Enhanced Tumor Synergistic Therapy by Injectable Magnetic Hydrogel Mediated Generation of Hyperthermia and Highly Toxic Reactive Oxygen Species

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Supporting Information

ABSTRACT: Nanoparticle-mediated tumor magnetic induction hyperthermia has received tremendous attention. However, it has been a challenge to improve the efficacy at 42 °C therapeutic temperatures without resistance to induced thermal stress. Therefore, we designed a magnetic hydrogel nanozyme (MHZ) utilizing inclusion complexation between PEGylated nanoparticles and α-cyclodextrin, which can enhance tumor oxidative stress levels by generating reactive oxygen species through nanozyme-catalyzed reactions based on tumor magnetic hyperthermia. MHZ can be injected and diffused into the tumor tissue due to shear thinning as well as magnetocaloric phase transition properties, and magnetic heat generated by the Fe₃O₄ first gives 42 °C of hyperthermia to the tumor. Fe₃O₄ nanozyme exerts peroxidase-like properties in the acidic environment of tumor to generate hydroxyl radicals (•OH) by the Fenton reaction. The hyperthermia promotes the enzymatic activity of Fe₃O₄ nanozyme to produce more •OH. Simultaneously, •OH further damages the protective heat shock protein 70, which is highly expressed in hyperthermia to enhance the therapeutic effect of hyperthermia. This single magnetic nanoparticle exerts dual functions of hyperthermia and catalytic therapy to synergistically treat tumors, overcoming the resistance of tumor cells to induced thermal stress without causing severe side effects to normal tissues at 42 °C hyperthermia.

KEYWORDS: magnetic nanoparticles, supramolecular hydrogel, nanozyme, hyperthermia, breast cancer

As a promising tumor treatment strategy, magnetic induction hyperthermia has brought breakthroughs in cancer treatment, especially the magnetic heat treatment based on magnetic nanomaterials.1,2 It uses magnetic nanoparticles as a heating medium and uses intravenous injection or in situ intervention as the delivery route to tumor. Under the application of a noninvasive external alternating current magnetic field (ACMF), the magnetic medium heats up to effects due to the Neel relaxation and Brownian relaxation, causing the tumor tissue to rapidly reach a certain temperature to kill or induce tumor cell apoptosis.3−5 Generally, there are mainly two temperature windows for tumor magnetic induction hyperthermia.6 The magnetic induction thermal ablation treatment with tumor tissue temperature above 50 degrees can remove tumors very effectively and quickly, but it also easily causes damage to surrounding normal tissues.7,8 The lower-temperature range around 40−45 °C can induce cancer cell death without normal cells being damaged but is insufficient because cells always obtain resistance to induced thermal stress.9,10 Many attempts have been made to develop an efficient heating medium or strategy to overcome the limitations of conventional hyperthermia associated with relatively high tumor recurrence. For
example, developing nanoparticles doped with the metal elements zinc, cobalt, and manganese contributes to enhance the heating efficiency. Combining with other treatment modalities is evidenced to amplify the efficacy of hyperthermia, such as photodynamic therapy and chemotherapy. Designing a synergistic therapy platform by integrating multiple heating capability strategies, such as magnetic hyperthermia, photothermal therapy, and magneto-ultrasonic heating, will improve the therapeutic outcome in clinical conditions. However, magnetic hyperthermia is still limited to the complexity of the treatment system design and subsequent tumor recurrence with heat tolerance of tumor at a 42 °C therapeutic temperature, where healthy tissue can avoid being damaged. Therefore, it is urgent to further explore different tumor treatment under conventional thermotherapy to improve the magnetic hyperthermia efficacy and avoid unexpected damage.

