Banoxantrone Coordinated Metal-Organic Framework for Photoacoustic Imaging-Guided High Intensity Focused Ultrasound Therapy

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Research

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Abstract

Background

Photoacoustic imaging (PA) with high spatial resolution has great potential as desired monitoring means in the high intensity focused ultrasound (HIFU) surgery of tumor. However, its penetration depth in the tissue does not meet the clinical needs. Nanomedicine provides a new opportunity for PA imaging to guide HIFU surgery. Our studies found that hypoxic heterogeneity of tumor was effectively reversed by HIFU.

Methods

Herein, specific metal-organic framework nanosystem, constructed by coordination of banoxantrone (AQ4N) and Mn^{2+} , is designed based on HIFU to reverse hypoxic heterogeneity of tumor.

Results

It could provide exogenous light-absorbing substances, thus improves the penetrability of PA imaging signal through the deep tissue and achieves clearer PA imaging for guiding HIFU surgery. In turn, AQ4N, in the hypoxic homogenous environment of tumor provided by HIFU, is activated sequentially to specifically treat the residual hypoxic tumor cells.

Conclusions

This strategy addresses the dissatisfaction of PA imaging-guided HIFU therapy and is promising for translation into a clinical combination regimen.

Introduction

Malignant tumors seriously threaten human life and health[1-3]. Accurate diagnosis and effective treatment of tumors are highly desirable[4]. High intensity focused ultrasound (HIFU), which causes irreversible solidification necrosis of tumor tissue in the focal area without damaging surrounding structures, provides a technology for accurate treatment of tumors. As reported by the 2021 State of the Field Report of Focused Ultrasound Foundation, 152 indications have been put into clinical application or study, showing good efficacy and safety. HIFU therapy has formed a vigorous development trend and been described as a revolutionary therapeutic technology. Meanwhile, it also encounters the bottleneck problem which affects its further development. For instance, real-time image monitoring is the "eye" during the HIFU surgery, but the need from the clinic shows that the existing "eye" (B-mode ultrasound) is not bright enough to see clearly and there is still the possibility of residual tumor tissue. Therefore, the future development direction of HIFU needs to solve the problem of inaccurate diagnosis.

Photoacoustic (PA) imaging could accurately and rapidly track the light-absorbing substances in the body, so as to obtain images of tumor tissue morphology, structure and metabolic function with high spatial resolution and high contrast[5, 6]. Thus, it might represent a superb candidate as ideal monitoring means. However, due to limited types and poor light absorption of intrinsic light-absorbing substances (mainly melanin and hemoglobin), its penetration depth in the tissue does not meet the clinical needs[7]. Its applications in HIFU are thus extremely restricted. The rapid development of nanomedicine provides a new opportunity for PA imaging to guide HIFU surgery[8-10]. Nanodelivery systems could target tumor tissue with exogenous light-absorbing substances, thus help to improve the imaging depth and achieve clearer PA imaging[11, 12]. Moreover, nanodelivery systems are cleverly designed to provide therapeutic function for residual tissue after HIFU ablation[13, 14]. Thus, the combined usages of PA imaging and exogenous contrast agent make it possible to guide HIFU accurate ablation of malignant tumors and comprehensively treat postoperative residues.

The changes of tumor tissue characteristics and environment during HIFU surgery are the fundamental basis for the design of HIFU-specific theranostic nanosystem. Numerous studies have shown that hypoxia is a prominent feature of most solid tumors[15, 16]. However, hypoxia levels in the tumor tissue are with heterogeneity[17, 18]. HIFU is a non-invasive treatment that selectively destroys tumor trophoblast vessels and capillary network in the focal area through thermal and cavitation mechanism[19]. Thus, it could block tumor blood supply and effectively reverse hypoxia heterogeneity. HIFU is expected to provide a new idea for therapy strategies based on tumor hypoxia.

Currently, the most important type of hypoxic-targeted therapy for tumors is hypoxia-activated prodrugs[20, 21]. Banoxantrone (AQ4N), a representative hypoxia-activated prodrug, has been widely used in preclinical studies and clinical trials of tumors. In AQ4N active metabolic process, low toxic AQ4N, only in the lack of oxygen microenvironment, could be reduced to highly toxic AQ4[22]. AQ4, a specific inhibition of DNA topoisomerase α , leads to DNA damage and cell death. AQ4N has achieved satisfactory results in phase I clinical trials[23, 24]. However, no progress has been made in phase II clinical trials. Further study on the phase I clinical trial data suggests that there is heterogeneity in the hypoxia level of tumor tissues, and the failure of AQ4N is mainly caused by insufficient hypoxia level in some areas of tumor[25, 26]. In addition, our studies suggest that AQ4N has strong acoustic signal. Therefore, it is of great potential to design AQ4N-loaded specific theranostic nanosystem based on HIFU to reverse hypoxia heterogeneity of tumor.

