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

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Photoacoustic (PA) imaging 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 is insufficient for achieving accurate intraoperative navigation, leading to residual tumor tissue. Nanomedicine provides a new opportunity for PA imaging to guide HIFU surgery. Studies have found that the hypoxic heterogeneity of tumor is effectively reversed by HIFU. Herein, a specific metal—organic framework nanosystem, constructed by coordination of banoxantrone (AQ4N) and Mn$^{2+}$, is designed based on HIFU to reverse the hypoxic heterogeneity of tumors. It can provide exogenous light-absorbing substances, thus improving the penetrability of PA imaging signal through the deep tissue and achieving clearer PA imaging for guiding HIFU surgery. In turn, AQ4N, in the hypoxic homogenous environment of the tumor provided by HIFU, is activated sequentially to specifically treat the residual hypoxic tumor cells. This combination treatment manifests higher tumor suppressors activation and lower expression of genes related to tumor progression. This strategy addresses the dissatisfaction with PA imaging-guided HIFU therapy and is promising for translation into a clinical combination regimen.

1. 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 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 has 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 helping to improve the imaging depth and achieve clearer PA imaging.[11,12] Moreover, nano delivery 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 in 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 networks in the focal area through thermal and cavitation mechanisms.[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.\textsuperscript{[22]} AQ4, a specific inhibition of DNA topoisomerase II, leads to DNA damage and cell death. AQ4N has achieved satisfactory results in phase I clinical trials.\textsuperscript{[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.\textsuperscript{[25,26]} In addition, our studies suggest that AQ4N has a strong PA 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/Mn\textsuperscript{2+} metal-organic frameworks (AMMOFs) nanosystem for PA imaging-guided HIFU surgery. This nanosystem could efficiently target the tumor site and provide exogenous light-absorbing substances for PA imaging. 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. Meanwhile, RNA-seq analyses reveal that this strategy significantly increases the gene expression of tumor suppressor, apoptotic and Fanconi anemia pathway while downregulating cell proliferation-related genes. In consequence, a remarkable in vivo antitumor effect is achieved by AMMOFs+HIFU. This design solves the core problems of existing HIFU surgery, might provide insights into non-invasive tumor therapy.

2. Results and Discussion

2.1. Preparation and Characterization of AMMOFs

The schematic illustration of the detailed preparation route of AMMOFs was presented in Figure 1. First, ordered AQ4N/Mn\textsuperscript{2+} 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, the 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 Mn\textsuperscript{2+} 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 < 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 618 nm, further confirming the successful fabrication of AMMOFs (Figure 2c). XRD pattern confirms that AMMOFs have a 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 Mn\textsuperscript{2+} 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 exhibit favorable stability in the PBS containing 10% FBS during 24 h at 37 °C, even in the lower concentration 0.1 mg mL\textsuperscript{−1} (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 applications that could guarantee its safety during delivery process, while realizing on-demand AQ4N release at the target site.

2.2. 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. To further analyze intracellular drug distribution, we calculated the Pearson correlation coefficient (PCC) and Overlap coefficient (OC) between the fluorescence signals from AQ4N and DAPI in confocal images, which showed similarly high results in free AQ4N and AMMOFs (PCC = 0.7922, 0.8176 and OC = 0.9310, 0.9635, respectively), indicating that AMMOFs could enter the tumor cells and specifically accumulate in the nucleus. Further, we quantitatively 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.\textsuperscript{[22]} To validate it, the in vitro selective cytotoxicity of AQ4N was evaluated in normoxic or hypoxic conditions. As shown in CCK-8 assay, AMMOFs exhibit significantly more cytotoxicity in hypoxic environment. The half maximal inhibitory concentration (IC\textsubscript{50}) of AMMOFs in the normoxic and hypoxic conditions are 25.06 and 0.119 mg L\textsuperscript{−1} (Figure 3c), respectively, further confirming its effect in the hypoxic environment. The IC\textsubscript{50} values of free AQ4N in the normoxic and hypoxic conditions are 37.46 and 6.78 mg L\textsuperscript{−1}, both are higher than IC\textsubscript{50} of AMMOFs. Sudden overdoses of ions in the cytosol or other intracellular compartments often induce programmed cell death.\textsuperscript{[27]} Thus, the lower IC\textsubscript{50} of AMMOFs might be related to the contribution of Mn\textsuperscript{2+}.
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 ≈99%), while nearly no cell apoptosis in the control groups and fewer 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 conditions, which indicates 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 levels within tumors after HIFU irradiation.