The tumor microenvironment (TME) has complex biological microstructures and exhibits many features, such as acidosis and higher intracellular glutathione (GSH). The rapid metabolism of cancer cells and insufficient blood supply lead to elevated hydrogen peroxide (H$_2$O$_2$) levels in tumors. Active tumor conversion of H$_2$O$_2$ to its downstream reactive oxygen species (ROS) through disproportionation can cause oxidative damage to lipids, proteins, and DNA. In our previous study, we revealed that Fe$_3$O$_4$ nanoparticles exhibit double enzyme activity in a pH-dependent manner in vitro and in vivo and catalytically decompose H$_2$O$_2$ into nontoxic H$_2$O and O$_2$ under neutral or alkaline pH conditions, exhibiting catalase activity and scavenging ROS. Under acidic conditions, they can catalyze the disproportionation of H$_2$O$_2$ to produce highly toxic reactive oxygen radicals (*OH), showing peroxidase-like activity. Therefore, Fe$_3$O$_4$ nanoparticles are considered to be potential nanoenzymes for treating tumors. However, the concentration level of H$_2$O$_2$ in tumor cells is not high enough to initiate the catalysis of Fe$_3$O$_4$ nanozyme to generate an effective amount of hydroxyl radicals, which limits the capability of Fe$_3$O$_4$ nanoparticles to exert their peroxidase activity in a tumor acidic environment. Hence, increasing the concentration of H$_2$O$_2$ in tumor tissue is essential for the initiation of catalytic reaction of Fe$_3$O$_4$ nanozyme. Meanwhile, the rapid growth of incomplete blood vessels in tumor tissues leads to an oxygen-deficient environment within solid tumors, which limits numerous in vivo enzymatic reactions that require oxygen to participate. Furthermore, hydroxyl radicals have an extremely short lifetime and diffusion distance, which makes them exert a killing effect on nearby cells.

Herein, we present the design of a magnetic hydrogel nanozyme (MHZ) self-assembled by PEGylated nanoparticles and α-cyclodextrin (α-CD) through inclusion complexation for enhanced tumor synergistic therapy by generation of 42 °C hyperthermia and highly toxic reactive oxygen species. The methoxy poly(ethylene glycol) (MPEG) blocks on the surface of poly(lactic-co-glycolic acid) (PLGA) nanocapsules and perfluorooctyl bromide (PFOB) nanoemulsion act as guest molecules for the crystalline inclusion complex with a cyclic oligosaccharide host molecule, α-CD. The MHZ composite of hierarchical structure enables loading of glucose into the center of the PLGA nanocapsule and D-mannitol as well as glucose oxidase (GOD) into the aqueous bulk of the gel, which also contains Fe$_3$O$_4$@PEI nanoparticles. The MHZ can be easily injected to the tumor site through a needle due to its shear-thinning property and diffusion into the tumor cell gap as it presents a magnetocaloric gel–sol transition when exposed to ACMF. Furthermore, D-mannitol promotes the diffusion of...
nanoparticles in tumor tissues by shrinking cells through dehydration. We expect that the effective diffusion of the various components in MHZ will result in a wider range of production of •OH in the tumor tissue and more significantly oxidative damage. At this time point, successive enzymatic reactions are initiated: First, in the presence of oxygen carried by PFOB nanoemulsions, GOD reacts with glucose to form excess H₂O₂. Second, Fe₃O₄@PEI nanoparticles play a mimicking enzyme function in the slightly acidic environment of the tumor and catalyze the production of •OH by H₂O₂ through the Fenton reaction. The magnetic heat generated by the Fe₃O₄ nanoparticles first gives a 42°C mild hyperthermia treatment to the tumor tissue. At the same time, the hyperthermia promotes the activity of the Fe₃O₄ nanozyme and increases the production of •OH, which further damages the heat shock protein (HSP) 70, which is highly expressed in hyperthermia simultaneously. Magnetic induction hyperthermia and pro-oxidative synergistic treatment based on magnetic nanoparticles showed promising anticancer effects, and 42°C thermotherapy can eliminate subcutaneous tumors of mouse breast cancer without obvious damage to normal tissues (Scheme 1).