Under the guidance of the above ideas, we develop AQ4N/Mn²⁺ metal-organic frameworks (AMMOFs) nanosystem for PA imaging guided HIFU surgery. This nanosystem could efficiently target at the tumor site and provide exogenous light-absorbing substances for PA. In the subsequent HIFU surgery, the penetrability of PA signal through the deep tissue is improved and clearer PA imaging is achieved for guiding HIFU surgery. In addition, in the hypoxic homogeneous environment obtained after HIFU surgery, AQ4N is activated sequentially and effectively carries out specific treatment for postoperative residual tissue. In consequence, remarkable *in vivo* antitumor effect is achieved by AMMOFs. This design solves the core problems of existing HIFU surgery, might provide insights into non-invasive tumor therapy.

Results And Discussion

Preparation and characterization of AMMOFs

The schematic illustration of the detailed preparation route of AMMOFs was presented in Figure 1. Firstly, ordered AQ4N/Mn²⁺ coordination compound was produced through the coordination between the C-O-Mn and C=O-Mn bonds. After TEA addition, part of hydrophilic AQ4N hydrochloride was converted to hydrophobic AQ4N, balance of hydrophilic and hydrophobic further contributed to self-assembly of nanosystem. Besides, π stacking between AQ4N also promotes the formation of aggregates with stable structures. Through adjusting ratios of formulation, we obtained optimal AMMOFs by coordination of Mn²⁺ and AQ4N at a molar ratio of 1:5, which resulted in high drug loading content (DLC) (87.27%), low polydispersity (PDI<0.20) and regular sphericity with size around 100 nm (Figure 2a, b). DLS and TEM measurements provide direct evidence for the successful fabrication of nanometer MOFs. The UV absorption spectrum of AMMOFs shows obvious characteristic peak of AQ4N at 610 nm, further confirming the successful fabrication of AMMOFs (Figure 2c). XRD pattern confirms that AMMOFs have good crystalline state (Figure 2d). The high drug loading capacity benefits from the structure composition without carrier material. Approximately uniform morphology benefits from ordered conjugation mode of Mn²⁺ and AQ4N in the MOFs.

Given the translational potential for *in vivo* studies, we evaluated the stability of AMMOFs in the mimic physiological environments. AMMOFs exhibits favourable stability in the PBS containing 10% FBS during 24 h, even in the lower concentration 0.1 mg/mL (Figure 2e), providing possibility for *in vivo* remarkable targeting effect by the enhanced permeability and retention (EPR) effects. The reason for this result is related to strong/weak interactions between drug ligands and metal ions. In PBS solution at pH 7.4, only little amount of AQ4N was released after 24 h incubation. But, in PBS solution at pH 4.5, AQ4N release dramatically increased to 94.91% (Figure 2f), exhibiting the pH-responsive structure dissociation. Because metal ions are Lewis acids and bind the ligands which are Lewis bases, formative coordination bonds are sensitive to pH changes. pH-responsive drug release property is important in biomedical application that could guarantee its safety during delivery process, while realizes on-demand AQ4N release at the target site.

Subcellular distribution, cellular uptake, cytotoxicity and cell apoptosis *in vitro*

Confocal Laser Scanning Microscope (CLSM) was used to observe subcellular distribution behavior. Because AQ4 reacts with topoisomerase II of DNA in the cell nucleus, AQ4N distributed in the nucleus after release is vitally important to induce next apoptosis. As shown in Figure 3a, cells treated with AMMOFs have strong nuclear fluorescence, indicating efficient AQ4N release in the acid endosome environment of tumor cells owing to pH sensitivity of its coordination bonding. Next, we evaluated the cellular uptake efficiency of AMMOFs. After incubation for 4 h, we observed similar intensity of red fluorescence in AMMOFs and free AQ4N groups. Further, we quantificationally determined the

intracellular fluorescence signal of AMMOFs by flow cytometry (Figure 3b). The results are well in accordance with CLSM images. This demonstrates that AMMOFs are rapidly internalized into the cells.