2.3. In Vivo Pharmacokinetics, Biodistribution, and Intratumoral Release

To observe the intratumoral release of AQ4N, a frozen section of tumors 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 times postinjection almost kept constant. The results suggest that AQ4N assembled in the AMMOFs has good physiological stability and facilitates prolonging 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 laying a solid foundation for enhancing the antitumor efficacy.

2.4. 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⁻¹) 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 weakly 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 the penetration depth of PA in the tissue and be used for in vivo deep imaging.

In contrast, in the free AQ4N group, 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 the EPR effect have overcome the limitations of in vivo delivery of AQ4N. The results exhibit excellent PA imaging performance of AMMOFs, which shows that AMMOFs have great potential for enhancing guiding effect of HIFU surgery.

2.5. 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
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\(^{-1}\)). The nucleus of the cell was stained with DAPI (blue). Red fluorescence represented AQ4N. The scale bar is 50 \(\mu \text{m}\). b,d) Flow cytometry images representing uptake and apoptosis assay of 4T1 cells incubated with free AQ4N or AMMOFs for 4 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 conditions. 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\(^{-1}\)). 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\(^{-1}\) of AQ4N). The scale bar is 100 μm. 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\(^{-1}\) 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 partial pressure measured by optical oxygen partial pressure microelectrode. b, c) Oxygen saturation determined from PA imaging. d) CLSM imaging of hypoxia immunofluorescence staining within tumors after HIFU irradiation at pre, 1, 4, 12, and 24 h. The nuclei and hypoxia areas were stained by DAPI (blue) and HIF-1α (green), respectively. e) Average HIF-1α fluorescence in the cell calculated from CLSM images.

2.6. In Vivo Antitumor Efficacy

The in vivo antitumor efficacy of AMMOFs combined with HIFU was systematically assessed. Tumor-bearing nude mice were treated with PBS, free AQ4N, or AMMOFs (the dosage of AQ4N: 10 mg kg⁻¹). After 12 h of accumulation at the tumor site, mice were immersed in degassed water for HIFU irradiation at 100 W for 5 s (Figure 6a). As shown in Figure 6b, within the 15 days following the treatment, the tumor volumes of free AQ4N, HIFU, and AMMOFs groups showed increasing tendency in different degrees, 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, AMMOFs+HIFU 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),...
Figure 6. In vivo antitumor effect. a) Schematic illustration of AMMOFs therapeutic schedule. b) Tumor inhibition curves, c) Tumor weight, d) photographs of excised tumor tissues at the end of the treatment period, e) Histological observation of nude mice bearing 4T1-induced tumors administered with PBS, free AQ4N, AMMOFs, HIFU, and AMMOFs+HIFU (AQ4N dosage: 10 mg kg$^{-1}$ body weight). Data were presented as mean ± SD by t-test, *p < 0.05, **p < 0.01. The scale bar is 100 μm.
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 AMMOFs + HIFU group, while moderate levels of damage were in the other groups. PCNA and TUNEL-stained images followed the same trend and revealed pronounced apoptosis and DNA damage (Figure 6c).