RESULTS AND DISCUSSION

Synthesis and Characterization of MHZ. PLGA nanocapsules with clear spherical core–shell structure and spherical shape were synthesized by a double emulsion method. As shown in Figure 1a, the size of the PLGA nanocapsules was found to be about 50 nm by transmission electron microscopy (TEM). The white phase is an aqueous cavity with glucose inside, and the darker shell has an 8 nm thickness. The hydrodynamic size of the PLGA nanocapsule is about 66 nm (Figure S1). The PFOB nanoemulsion was prepared by ultrasonic emulsification and had a hydrodynamic size of 54 nm (Figure S2). The TEM image from Figure 1b shows the size was between 30 and 50 nm. The PFOB was an oil phase, in which oxygen was dissolved due to PFOB’s natural ability to carry oxygen. The dissolved oxygen concentration was 8.76 mg/L, which was significantly higher than that of pure water, at 5.1 mg/L (Figure S3). Mixing PLGA nanocapsules and PFOB nanoemulsions in a volume ratio of 1:1 constitutes solution A. Figure 1c shows the 8 nm Fe₃O₄ nanoparticle synthesized by high-temperature thermal decomposition with a saturation magnetization of 68 emu/g (Figure S4), which can be transferred from the organic phase to the aqueous phase by the ligand exchange method using dimercaptosuccinic acid (DMSA). Then the cationic polymer polyethyleneimine (PEI) was modified on the surface of the nanoparticles by electrostatic adsorption. An Fe₃O₄@PEI colloidal solution dissolved in α-CD, D-mannitol, and GOD formed solution B. Solution A and solution B were uniformly mixed at a volume ratio of 1:1 and allowed to stand for 30 min to obtain the MHZ, which has a typical hydrogel porous structure (Figure 1g). From Figure 1e and f, we can find three features of an MHZ: First, an MHZ maintains its hydrogel state at room temperature and a body temperature of 37°C, while it becomes a sol state and then starts to flow when the temperature reaches 42°C. As shown in Figure 1e, a glass capillary containing MHZ is immobilized in an ACMF coil. When an ACMF is applied, the temperature rises from room temperature to 37°C and MHZ remains in the original position. Then, the MHZ flows to the bottom of the glass tube when the temperature continues to rise to 42°C. Second, the MHZ has shear thinning properties that can be injected (Figure S5) and written into a desired pattern by a syringe. Furthermore, the Fe₃O₄ particles are uniformly supported in the hydrogel (inset of Figure 1g) and will exert a peroxidase-
like activity to catalyze the H$_2$O$_2$ produced by glucose and glucose oxidase to oxidize 3,3′,5,5′-tetramethylbenzidine (TMB), which turns blue (Figure 1f) when MHZ was written on a TMB-containing gellan gum gel substrate in an acidic environment (Figure S6).

The mechanism of hydrogel formation detected by XRD shows that the 2θ value of the main peak exhibited by MHZ is 19.7 degrees, which corresponds to the characteristic peak of the complex formed by MPEG and cyclodextrin, indicating that the hydrogel binding site is the composite of an MPEG chain on the surface of the nanoparticle and α-CD.32,33 Rheological experiments were performed to reveal the viscoelasticity and gelation process of the MHZ under a small-amplitude oscillatory shear. Figure 2b and c show the measurements of viscosity and modulus for the MHZ during temperature jumps from 20 °C to 42 °C. On heating from 20 °C to 42 °C and subsequent cooling to 20 °C, the viscosities underwent a cycle variation of high–low–high values and recover more than 85% finally, reflecting satisfactory thermosteroviscosity of the MHZ. Viscosities obtained at specific temperatures under the rotating speed over a range of 20–100 s$^{-1}$ revealed the shear-thinning properties of MHZ. (Figure S7). At 37 °C, the gel still maintains a certain viscosity and elastic modulus, which lays the foundation for subsequent retention after injection into tumor tissues. As we know, when a nanoparticle colloidal solution was administered directly into the tumor, the small size of the particles and the high osmotic pressure of the tumor cause NPs to easily leak out from the pinhole created by the syringe and escape from the tumor tissues.7,34 however, our injectable MHZ can be injected not only like magnetic fluid colloidal solutions but also without uncontrolled leakage. The value of elastic $G'$ was near the loss moduli $G''$ at 42 °C, indicating the temperature of the phase transition point, which can also be found in Figure S8, and the elastic and loss moduli $G'$ and $G''$ at different temperatures exhibited the characteristic viscoelastic behavior of an MHZ. The value of $G'$ was larger than $G''$ over the time range investigated at 25 °C (room temperature) and 37 °C (physiological temperature), while similar values of $G'$ and $G''$ were present at $T_{g_{ls}}$ (42 °C), indicating the properties of retention and solvation, respectively. This is consistent with the fact that α-CD can thread onto the MPEG polymer, bringing about the formation of reversible physical cross-links, and a temperature higher than $T_{g_{ls}}$ induced dissociation, leading to the collapse of the network. MHZ with low viscoelasticity at $T_{g_{ls}}$ (42 °C) contributes to the liquid diffusion during the magnetically induced heating under ACMF. The proper magnetic induction heating ability is crucial for the MHZ for follow-up functions. The magnetic induction heating experiments of single magnetic nanoparticles and MHZ show that the different environments do not affect the heating ability of the nanoparticles markedly. The specific absorption rate (SAR) value was found to be 96 w/g Fe by calculation, and the thermal images and the temperature rise curve showed consistent effects. Furthermore, the components of the MHZ have good biocompatibility and can be easily cleared from the body due to the low molecular weight of the polymer.35