Inactive AQ4N undergoes enzymatic reduction under hypoxia and the reduced product (active AQ4) possesses strong topoisomerase inhibition[22]. To validate it, the *in vitro* selective cytotoxicity of AQ4N was evaluated in the normoxic or hypoxic conditions. As shown in CCK-8 assay, AMMOFs exhibit significantly more cytotoxicity in hypoxic environment. The half maximal inhibitory concentration (IC₅₀) of AMMOFs in the normoxic and hypoxic conditions are 25.06 and 0.119 mg/L (Figure 3c), respectively, further confirming its effect in the hypoxic environment. The IC₅₀ values of free AQ4N in the normoxic and hypoxic conditions are 37.46 and 6.78 mg/L, both are higher than IC₅₀ of AMMOFs. Sudden overdoses of ions in the cytosol or other intracellular compartments often induce programmed cell death[27]. Thus, lower IC₅₀ of AMMOFs might be related to the contribution of Mn²⁺.

To compare the therapeutic effect, flow cytometric apoptosis and live/dead cytotoxicity kit were conducted to distinguish cell viability (Figure 3d, e). It demonstrates that cells treated with AMMOFs under hypoxia show the most significant apoptosis ratio (up to about 99%), while nearly no cell apoptosis in the control group and less apoptosis rates in the AMMOFs groups under normoxia. As shown in Figure 3e, negligible red signal and widespread green signal were observed in the groups under normoxic condition, which indicate high cell viability. Comparatively, obvious red signal and less green signal was clearly observed under hypoxic condition. And the live/dead staining results corroborated the results of flow cytometry and CCK-8 assay, which highlighted that low oxygen tension completely activated chemotherapeutic activity of AQ4N. Therefore, we reasoned that the antitumor activity of AQ4N *in vivo* could be enhanced by improved hypoxia level within tumors after HIFU irradiation.

***In vivo* pharmacokinetics, biodistribution and intratumoral release**

To observe the intratumoral release of AQ4N, frozen section of tumors was displayed a precise location of AQ4N by CLSM imaging (Figure 4a). AQ4N fluorescence from the CLSM images was calculated (Figure 4b). The CLSM images result shows that nuclear signals of AQ4N in the AMMOFs group are stronger than that of free AQ4N. It reveals that AMMOFs significantly improve *in vivo* distribution in the tumors and efficiently release AQ4N from AMMOFs in the tumor cells.

The stability of AMMOFs *in vivo* was tested by plasma pharmacokinetics. As represented in Figure 4c, free AQ4N was rapidly cleared from the blood, after 24 h, only little amount of free AQ4N was in the plasma compared with 55.64% of AMMOFs. More importantly, the AMMOFs in blood at different time post injection almost kept constant. The results suggest that AQ4N assembled in the AMMOFs has good physiological stability and facilitates to prolong the blood circulation time by taking advantage of nanotechnology.

Subsequently, *in vivo* quantification of AQ4N distribution in the tumors of 4T1 xenografted nude mice was conducted by tissue distribution method (Figure 4d). Surprisingly, AQ4N in the tumor reached 20.08% of injected dose at 12 h and 12.72% of injected dose at 24 h. The results demonstrate that

the AMMOFs could significantly enhance the *in vivo* accumulation of AQ4N in the tumor. This property could avoid the needless distribution and release in normal tissues, thus lays a solid foundation for enhancing the antitumor efficacy.

***In vitro* and *in vivo* PA imaging of AMMOFs**

To assess *in vitro* contrast-enhanced PA imaging capability of AMMOFs, the PA signal at 690 nm was measured. The PA phantom images show that AMMOFs exhibit significant enhancements in the PA mode. And PA signal intensity increased with AQ4N concentration (0.10, 0.25, 0.50, 1.00 and 2.00 mg/mL) with linear relationship (Figure 4e). The *in vitro* imaging evaluations demonstrate the high PA imaging potential of AMMOFs.

We carefully evaluated the penetration depth of AMMOFs covered by pig skin under 690 nm laser irradiation. Compared with PBS group, PA signal intensity of AMMOFs at 0.25 cm depth was very strong and decreased gradually with increasing depth of pig skin tissue, but was still clearly visible at a depth of 2.5 cm. The normalized curve of signal-to-background ratio is shown in Figure 4f. This result suggests that AMMOFs could enhance penetration depth of PA in the tissue and be used for *in vivo* deep imaging.