2.7. Transcriptomic Analysis

We further studied the molecular mechanisms of the combined effect of AMMOFs and HIFU, and the transcriptomics change of tumor tissues after different treatments was compared by RNA-seq. A total of 18 584 genes in tumor tissues were measured after treatment with PBS, AMMOFs, and AMMOFs + HIFU (Figure 7a). Among these, 338 genes were found to be differentially expressed ([log2-fold change] ≥ 1 and p-value < 0.05) between control (treated with PBS) and AMMOFs + HIFU. The heat map showed most of the differentially expressed genes (DEGs) were up- or down-regulated in AMMOFs + HIFU group (Figure 7b). The volcano plots were drawn by quantitative analysis (Figure 7c; Figure S1, Supporting Information) and showed Bmp5, Adamt15, Sfrp1, Ccn5, Tnxb, and Chl1 were up-regulated which could inhibit tumor growth in mammary tissues, while the genes related to cancer cell proliferation and progression (Wnt2, F1rt3, Fxyd3, Hoxb9, Cx3cr1, and S100a14) were down-regulated in AMMOFs + HIFU group compared to control. Sequentially, the gene-regulated pathway was analyzed by the Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis. As shown in Figure 7d and Figures S2 and S3 (Supporting Information), the up-regulated genes played an important role in the processes of cellular, metabolism, genetic information processing, and organismal systems, etc. The DEGs were also classified into “biological process”, “cellular component”, and “molecular functions” categories by gene ontology (GO) analysis (Figure 7e; Figure S4, Supporting Information).

Moreover, GSEA revealed a positive gene enrichment for Fanconi anemia pathway (NES = 1.82, FDR = 5.89e-3), mismatch repair (NES = 1.98, FDR = 9.95e-4) and DNA replication (NES = 2.24, FDR = 0.0) in the AMMOFs + HIFU group compared to control. Sequentially, the gene-regulated pathway was analyzed by the Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis. As shown in Figure 7d and Figures S2 and S3 (Supporting Information), the up-regulated genes played an important role in the processes of cellular, metabolism, genetic information processing, and organismal systems, etc. The DEGs were also classified into “biological process”, “cellular component”, and “molecular functions” categories by gene ontology (GO) analysis (Figure 7e; Figure S4, Supporting Information).

Importantly, based on Figure 7g, the expression of CRT and cleaved caspase-3 was also up-regulated by AMMOFs, while caspase-3 was down-regulated. Mechanistically, these above kinases down- and up-regulated by AMMOFs further up-regulated the expression of cell apoptosis-related proteins (CRT and cleaved caspase-3), leading to the cell apoptosis of 4T1 cells. In addition, compared with control and AMMOFs, AMMOFs + HIFU also prominently enhanced the expression of γH2A.X (a DNA damage-associated marker). All these results indicate that AMMOFs + HIFU treatment significantly induces 4T1 cell apoptosis and inhibits tumor metastasis compared with control and AMMOFs treatment.

Overall, these results reveal that sequentially activated tumor therapy with AMMOFs + HIFU is a promising strategy. Via this approach, the first applied AMMOFs and PA-guided HIFU could not only accurately ablate tumor tissue, but also generate severe hypoxic environment of tumor by 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 in enhanced HIFU-based tumor theranostics.

2.8. In Vivo Toxicity Test

The 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 8a), the hemolysis rate was below 5% due to negligible destruction of blood cells. Blood biochemistry and complete blood panel analysis were conducted on day 15. As shown in Figure 8c–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 8b). The great biocompatibility might be attributed to low toxic AQ4N under the normoxia environment and nontoxic Mn2+ 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 agents based on HIFU.

3. Conclusion

In summary, we rationally designed and constructed MOFs nanosystem (AMMOFs) by coordination of hypoxia-activated prodrug (AQ4N) and metal ions (Mn2+). The AMMOFs show several noteworthy features as multifunctional theranostic nanoplatforms: 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.
Figure 7. Transcriptomic analysis of the 4T1 tumor tissues in vivo after different treatments. a) Venn diagram showed the number of genes transcribed in each treatment group. b) Heat map of differentially expressed genes (DEGs) in 4T1 tumor tissues after various treatments ($n=3$). c) Volcano plot of DEGs in 4T1 tumor tissues after treatment with control and AMMOFs+HIFU. d) KEGG enrichment analysis and e) GO enrichment classification of the DEGs between AMMOFs+HIFU and control group. f) Gene set enrichment analysis (GSEA) revealed the positive enrichment of the DNA replication, mismatch repair, and Fanconi anemia pathway, and negative enrichment of the lysosome and ribosome in AMMOFs+HIFU compared to those treated with control. g) Western blotting plots showed the DNA damage and apoptosis-associated proteins in 4T1 tumor tissues after different treatments.
Figure 8. In vivo toxicity test. a) Hemolysis assay of AMMOFs at different concentrations (1.25, 2.5, 5.0, 10, and 20 mg L$^{-1}$). b) Histological analysis of major organs by H&E staining after different treatments. The scale bar is 100 μm. c–j) Complete blood count of healthy Balb/c mice at 15th day postinjection of AMMOFs. Mice injected with PBS were used as a control.