The physical cross-linking force of MHZ comes from inclusion complexation structures between the MPEG blocks and host molecule α-CD. Therefore, the key factor in MHZ synthesis is mainly the preparation of PEG-modified nanoparticles. PLGA nanocapsules and PFOB nanoemulsions can be facilely prepared by the double-emulsion method and the ultrasonic emulsiﬁcation method, respectively. Due to the loading ability of the aqueous bulk of the gel, the addition of the remaining ingredients can be done by simple mixing. By adjusting the concentration ratio of MPEG and CD and the
reaction time, we also easily obtained MHZ systems with different gel–sol transition temperatures (Figure S9), which ensures the reproducibility of our hydrogel system.

**In Vitro Characterizations of the Catalytic Performances of MHZ.** As illustrated in the above section on the mechanism of the magnetothermally driven continuous treatment mode (Scheme 1), MHZ initially catalyzes the transition of glucose into \( \text{H}_2\text{O}_2 \) biologically through the wrapped GOD in the presence of oxygen carried by the PFOB nanoemulsion. Immediately, \( \text{Fe}_3\text{O}_4 \) nanoparticles catalyze the production of highly toxic \( \cdot\text{OH} \) by \( \text{H}_2\text{O}_2 \) through mimicking an enzyme function under the tumor’s slightly acidic environment. In order to monitor the production of radical species, we offer TMB as the chromogenic substrate under the catalysis by \( \text{Fe}_3\text{O}_4 \) NPs in an acidic environment, which produces a blue color measurable at a wavelength of 650 nm. The time-course absorbance upon the addition of \( \text{H}_2\text{O}_2 \) into \( \text{Fe}_3\text{O}_4 \) nanoparticles in sodium acetate buffer solution (20 mM, pH = 5.2) is plotted in Figure 3a and shows the ability of different surface-modified \( \text{Fe}_3\text{O}_4 \) nanoparticles to catalyze the chromogenic changes of \( \text{H}_2\text{O}_2 \) additions (photographs in the inset). PEI-modified nanoparticles have the highest catalytic performance, and PEG-modified nanoparticles exhibit the lowest capacity. The main reason may be that the dense PEG shell hinders the opportunity for the substrate to diffuse to the surface of the nanoparticle (Figure S10).

The temperature-course absorbance plotted in Figure 3b shows the comparison of capacity catalyzed by MHZ at different temperatures. As the temperature increases, the catalytic performance of MHZ shows an increasing trend, indicating the important contribution of temperature to the catalytic performance of nanozymes. Glucose (8, 4, 2, 1, 0.5, 0.25, 0.125, and 0.0625 mM) served as the reactant in the assays of MHZ under different temperatures, respectively (Figure 3c). The release of glucose from the PLGA nanocapsule can be accelerated with the temperature increase (Figure S11). In the range of 0 to 2 mM, with the increase of the glucose concentration in the PLGA, the hydroxyl radicals generated by MHZ showed a significant increase, and the increase of the reaction temperature played a positive role in the catalytic reaction. The time-course absorbance under simulated conditions (42 °C, pH = 5.2) reflects the catalytic reaction in tumor tissue during hyperthermia. From these results, it can be known that the MHZ could provide a moderate and sufficient catalytic therapeutic effect toward cancer cells.