In contrast, in the AQ4N groups, PA signals were almost invisible after 24 h injection (Figure 4g, h). This increase in PA signal is believed to be owing to the gradual accumulation of AMMOFs in the tumor. No significant PA signal increase of free AQ4N is due to the rapid pharmacokinetics. Nanoencapsulation strategies by EPR effect have overcome the limitations in *in vivo* delivery of AQ4N. The results exhibit excellent PA imaging performance of AMMOFs, which show that AMMOFs has great potential for enhancing guiding effect of HIFU surgery.

Tumor hypoxia evaluation

The significant upregulation of tumor hypoxia by HIFU provides rationale for carrying out therapeutic efficacy of AMMOFs. To evaluate the HIFU-induced hypoxia status within tumors, PA imaging, optical oxygen partial pressure microelectrode and hypoxia probe immunofluorescence staining were performed in 4T1 xenografted nude mice. PA imaging and optical oxygen partial pressure microelectrode were utilized to qualitatively observe and quantitatively test oxygen saturation of blood inside tumors (Figure 5a, b). The oxygen saturation significantly decreased after HIFU irradiation for 24 h. In the immunofluorescence staining images, hypoxia-inducible factor 1 α (HIF-1 α) was remarkably upregulated in the HIFU group compared with the control group (Figure 5d, e). The results of PA imaging, oxygen partial pressure microelectrode and HIF-1 α staining assays were strikingly coincide, demonstrating that HIFU could cut off the oxygen supply within tumors and create profound hypoxia within tumor microenvironment. It provides convincing evidence that HIFU activated sequentially the therapeutic function of AQ4N.

***In vivo* antitumor efficacy**

The *in vivo* antitumor efficacy of AMMOFs plus HIFU was systematically assessed. Tumor-bearing nude mice were treated with PBS, AQ4N or AMMOFs (the dosage of AQ4N: 10 mg/kg). After 24 h of accumulation at the tumor site, mice were immersed in degassed water for HIFU irradiation. As shown in Figure 6b, within the 15 days following the treatment, the tumor volumes of HIFU+PBS, HIFU+AQ4N and AMMOFs groups showed increasing tendency in different degree, verifying the limited activation of AQ4N, fast excretion of AQ4N in its free form and insufficient treatment of HIFU in solid tumors. Consistent with the proposed scenario, HIFU+AMMOFs showed the most efficient therapeutic effect (Figure 6c). It was noteworthy that the difference between groups was obvious.

To further assess the therapeutic performance, the histological changes and apoptosis levels of tumors were analyzed by histological analysis including hematoxylin and eosin (H&E), proliferating cell nuclear antigen (PCNA) and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays. In the H&E staining images, severe morphology change and necrosis were observed in the tumor of HIFU+AMMOFs group, while moderate levels of damages in the other groups. PCNA and TUNEL-stained images followed the same trend revealed pronounced apoptosis and DNA damage (Figure 6e).

Overall, these results reveal that sequentially activated tumor therapy with HIFU+AMMOFs is a promising strategy. Via this approach, the firstly applied AMMOFs and PA guided HIFU could not only accurately ablate tumor tissue, but also generate severe hypoxic environment of tumor through inducing tumor blood vasculature damages for further activation of AQ4N to kill postoperative residual cells. Ample evidence indicates that AMMOFs could act as an important role for enhanced HIFU-based tumor theranostics.

***In vivo* toxicity test.**

Systemic toxicity of AMMOFs was investigated for their potential translation in the future. Hemolysis is an important indicator for evaluating the biocompatibility of nanosystem. In the hemolysis experiment (Figure 7a), the hemolysis rate was below 5% due to negligible destruction of blood cell. Blood biochemistry and complete blood panel analysis were conducted on day 15. As shown in Figure 6c-j, primary indicators were all in the normal reference range, implying avoidance of any disorders or disease conditions. Meanwhile, H&E staining of major organs shows no pathological abnormalities (Figure 7b). The great biocompatibility might be attributed to low toxic AQ4N under the normoxia environment and nontoxic Mn^{2+} of nanomedicine. These results highlight their potential application for postoperative tumor-specific PA imaging and highly efficient therapy with negligible systemic toxicity. Taken together, the above antitumor efficacy and toxicity test demonstrate the safety, effectiveness and suitability of AMMOFs as theranostics agent based on HIFU.

