM/D, HM/D-I-BL, I-BL+Laser, HM/D+Laser, and HM/D-I-BL+Laser. Tumor volumes of each group were $\sim 100\text{ mm}^3$ before treatments. Tumors were irradiated with 808 nm laser for 5 min (1 W/cm^2) after 2 h injection of the NPs. The therapeutic efficacy of HM/D-I-BL+Laser-enhanced chemotherapy and phototherapy was further evaluated by monitoring tumor weight and volume. On day 15, the tumors of TNBC bearing-mice treated with HM/D-I-BL+Laser showed significant regression ($p<0.001$) (Figure 5C). Besides, tumor regressions induced by HM/D and HM/D+L groups were remarkably weaker than that of HM/D-I-BL group, suggesting the active targeting ability of HM/D-I-BL. However, the tumor weight did not differ between HM/D-I-BL and I-BL plus laser irradiation groups, probably due to from the effective chemotherapy or phototherapy (Fig. 5D). The results of tumor volumes among seven groups were consistent with that of tumor weight (Fig.

5E). These findings due to the hypoxia alleviation of tumor tissue mediated by MnO_2 plus chemotherapy and phototherapy is important for the remarkably improved therapeutic efficacy of HM/D-I-BL plus laser irradiation.

The high-quality pathological H&E tumor slices on day 15 after treatments are shown in Fig. 5G. The corresponding H&E and TUNEL sections showed larger tumor necrosis area and more cell apoptosis with HM/D-I-BL plus laser irradiation treatment, which suggested more complete tumor damage with HM/D-I-BL+Laser enhanced chemotherapy and phototherapy effect. These results suggest that HM/D-I-BL may be a powerful chemical and photo agent, superior to HM/D and I-BL plus laser irradiation, because HM/D-I-BL significantly improved tumor hypoxia resulting from MnO_2 -triggered H_2O_2 decomposition and increased the concentration of drugs in the tumor site induced by biomimetic modification.

To evaluate the potential toxicity of HM/D-I-BL treatment, the body weight loss of mice was used as an indicator for treatment-induced toxicity (Fig. 5F). The result showed the body weight of mice did not differ with various treatments. To further assess the *in vivo* safety of HM/D-I-BL, the main organs of mice on day 15 post-treatment were excised and analyzed. H&E-stained sections of the five tissues (heart, liver, spleen, lung, and kidney) showed no apparent lesion (no necrosis, edema, inflammatory infiltration or hyperplasia) with saline or the NPs injection (Fig. S9), suggesting chemotherapy and phototherapy for TNBC with HM/D-I-BL treatment did not induce significant damage in major organs. Therefore, the FI and MRI monitored chemotherapy and phototherapy synergistic therapy mediated by HM/D-I-BL with advanced tumor inhibition effect and good biocompatibility was promising in anti-TNBC treatments.

Conclusions

We successfully constructed intelligent multifunctional nuclear membrane nanocomposites (HM/D-I-BL). We demonstrated that HM/D-I-BL can specifically could target tumors and deliver drugs into the homology-tumor site *in vitro* and *in vivo* after biomimetic modification, which could effectively address the lacking targeted treatment issue of TNBC. In addition, HM/D-I-BL exhibits an excellent performance in alleviating tumor hypoxia after laser irradiation via interaction with TME, which could effectively enhance the chemotherapy and phototherapy against TNBC. In the presence of GSH, the MnO_2 of HM/D-I-BL can be reduced to Mn^{2+} exhibiting high Fenton-like activity and T1 relaxivity, which could be used as an ideal agent for tumor CDT and MR imaging. Moreover, DOX combined with ICG realized fluorescence image-guided multimodal therapy for TNBC. All these make HM/D-I-BL a promising agent for biomedical applications and can make outstanding contributions to TNBC.

Declarations

Acknowledgements

Not applicable.

Authors' contributions

QL and AW provided financial support and participated in the supervision and coordination of the study; MW and TC conceived and designed the experiments; MW, TC, LW, OA, XM and JX performed most of the experiments; JH, QF and JC analyzed the data; AW, MW and TC contributed to the writing, review and revision of the manuscript. All authors read and approved the final manuscript.

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Ethics approval and consent to participate

All animal experiments were conducted under the Regional Ethics Committee for Animal Experiments of Ningbo University, China [permit no. (Zhe) 2019- 0005].

Consent for publication

All authors agreed to submit this manuscript.

Availability of data and materials

All data generated or analyzed during this study are included in the article.