**Ex Vivo Diffusion Performance of MHZ in Tumor Tissues.** Generally, an implanted hydrogel often exists as an isolated point in tumor tissue. The heat generated by the hydrogel that was used as the magnetic medium under the ACMF has a long diffusion range and can better cover the area of the tumor tissue, while the killing range of hydroxyl radicals produced by the Fenton reaction is only a short distance. In order to obtain a better therapeutic effect when combining heat and hydroxyl radicals, it is important to increase the contact area of the hydrogel within the tumor tissue. The hydrogel we designed has the particular characteristic of a temperature-sensitive phase transition. MHZ becomes its sol state and begins to flow and diffuse between tumor cells when the temperature reaches the 42-degree phase transition point. Moreover, the addition of D-mannitol enlarges the cell gap, further promotes the diffusion of MHZ components in the tumor tissue, and increases the reaction range within the tumor tissue for a better therapeutic effect.

To verify the diffusion ability of MHZ after being injected into tumor tissue, the ex vivo isolated tumor tissue is cut off to form a central groove, where MHZ is injected followed by an ACMF. After 5 min, the MHZ was heated to its phase transition temperature of 42 °C. At this time point, MHZ began to change from a gel to a sol and gradually diffuses and penetrates. As can be seen from Figure 4a, the part of the
MHZ that is in contact with the tumor tissue gradually penetrates into the tumor tissue, and no clear boundary between the tumor tissue and MHZ can be found. In order to verify the diffusion of the MHZ in the tumor tissue at the microscopic level, we first observed the effect of cell gap enlargement by the application of D-mannitol. It can be seen from the HE staining in Figure 4b that the distance between the tumor cells is significantly enlarged before and after the treatment of D-mannitol. The microstructure of the HE slice is obviously lost after processing, and the distance between cells has changed from 7–10 μm to 9–20 μm. Then we injected D-mannitol-loaded MHZ into the tumor tissue. The experiment was carried out by applying an ACMF for 10 min and then staining with Prussian blue. As shown in Figure 4c, we can see the blue region representing the iron element is loosely distributed and diffused in the experimental group compared to the concentration distribution of blue area of the control group where no ACMF and D-mannitol were applied. In order to...
to observe the effect in vivo, MHZ was injected in two parts into the tumor site in 4T1 tumor-bearing mice and subjected to MRI of a T2-weighted pattern. From Figure 4d, we found that the MHZ injection sites began to merge and disperse continuously under the application of ACMF, reflecting that MHZ had the ability to spread as much as possible to the entire tumor area. These results indicated that the Fe3O4 nanoparticles were significantly diffused to the tumor tissue, which also enabled the MHZ to produce a larger range of ROS in subsequent enzymatic reactions to kill the tumor cells.

**In Vivo Therapy with MHZ.** The excellent in vitro efficacy of catalysis and heating as well as the diffusion performance of MHZ may imply its potentially high in vivo therapeutic outcome. To verify this, the therapeutic performance of MHZ was examined on a 4T1 breast tumor xenograft on BALB/c mice. The MHZ, nanozyme, hyperthermia, and control (saline) groups were administrated intratumorally to investigate the treatment effects, respectively (Figure 5). Magnetic hyperthermia and pro-oxidative therapy were performed simultaneously in the MHZ group. The nanozyme group was treated with simple pro-oxidation therapy without ACMF, the hyperthermia group was treated by magnetic hyperthermia alone without the increased production of H2O2 by GOD, and the control group was a blank control group with saline.