Materials And Methods

Materials, Cells, and Animals

Manganese chloride and banoxantrone dihydrochloride (AQ4N) were purchased from Sigma-Aldrich (Milwaukee, USA). 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI), HIF-1 α antibody, FITC goat anti-rabbit IgG secondary antibody, Calcein acetoxymethyl ester (Calcein-AM), Annexin V-FITC and propidium iodide (PI) were obtained from Beyotime Biotechnology Co., Ltd (China). All compounds were of analytical purity grade and utilized exactly as supplied, with no additional purification. Before usage, all solvents were dried and distilled entirely.

Mice breast adenocarcinoma cells (4T1) were obtained from the Chinese Academy of Science cells Bank (Shanghai, China) and cultured in RPMI-1640 supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C in a humidified 5% CO₂ incubator according to ATCC recommended conditions. An incubator (Thermo Scientific, USA) containing 21% O₂, 5% CO₂ and 74% N₂ was used to cultivate cells under normoxic conditions. In a self-built sealed aeration tank infused with a gas combination of 2% O₂, 5% CO₂, and 93% N₂, hypoxic (2% O₂) cultivation was achieved. All studies were carried out while the cells were in the logarithmic phase of their development.

Female BALB/c nude mice (4-6 weeks old, 20-25 g), BALB/c mice and Sprague-Dawley rats were supplied by the Laboratory Animal Center, Chongqing Medical University, China. All animal experiments were conducted under the guideline assessed and authorized by Chongqing Medical University's ethical committee.

Preparation of AMMOFs

Briefly, AQ4N (2 mg) was dissolved in deionized water (1 mL), and MnCl₂ (100 μ L, 1 mg/mL) was added to the preceding solution and agitated for 4 h. The aforesaid mixture was then stirred with 400 μ L TEA at room temperature and protected from light. After 12 h, by vigorous stirring, the mixture was dropped into 10 mL deionized water. Free AQ4N and MnCl₂ were removed by dialysis. The resulting solution was lyophilized and stored at -20 °C.

UV spectroscopy (PerkinElmer, UK) with absorbing wavelengths of 610 nm was used to measure the amount of AQ4N in the AMMOFs. Loading capability (LC) was calculated as shown in the expression below:

$$LC \text{ (wt \%)} = \frac{\text{loaded-drug weight}}{\text{loaded-drug nanoparticles weight}} \times 100\%.$$

Characterization of AMMOFs

DLS on a Zetasizer Nano (Malvern, UK) and TEM were used to determine the size, zeta potential, and shape of AMMOFs (JEM-1200EX, Japan). The AMMOFs solution (1 mL, 1 mg/mL) was measured in a glass cuvette at 25 °C with a scattering angle of 173°. In order to test the stability, AMMOFs was kept at 37°C and monitored for 24 h in phosphate buffered saline (PBS) containing 10% fetal bovine serum (FBS). The *in vitro* release behavior of AQ4N from the AMMOFs was examined through being sealed in

dialysis bags after exposing to two distinct types of PBS: PBS (pH=4.5) and PBS (pH=7.4) as the medium of release. The release of AQ4N was measured using UV spectrophotometer at the appropriate time points. All measurements were made in 3 replicates and values are expressed as mean $\pm$ standard deviation (n=3).

Intracellular drug release, distribution and *in vitro* cytotoxicity of AMMOFs

4T1 cells (4×10^4 per well) were cultured for 24 h to adhere to the wall and then treated with new media containing free AQ4N or AMMOFs formulas at a concentration of 10 mg/L for an additional 4 h. Cells were then gently washed three times in PBS, stained with DAPI for 10 minutes, and visualized by confocal laser scanning microscopy (CLSM, Leica, Germany) at 640 nm excitation wavelengths.

4T1 cells (4×10^5 per well) were cultured for 24 h to adhere to the wall and then treated with new media containing free AQ4N or AMMOFs formulas at a concentration of 10 mg/L for an additional 4 h. Cells were then gently washed three times in PBS, harvested with trypsin, and centrifuged (1000 rpm, 5 min). Flow cytometry (Beckman Coulter Cytomics FC-500, USA) with excitation wavelengths of 650 nm was used to analyze the cells after being resuspended in PBS.

4T1 cells (4×10^5 per well) were cultured for 24 h to adhere to the wall and then treated with new media containing free AQ4N or AMMOFs formulas at a concentration of 10 mg/L for an additional 48 h. Cells were then gently washed three times in PBS, harvested with trypsin, and centrifuged (1000 rpm, 5 min). Flow cytometry (Beckman Coulter Cytomics FC-500, USA) with excitation wavelengths of 488 and 650 nm was used to analyze the cells after being resuspended in PBS.