Competing interests

The authors declare no competing financial interest.

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Schemes

Scheme 1 is available in the Supplemental Files section

Figures

Figure 1

Characterizations of HM/D-I-BL. TEM images of (A) HM and (B) HM/D-I-BL. (C) Size distribution histogram and (D) zeta potential of HM/D-I-BL. (E) Pore-size distribution curve and N₂ adsorption/desorption isotherms (inset) of HM. (F) UV-Vis absorbance spectra of HM, DOX, ICG, I-BL, HM/D, and HM/D-I-BL. (G) SDS-PAGE protein analysis of marker, 4T1 cell, 4T1 cell membrane (m4T1 cell), and HM/D-I-BL. (H) DOX loading weight ratio of HM/D-I-BL at different drug feeding weight ratios. (I) The influence of laser irradiation on DOX leakage (808nm laser, 1W/cm², 5min).

Figure 2

In vitro homotypic targeting and immune escape abilities assessment. Cellular uptake (A) and flow cytometry analysis (B) of 4T1 cells after 2 h incubation with HM/D-I-L and HM/D-I-BL. (C) The homologous targeting ability was verified by flow cytometry using different cell lines after 2 h incubation with HM/D-I-BL. Cellular uptake (D) and flow cytometry analysis (E) of RAW264.7 cells after 2 h incubation with HM/D-I-L and HM/D-I-BL. The ICG concentration was 20 μ g/ml.

Figure 3

In vitro experiments with HM/D-I-BL. (A) A schematic illustration of HM/D-I-BL for TME-responsive drug delivery and oxygen-elevated chemotherapy and phototherapy. (B) O₂ generation at different Mn concentrations (from 0 to 7.50 $\mu\text{g/mL}$) from H₂O₂ (10 mM) and GSH solutions (10 mM) with HM. O₂ generation at different Mn concentrations from H₂O₂ and GSH solutions with HM/D-I-BL in the absence (C) or presence (D) of laser irradiation (808 nm laser, 1 W/cm²). (E) *In vitro* UV absorbance of DPBF at 410 nm after laser irradiation. (F) The fluorescence images of ROS by DCFDA staining in 4T1 cells treated

with various formulations. Nuclei were stained with DAPI (blue). (G) UV-Vis absorbance spectra and photographs of MB after incubating with HM/D-I-BL in the presence or absence of 1 mmol/L GSH for 30 min. *In vitro* cytotoxicity was quantified by CCK-8 assay 24 h after (H) chemotherapy and (I) phototherapy. The concentration of ICG was 20 $\mu\text{g/mL}$. * $p < 0.05$, ** $p < 0.01$ versus the control.

Figure 4

Dual-modal imaging of HM/D-I-BL. (A) *In vitro* T1-weighted MR images of HM/D-I-BL under various concentrations at different pH (6.5 and 7.4) with or without GSH (10 mM). (B) Longitudinal relaxation rate (r_1) of HM/D-I-BL. (C) *In vivo* T1-weighted MR images and (D) corresponding quantified T1-MR signals in tumor of 4T1 tumor-bearing mice after i.v. injection of HM/D-I-L or HM/D-I-BL at different times. (E) *In vivo* fluorescence images and (F) corresponding semi-quantification analysis in tumor of 4T1 tumor-bearing mice taken at different time points post i.v. injection of HM/D-I-L or HM/D-I-BL (three mice per group). (G) *Ex vivo* fluorescence images and (H) corresponding semi-quantification analysis of major organs and tumor dissected from mice at 24 h. Error bars indicate standard deviations of three independent measurements. * $p < 0.05$, ** $p < 0.01$ versus the control.

Figure 5

The photothermal characters of HM/D-I-BL. (A) *In vivo* infrared thermal images of 4T1 tumor-bearing mice after i.v. injection with saline, HM/D-I-L and HM/D-I-BL with the NIR therapy (808 nm laser, 1 W/cm²). (B) The temperature changes of different solutions after laser irradiation. The tumor photographs (C), weight (D) and volumes (E) of 4T1 tumor-bearing mice after different treatments. (F) The body weight of 4T1 tumor-bearing mice after different treatments. (G) H&E and TUNEL staining of the tumor sections from seven groups treated with control, I-BL, HM/D, HM/D-I-BL, I-BL+L, HM/D+L, and HM/D-I-BL+L, respectively. The bar indicates 20 μ m. *p < 0.05, **p < 0.01 versus the control.

Supplementary Files

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