The entire treatment was divided into two doses, and the second injection was performed on the 12th day after the first injection. For the MHZ group and the hyperthermia group, the ACMF was applied immediately after the injection of the MHZ (Figure S12). The irradiation time of the ACMF was 15 min, which consisted of a 5 min temperature rise to 42 °C and 10 min of maintenance time. Our ACMF generator is a current-adjustable instrument in a range of 1−28 A with a fixed frequency of 410 kHz, in which the ACMF coil has three turns. The corresponding magnetic field strength $H$ can vary from 0.1 to 2.8 kA/m. In this experiment, we select a current of 12 A to generate a magnetic field strength of 1.2 kA/m to heat the tumor to a temperature of 42 °C.

The temperatures described herein are all tumor surface temperatures that were obtained by thermal imaging. As the depth increases, there is a difference between the internal temperature and the surface temperature. We used a FOT fiber optic sensor to measure the temperature inside the tumor. The height of the tumor was about 4 mm, and we inserted the fiber probe to a depth of 2 mm from the surface of the tumor. By comparing the temperature with the thermal imaging (Figure S13), we found that the temperature of the tumor center was 0.4−0.6 degree higher than the surface, which was consistent with our previous simulated results. This also shows that the tumor temperature is still controlled below 43 degrees, in line with our original intention to break through the bottleneck of tumor treatment based on mild hyperthermia.

The heat generated by the magnetic nanoparticles caused a 42 °C hyperthermia in the tumor tissue and promoted the components of the MHZ to fully diffuse in the tumor space. To heat the tumor to a target temperature of 42 °C, we use different currents and times to achieve it. The current we used in the 42 °C hyperthermia was 12 A, and we maintained this temperature by changing the current in the range 1−12 A with a change rate of 1 A/s. By adjusting the current and duration at different currents, we ensure that the surface temperature of the tumor captured by the thermal image is within the range of 41.5−42.5 degrees to obtain an accurate comparison.

Immediately, Fe3O4 nanozymes exert a stronger catalytic activity at 42 °C to catalyze the production of hydroxyl radicals by hydrogen peroxide produced by GOD and glucose to kill tumors. The other two groups (hyperthermia and control group) have the same application parameters as the ACMF with MHZ group. After 7 days of treatment, it was found that tumor growth was significantly inhibited in the experimental group compared to the control group. In order to further
Figure 7. (a) The relative tumor volumes of different groups after treatment (inset: eliminated tumors). (b) Long-term survival rates of mice bearing 4T1 tumors after several treatment processes as indicated. Measurement for each treatment was repeated in triplicate, and the results are presented as mean ± SD (**, P < 0.01, ***., P < 0.001, n = 6).

After the therapeutic processes, tumors of mice in all groups are dissected and are shown in Figure 7a, which demonstrates that tumor growth could be effectively destroyed after the administration of MHZ intratumorally. A survival study was performed for mice following MHZ therapy and other control therapies. Compared to the control groups, the MHZ group had a significant survival advantage, with 100% survival for at least 50 days (Figure 7b). MHZ therapy has obvious advantages in therapeutic effects compared with other therapies. During 50 days of the therapeutic period, the body weights of mice in all groups show no obvious variations (Figure S15), indicating the high biocompatibility of the intratumoral injection of MHZ during the treatment. Prussian blue/nuclear fast red (PB&NFR) double staining and hematoxylin and eosin (H&E) assays of the major organs from all experimental groups were also carried out to further evaluate the long-term in vivo safety. H&E staining of the major organs on day 28 (Figure S16) shows no obvious damage when compared to the control groups. From PB&NFR double staining (Figure S17), there are no blue spots representing the MNPs dispersed in main organs, further demonstrating the high biosafety of the sequential MHZ during the therapeutic process.

This technique has great potential for more types of solid tumors due to its broad spectrum of killing effect on tumor cells, which does not depend on the type of cell line. However, it is a challenge to apply to all types of tumors because of the complexity of the tumors. The treatment of nonlocalized tumors with regional metastases or poorly defined tumors is still difficult to perform. Despite this, combined with the currently developed interventional techniques and image-guided techniques, more types of cancer delivery and treatment will be achieved.