4T1 cells (4×10^3 per well) were seeded in 96-well plates for 24 h to adhere to the wall and then incubated for 48 h at 37°C under normoxic (21%) or hypoxic (2%) conditions. Cells were cultured for another 48 h after the culture media was replaced with new media containing AQ4N formulas with appropriate AQ4N concentrations: 0.01, 0.1, 1, 10, and 100 mg/L. After that, the relative cell viabilities were determined by using the standard Cell Counting Kit-8 (CCK8) treatment.

Live/dead cell staining of 4T1 cells was performed by Calcein-AM/PI double stain kit. Briefly, 4T1 cells (4×10^4 per well) were seeded in 6-well plates and incubated in normoxic or hypoxic atmosphere as described above. Free AQ4N or AMMOFs formulas were added to the plate at a concentration of 50 mg/L, respectively. Cells were washed thrice with PBS after 48 h of incubation and stained with 0.3 mL assay buffer containing Calcein-AM and PI. 4T1 cells were incubated at 37°C for 15 min and observed by CLSM. Calcein-AM labeled live cells green, while PI stained dead cells red.

***In vivo* pharmacokinetic study, biodistribution and intratumoral release**

In the plasma pharmacokinetic study, Sprague-Dawley rats were randomly separated into 2 groups (6 mice in each group), and free AQ4N or AMMOFs at 25 mg/kg (AQ4N/body weight) were intravenously administered. Blood samples were taken out from the orbit at different intervals and centrifuged (5000

rpm, 10 min). The supernatant plasma was mixed with acetonitrile for precipitating the proteins. Finally, the organic layer was recovered, concentrated, and the AQ4N levels were determined by UV spectrophotometer.

4T1 xenografted nude mice were injected intravenously with 0.2 mL AQ4N formulas at 10 mg/kg (AQ4N/body weight) when the mean volume of tumors reached 200 mm³. The mice were then euthanized, and tumors were collected at various time intervals. For evaluation of intracellular drug release, tumors were dissected and frozen in OCT tissue compound on dry ice before being sectioned into 10 µm slices. CLSM was used to scan tumor slices that had been fixed with acetone and stained with DAPI. For quantitative biodistribution analysis, at various intervals of time, the mice (6 mice in each group) were sacrificed, and 1 mL hydrochloride acid was used to dissolve 0.1 g tumor tissue homogenate (2.0 M). The resulting mixture was then centrifuged, and the AQ4N content of the supernatant was extracted with chloroform/isopropanol (3:1 v/v) and measured using a UV spectrophotometer[13].

***In vitro* and *in vivo* PA imaging of AMMOFs**

Small-animal PA imaging equipment (Vevo®LAZR, Visual Sonics, Toronto, Canada) was used with a laser emission probe (laser excitation between 680 and 1000 nm with an excitation depth of about 2 cm), a diagnostic US probe, and a computer processing system to verify the feasibility of PA imaging of AMMOFs. Following the preparation, AMMOFs solutions were diluted to various concentrations (0.10, 0.25, 0.50, 1.00 and 2.00 mg/mL) using agar phantom (1% agar w/v in distilled water). The PA imaging equipment was used to examine the produced agar columns.

Confocal dishes containing AMMOFs solution (2 mg/mL) were superimposed with various thicknesses (0.25, 0.50, 1.00, 1.50, 2.00 and 2.50 cm) of pig skin, respectively. The penetration depth of PA+AMMOFs (2 mg/mL) in the tissue was performed using PA imager with an excitation wavelength of 690 nm, and the normalized curve of photoacoustic signal versus irradiation depth was calculated.

In 4T1 tumor xenografted nude mice, PA imaging was used to monitor *in vivo* AMMOFs guidance. 4T1 xenografted nude mice with tumor size of approximate 200 mm³ were injected intravenously with 0.2 mL AQ4N formulas at 10 mg/kg (AQ4N/body weight). Images were determined by PA imager with an excitation wavelength of 690 nm to record the PA signals and collect c-scan images in PA modes at 8, 12, and 24 h.

Tumor hypoxia evaluation

4T1 xenografted nude mice with tumor size of approximately 200 mm³ were immersed in degassed water for HIFU irradiation. After 24 h, the mice were anesthetized with 1% pentobarbital and placed on PA imaging system. Oxygen saturation (sO₂) was used to evaluate the hypoxia intensity. sO₂ was calculated using the following equation:

$$\text{Oxygen saturation (sO}_2\text{)} = \frac{M(\text{HbO}_2)}{M(\text{Hb}) + M(\text{HbO}_2)} \times 100\%$$

where M(Hb) and M(HbO₂) represent the mean intensity value of Hb and HbO₂ in tumor regions detected by oxygenated hemoglobin signal.