4. Experimental Section

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-1α 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 (No. 2018020).

Preparation of AMMOFs: Briefly, AQ4N (2 mg) was dissolved in deionized water (1 mL), and MnCl2 (100 μL, 1 mg mL$^{-1}$) was added to the preceding solution and agitated for 4 h. The aforesaid mixture was then stirred with 400 μL TEA at room temperature and protected from light. After 12 h, by vigorous stirring, the mixture was dropped into 10 mL of 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 618 nm was used to measure the amount of AQ4N in the AMMOFs. Loading capability (LC) was calculated as shown in the expression below:

$$LC_{(\text{wt}\%)} = \frac{\text{loaded – drug weight}}{\text{loaded – drug nanoparticles weight}} \times 100\% \quad (1)$$

Characterization of AMMOFs: DLS on a Zetasizer Nano (Malvern, UK) and TEM (JEM-1200EX, Japan) were used to determine the size and shape.
of AMMOFs. The AMMOFs solution (1 mL, 1 mg mL\(^{-1}\)) was measured in a glass cuvette at 25 °C with a scattering angle of 173°. In order to test the stability, AMMOFs were 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 by being sealed in dialysis bags after exposure 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 a UV spectrophotometer at the appropriate time points. All measurements were made in three replicates and values are expressed as mean ± standard deviation (n = 3).

Intracellular Drug Release, Distribution, and In Vitro Cytotoxicity of AMMOFs: 4T1 cells (4 × 10^5) were seeded 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\(^{-1}\) for an additional 4 h. Cells were then gently washed three times in PBS, stained with DAPI for 10 min, and visualized by confocal laser scanning microscopy (CLSM, Leica, Germany) at 640 nm excitation wavelengths.

4T1 cells (4 × 10^5) 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\(^{-1}\) 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) 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\(^{-1}\) 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 488 and 650 nm was used to analyze the cells after being resuspended in PBS.[11]

4T1 cells (4 × 10^5) 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 free AQ4N or AMMOFs formulas at a concentration of 10 mg L\(^{-1}\). After that, the relative cell viabilities were determined by using the standard Cell Counting Kit-8 (CCK-8) treatment.[32]

Live/dead cell staining of 4T1 cells was performed by Calcein-AM/PI double stain kit. Briefly, 4T1 cells (4 × 10^5) per well were seeded in six-well plates and incubated in a normoxic or hypoxic atmosphere as described above. Free AQ4N or AMMOFs formulas were added to the plate at a concentration of 30 mg L\(^{-1}\), 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 two groups (six mice in each group), and free AQ4N or AMMOFs at 25 mg kg\(^{-1}\) (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\(^{-1}\) (AQ4N/body weight) when the mean volume of tumors reached 200 mm\(^3\). 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 (six mice in each group) were sacrificed, and 1 mL hydrochloric acid was used to dissolve 0.1 g tumor tissue homogenate (2.0 mL). 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 Vivo and In Vivo PA Imaging of AMMOFs: Small-animal PA imaging equipment (VeoLoZAR, Visual Sonics, Toronto, Canada) was used with a laser emission probe (laser excitation between 680 and 1000 nm with an excitation depth of ~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 (0.1, 0.25, 0.50, 1.00, and 2.00 mg mL\(^{-1}\)) 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\(^{-1}\)) 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\(^{-1}\)) in the tissue was measured using a 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 a tumor size of ~200 mm\(^3\) were injected intravenously with 0.2 mL AQ4N formulas at 10 mg kg\(^{-1}\) (AQ4N/body weight). Images were determined by a 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 a tumor size of ~200 mm\(^3\) were immersed in degassed water for HIFU irradiation. After 24 h, the mice were anesthetized with 1% pentobarbital and placed on a PA imaging system. Oxygen saturation (SO\(_2\)) was used to evaluate the hypoxia intensity. SO\(_2\) was calculated using the following equation:

\[
\text{Oxygen saturation (SO}_2\text{)} = \frac{M(HbO_2)}{M(Hb) + M(HbO_2)} \times 100\%
\]

where \(M(Hb)\) and \(M(HbO_2)\) represent the mean intensity value of Hb and HbO\(_2\) in tumor regions detected by oxygenated hemoglobin signal.

4T1 xenografted nude mice with a tumor size of ~200 mm\(^3\) 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 a tumor size of ~200 mm\(^3\) were immersed in degassed water for HIFU irradiation. The mice were euthanized before and 1, 4, 12, and 24 h after treatment, 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 the anti-rabbit anti-HIF1α (Santa Cruz Biotechnology, CA, USA) secondary antibody conjugated to Anti-HRP. 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 mm\(^3\), 4T1 xenografted nude mice were randomly divided into five groups (six mice in each group). Mice were intravenously administered with PBS, free AQ4N, or AMMOFs at 10 mg kg\(^{-1}\) (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 at 100 W for 5 s. 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.[13]

RNA-Seq Analysis: Total RNA was extracted from the different treated cancer tissues utilizing TRIzol (Invitrogen). The Nano Drop and Agilent 2100 bioanalyzer (Thermo Fisher Scientific) was used for qualifying and quantifying total RNA. RNA-seq libraries were sequenced with the sequence reads.
paired-end option using a Sequencer at BGI (Shenzhen) as per the manufacturer’s recommended protocol. DESeq2 was used to perform differential expression analysis, reducing the effect of gene length and library size from each sample. All the visualization and pathways enrichment were implemented on these processed data.

**Western Blotting:** For western blotting, ≈50 mg of each tissue was ground into powder in the homogenizer for selecting total proteins. Subsequently, total proteins were separated by SDS/PAGE loading buffer and transferred to Immobilon-P membrane. Primary antibodies were applied in 5% BSA and incubated overnight at 4 °C. After being extensively washed by TBST, the membranes were incubated with Goat anti-mouse/rabbit poly-HRP secondary antibodies (1:5000) for 1 h at room temperature and followed with Enhanced Chemiluminesence (NCM Biotech). β-actin antibodies were used for normalization and accurate quantification.

The primary antibodies were used including β-actin (1:5000, Proteintech), CRT (1:1000, Abcam), Caspase-3 (1:1000, Proteintech), cleaved caspase-3 (1:5000, Abcam), γ-H2AX (1:1000, Abcam).

**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 AMO-Fs (1.25, 2.5, 5, 10, and 20 mg L⁻¹) 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. Supernatant samples (0.1 ml) 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:

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

(3)

The identical circumstances (in vivo antitumor therapy) were applied to healthy female BALB/c mice. Fifteen days after injection, the mice were anesthetized 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), 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 ± standard deviation (SD) of at least three studies. Data analysis was performed using Origin 8.0 and GraphPad Prism 8.0. 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.

**Supporting Information**

Supporting Information is available from the Wiley Online Library or from the author.

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**Conflict of Interest**

The authors declare no conflict of interest.

**Author Contributions**

T.S. and J.L. contributed equally to this work. T.S. and J.L. carried out the experiments and analyzed the data. C.Z. assisted with cell experiments, C.L. and X.L. assisted in the analysis of animal experimental data. H.L. proposed the concepts and supervised the project. T.S. and J.L. interpreted the data and wrote the manuscript with further editing by H.L.

**Data Availability Statement**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

**Keywords**

HIFU, hypoxia-activated, nanomedicines, photoacoustic imaging, tumor theranostics

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