The clinical studies of magnetic fluid hyperthermia demonstrated the feasibility and efficacy of this approach. For the practical applicability of MHZ in the clinic, the following four key factors need to be considered. First is the accuracy of the injection position: This process requires real-time image guidance. The Fe₃O₄ nanoparticles in MHZ can be used as magnetic resonance contrast agents, which can guarantee the accurate injection of MHZ. Second is no leakage to the surrounding tissues: MHZ can be injected due to its shear-thinning characteristics and maintain its gel state at body temperature, which lay the foundation for the restriction of the MHZ in tumor tissue without diffusion and leakage. Third, a temperature feedback control system that adjusts the ACMF parameters in real time may be needed. Finally, biosafety and controllable side effects are required: The component of MHZ showed good biocompatibility, and 42-degree hyperthermia did not cause obvious side effects such as edema and inflammation. We believe MHZ will have great potential for in vivo synergistic therapy in the future.
CONCLUSION

In summary, we have presented a smart injectable supramolecular hydrogel composed of PEGylated nanoparticles and α-CD for sequential tumor therapy, which show advantages in overcoming intratumoral diffusion disorder and a synergistic effect of therapy. We have demonstrated that our temperature-responsive hydrogel system driven via magnetic heating generated by Fe3O4 nanozyme is a powerful platform for combination with hyperthermia and catalytic therapy. MHZ underwent a phase transition sensitively at 42 °C and diffused throughout the tumor tissue with the participation of D-mannitol. A single Fe3O4 magnetic nanoparticle exerts the dual effects of magnetic heating and nanozyme concurrently in the hydrogel system. The obtained results confirmed that the catalysis of the Fe3O4 nanozyme is effective for tumor treatment due to an inhibition of growth and elimination at a 42 °C therapeutic temperature, which indicated that the resistance of tumor cells to induced thermal stress can be removed without causing severe side effects to normal tissues at lower temperature hyperthermia. The developed system provides a versatile platform for synergistic treatment of solid tumors more safely and precisely. Furthermore, the proposed strategy may contribute to further discoveries and applications of multifunctional nanotherapeutic agents in various biomedical fields.

METHODS

Preparation of Glucose-Loaded PLGA Nanoparticles by Double-Emulsion Solvent Evaporation. A glucose-loaded PLGA nanoparticle was prepared by a double-emulsion solvent evaporation method. The internal aqueous phase consists of 2 mg of glucose in 250 μL of deionized water; 250 mg of MPEG10K-PLGA11K powder was added to 5 mL of chloroform as the oil phase; 15 mL of a 1.0% (w/v) poly(vinyl alcohol) (PVA) solution was prepared as the external aqueous phase. First, the inner aqueous phase was added to the oil phase under sonication for 30 s in an ice bath. Then, it was added to 15 mL of 1% PVA and sonicated for 2 min in an ice bath to form a water-in-oil-in-water double-emulsion system. The mixture was added to 250 mL of 0.3% PVA and stirred at 500 r/min for 3 h to slowly evaporate the chloroform. The final reaction solution was centrifuged twice at 10 000 rpm to remove excess PVA and then stored in pure water at 4 °C.

Preparation of Oxygen-Carrying PFOB Nanoemulsion. A PFOB nanoemulsion was prepared by an ultrasonic emulsification method. A 20 mL amount of deionized water containing 100 mg of DSPE-MPEG2000 was added to a 100 mL three-necked flask as the aqueous phase. The water phase was heated in an oil bath to raise the temperature to 100 degrees and stirred at a rate of 600 rpm. At this time, 1 mL of PFOB as the oil phase was dropwise added. After 30 min of stirring, the nanoemulsion was concentrated by a 30K ultrafiltration tube to a desired volume and stored at 4 °C.