4T1 xenografted nude mice with tumor size of approximate 200 mm³ were immersed in degassed water for HIFU irradiation. After 24 h, the optical oxygen partial pressure microelectrode was embedded into the tumor (1 mm depth of tissue penetration) to detect the oxygen partial pressure.

4T1 xenografted nude mice with tumor size of approximate 200 mm³ were immersed in degassed water for HIFU irradiation. The mice were euthanized after 24 h, and tumors were collected. For evaluation of hypoxia, tumors were dissected and frozen in OCT tissue compound on dry ice before being sectioned into 10 μm slices. The slices were then fixed in cooled acetone for 20 min before being rinsed three times with PBS, blocked with 10% goat serum, and incubated overnight at 4°C with FITC goat anti-rabbit IgG secondary antibody conjugated to Anti-HIF-1 alpha. After that, the sections were rinsed three times with PBS. The nuclei of the cells were stained with DAPI as directed by the manufacturer. The slices were covered with coverslips and visualized by CLSM. Three randomly selected microscopic fields were quantitatively analyzed by the LAS X software.

***In vivo* antitumor efficacy**

When the tumors attained a mean volume of 200 mm³, 4T1 xenografted nude mice were randomly divided into 6 groups (6 mice in each group). Mice were intravenously administered with PBS, free AQ4N or AMMOFs at 10 mg/kg (AQ4N/body weight). After 12 h of AMMOFs accumulation at the tumor site, mice were anesthetized with 1% pentobarbital and then immersed in degassed water for HIFU irradiation. The treatment was repeated for three times at every 5 days interval. Sections were stained with hematoxylin and eosin (H&E), proliferating cell nuclear antigen (PCNA) and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays before being examined under an optical microscope (OLYMPUS BX50) and CLSM. Following that, the major organs (heart, liver, spleen, lungs, and kidneys) were taken for pathological studies[28].

***In vivo* toxicity test**

To extract red blood cells (RBCs), blood from Sprague-Dawley rats was centrifuged (3000 rpm, 10 min) at 4°C and washed three times with PBS. RBCs were mixed with various doses of AMMOFs (1.25, 2.5, 5, 10 and 20 mg/L) at 37°C for 4 h. After spinning the samples for 3 min at 1200 rpm, the supernatant was separated to assess the rate of hemolysis. 0.1 mL supernatant samples were added to 96-well plates, and absorbance at 541 nm was measured by a microplate reader (Infinite M200 Pro N, TECAN, Switzerland). RBCs suspended in PBS served as the negative control, whereas RBCs combined with deionized water served as the positive control. The rate of hemolysis was calculated as follows equation:

$$\text{Hemolysis (\%)} = \frac{\text{OD}(\text{sample}) - \text{OD}(\text{negative})}{\text{OD}(\text{positive}) - \text{OD}(\text{negative})} \times 100\%.$$

The identical circumstances (*in vivo* antitumor therapy) were applied to healthy female BALB/c mice. 15 days after injection, the mice were anaesthetised and blood samples were collected for routine blood biochemical testing and analysis of whole blood cell parameters including red blood cells (RBC), white blood cells (WBC), hemoglobin (HGB), hematocrit (HCT), and platelet (PLT). Blood aspartate aminotransferase (AST), alanine aminotransferase (ALT) and blood urea nitrogen (BUN) were also measured.

Statistical analysis

Data are reported as the mean $\pm$ standard deviation (SD) of at least three studies. Data analysis was performed using Origin 8.0 and GraphPad Prism 8.02. Differences between groups were tested using the Student two-tailed t-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). $p < 0.05$ was considered significant.

Conclusions

In summary, we rationally designed and constructed MOFs nanosystem (AMMOFs) by coordination of hypoxia-activated prodrug (AQ4N) and metal ions (Mn^{2+}). The AMMOFs shows several noteworthy features as multifunctional theranostic nanoplatform: 1) its sequential activation pattern could solve the core problems of existing HIFU surgery by enhanced intraoperative guiding effect of PA imaging and in turn specific treatment for postoperative residues based on HIFU-provided tumor hypoxic homogenous environment; 2) its excellent biocompatibility, well defined composition and facile fabrication technology would make it a promising candidate for future clinical translation. Therefore, it could improve the outcome of traditional HIFU surgery and has important clinical significance.