Oleic Acid (OA)-Coated Fe3O4 Nanoparticle Synthesis. The OA-coated Fe3O4 nanoparticles were successfully synthesized via a thermal decomposition procedure. In a typical experiment, 2 mmol of an iron precursor of Fe(acac)3 and 11 mmol of oleic acid were added to a 100 mL three-necked flask with 20 mL of a benzyl ether reaction solvent. The temperature of the reaction system was ramped to 220 °C at a heating rate of 3.3 °C/min. The nanoparticles were nucleated for 1 h and then heated at a rate of 3.3 °C/min to a growth temperature of 290 °C for 30 min. Nitrogen gas was continuously introduced during the experiment to remove oxygen from the system. The heat source was removed after the reaction was complete. The reaction system was transferred to a beaker and washed with ethanol three times to clear off residual oleic acid, benzyl ether, and unreacted precursors via magnetic separation. Finally, the magnetic Fe3O4 nanoparticles were fixed in chloroform and stored.

Preparation of Fe3O4@PEI Nanoparticles. We synthesize Fe3O4@PEI nanoparticles by the following two steps. First, we prepared the Fe3O4@DMSA nanoparticles using our previously reported ligand exchange method. Second, Fe3O4@PEI nanoparticles were prepared via electrostatic adsorption between cationic polymer PEI and negatively charged Fe3O4@DMSA nanoparticles. In a typical procedure, 30 mg of (Fe element) Fe3O4@DMSA colloidal solution was dropwise added into a 150 mg PEI solution (10 kDa) under 1200 rpm of mechanical stirring and 100 W of sonication at room temperature for 2 h. After that, the resultant solution was transferred into an ultrafiltration tube with a molecular weight cutoff of 100 kDa. The excess PEI was removed through centrifugation at 6000g 10 times. Then the final product was filtered through a 0.22 μm filter and stored at 4 °C.

Preparation of MHZ. The general protocol for the hydrogel preparation is as follows. The prepared glucose-loaded PLGA nanocapsules and the oxygen-carrying PFOB nanoemulsion were mixed into solution A in a volume ratio of 1:1. The obtained Fe3O4@PEI nanoparticles were mixed with α-CD, GOD, and D-mannitol into solution B, wherein the concentration of α-CD, GOD, and D-mannitol was 300, 2, and 1 mg/mL, respectively, and the iron element content in solution B was 12 mg/mL. Solutions A and B were mixed at a volume ratio of 1:1 and then allowed to stand for 30 min at room temperature to obtain MHZ.

In Vitro Heat Induction Measurements. The heat generation under ACMF application of Fe3O4@PEI nanoparticles and MHZ was measured by a moderate radio frequency heating machine (Shuangping SPG-06-II, China). The test tube was placed in the center of a water-cooled magnetic induction copper coil. The parameters of the machine and samples were as follows: frequency (f) = 410 kHz, magnetic field intensity = 1.2 kA m−1, solution volume = 1 mL, mass of Fe (mFe) = 6 mg. The heating ability of MHZ and Fe3O4@PEI nanoparticles can be quantified by specific absorption rate (SAR = C0(dT/dt)(mFe/m0)) values when an ACMF is applied, where C0 is the specific heat capacity of the water (4.18 kJ kg−1 K−1). In order to avoid possible errors, dT/dt is the slope of the heating curve in the initial 1 min; mFe is the mass of the colloidal solution, and m0 is the mass of the iron element in the suspension.

In Vivo Synergistic Therapy of Tumors. When the animal model is established 2 weeks later (tumor diameter reached ~0.5 cm), the 4T1 xenograft tumor mice were randomly divided into 4 groups: MHZ, nanozyme, hyperthermia, and control groups (n = 6 per group). Then, mice were in situ administrated with 30 μL of MHZ through a syringe after being anesthetized. The magnetic-induced hyperthermia experiments were conducted by the heating machine with the same parameters as the in vitro test. During the magnetic hyperthermia application, the tumors of mice were guaranteed to locate exactly in the central region of the water-cooled coil where ACMF possesses the highest field density. The duration of the magnetic hyperthermia application was 15 min. An infrared-thermograph (Fluke, Ti32, USA) was taken to measure the temperature of the tumor regions. Body weight, tumor volume, and survival rate of each mouse in the following days were all monitored.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsnano.9b06134.

Details of materials used; preparation of TMB-containing gellan gum hydrogel; material characterization; rheological experiments; in vitro glucose release; animal protocol; in vivo MRI imaging; ex vivo histological staining; statistical analysis and additional results (PDF)