Declarations

Authors' contributions

TY-S and JN-L performed all experimental work and wrote original draft. These authors contributed equally to this work. CZ, CY-L and XR-L conducted data analysis and manuscript revisions. HN-L and HJ-Z helped critically revise the manuscript and provided project guidance. All authors read and approved the final manuscript.

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Availability of data and materials

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

Ethics approval and consent to participate

This study has been approved by the ethics committee of Chongqing Medical University and conducted in accordance with ethical guidelines.

Consent for publication

All authors agree to be published.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Schematic illustrations of sequentially activated theranostics functions of AMMOFs for PA imaging-guided HIFU therapy.

Figure 2

Characterizations of AMMOFs. (a) The size distribution of AMMOFs by DLS. (b) The morphology of AMMOFs by TEM. (c) UV-vis absorption spectrum and (d) XRD patterns of AMMOFs. (e) The size stability of AMMOFs at 37°C in PBS with 10% FBS for 24 h ($n=3$). (f) *In vitro* drug releases from AMMOFs at different pH values ($n=3$).

Figure 3

Cellular uptake, intracellular drug distribution and cytotoxicity of AMMOFs. (a) CLSM images of 4T1 cells incubated with free AQ4N or AMMOFs for 4 h (AQ4N concentration 10 mg/L). The nucleus of the cell was stained with DAPI (blue). Red fluorescence represented AQ4N. The scale bar is 100 μ m. (b, d) Flow cytometry images representing uptake and apoptosis assay of 4T1 cells incubated with free AQ4N or AMMOFs for 4 h or 48 h based on Annexin V-FITC and PI staining. (c) Cytotoxicity of free AQ4N or

AMMOFs in 4T1 cells at various AQ4N concentrations after incubation for 48 h under normoxia and hypoxia condition. Data were presented as mean±SD by t-test, ***p < 0.001. (e) CLSM images of 4T1 cells incubated with AMMOFs or free AQ4N for 48 h (AQ4N concentration 50 mg/L). The live/dead cell was stained with Calcein-AM (green) &PI (red).

Figure 4

In vivo drug accumulation and PA imaging for *in vitro* and *in vivo* detection with AMMOFs. (a) CLSM images of excised tumor cryosections at 24 h postinjection of AMMOFs (10 mg/kg of AQ4N). The scale bar is 100 μ m. (b) Average AQ4N fluorescence in the cell calculated from CLSM images. (c) *In vivo* pharmacokinetics of female SD rats at 24 h postinjection of AMMOFs (10 mg/kg of AQ4N). (d) AQ4N content in the tumor tissue of 4T1-tumor-bearing mice after intravenous injection of AMMOFs at different time intervals. (e) *In vitro* PA images and signal intensities of AMMOFs treated with different concentrations under 690 nm excitation laser irradiation (n=3). (f) Representative PA images and Quantification of PA intensity of AMMOFs covered with various thicknesses (0.25, 0.50, 1.00, 1.50, 2.00 and 2.50 cm) of pig skin (n=3). (g) Representative PA images and (h) Quantification of PA intensity of tumors at 24 h postinjection of AMMOFs (n=3).

Figure 5

Tumor hypoxia evaluation after HIFU irradiation. (a) Oxygen saturation determined from PA imaging, (b) Oxygen partial pressure measured by optical oxygen partial pressure microelectrode and (c) CLSM

imaging of hypoxia immunofluorescence staining within tumors after HIFU irradiation at 24 h. The nuclei and hypoxia areas were stained by DAPI (blue) and HIF-1 α (green), respectively.

Figure 6

In vivo antitumor effect. (a) Schematic illustration of AMMOFs therapeutic schedule. (b) Tumor inhibition curves, (c) Tumor weight, (d) Histological observation of nude mice bearing 4T1-induced tumors

administrated with PBS, free AQ4N, AMMOFs, HIFU+PBS and HIFU+AMMOFs (AQ4N dosage: 10 mg/kg body weight). Data were presented as mean±SD by t-test, *p < 0.05, **p < 0.01. The scale bar is 100 μm.

Figure 7

In vivo toxicity test. (a) Hemolysis assay of AMMOFs at different concentration (1.25, 2.5, 5.0, 10 and 20mg/L). (b) Histological analysis of major organs by H&E staining after different treatments. The scale bar is 100 μm. (c) Complete blood count of health Balb/c mice at 15 d post injection of AMMOFs. Mice injected with PBS was used as control.

Supplementary Files

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