Supporting Information (SI) for

Enhancing Targeted Cancer Treatment by Combining Hyperthermia and Radiotherapy Using Mn-Zn Ferrite Magnetic Nanoparticles

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1. Experimental section

1.1 Synthesis of $Mn_{0.6}Zn_{0.4}Fe_2O_4$ nanoparticles

$Mn_{0.6}Zn_{0.4}Fe_2O_4$ (MZF) nanoparticles were synthesized according to the previously reported method[1]. 0.2 mmol $Fe(acac)_2$, 0.6 mmol $Mn(acac)_2$ and 0.4 mmol $Zn(acac)_2$ were mixed with 10 mmol 1,2-hexadecanediol, 3 mmol oleic acid and 3 mmol oleylamine in 20

mL benzyl ether under the protection of argon with the magnetic stirring. The mixture was heated to 200 ° C for 2 h, and then heated to 300 °C at 2°C/min for 90 min. After cooling to room temperature, 30 mL of ethanol was added to the product, and then centrifuged (8000 rpm, 10 min) to obtain a dark-brown precipitate. The filtrate was removed by centrifugation (8000 rpm, 10 min), 0.05 mL oleic acid and 0.05 mL oleylamine were added to the precipitate which was dissolved and dispersed with n-hexane. After centrifugation (8000 rpm, 10 min), any undispersed impurities were removed. After being reprecipitated with ethanol and centrifuged (8000 rpm, 10 min) to remove the solvent, the $Mn_{0.6}Zn_{0.4}Fe_2O_4$ (MZF) nanoparticles were obtained after drying under vacuum for 24 h.

1.2 Synthesis of MZF-NH_2

For functionalization of oleic acid (OA)-coated MZF, a chemical conjugation of hyaluronan was carried out[2]. Briefly, 5 mg MZF nanoparticles were dispersed in 1mL THF and then dispersion solution was added into 1mL of the NH_2-PEG_{2000}- PCL_{3400} polymer solution (10 mg/mL in THF). The mixture solution was dropped into 5mL deionized water followed by ultrasonication for 5 min. The mixture was dialyzed against deionized water (MWCO 3400) at room temperature for 24 h to obtain MZF-NH_2.

1.3 Synthesis of MZF-HA

Hyaluronic acid-modified MZF (MZF-HA) was synthesized via previous method with some adjustments[3]. Briefly, Hyaluronic acid (0.44g, 1 mmol), NHS (0.12g, 1 mmol) and EDC•HCl (0.21g, 1 mmol) were dissolved in 100ml PBS. The mixture was tstirred at 25°C in dark and nitrogen atmosphere protected for overnight. Subsequently, the mixture was

added with MZF-NH_2 and stirred for another 24 hours. The solvent(PBS) and unreacted hyaluronic acid were removed through dialysis for 3 days against deionized water. The resultant solution was lyophilized and MZF-HA was obtained.

2. Characterization techniques

The structural formation of MZF nanoparticles was characterized by X-ray diffraction (XRD) on a Rigaku D/Max-2550 X-ray powder diffractometer from $2\theta = 10°$ to 80° at a speed of 5° min-1. The morphology of MZF and MZF-HA was observed using a JEOL JEM-2010F transmission electron microscope (TEM) at an accelerating voltage of 120 kV. The size distributions and zeta potential values of MZF and MZF-HA were detected using Malvern Zetasizer Nano-ZS90 at 25℃. Attenuated total reflection Fourier transform infrared (ATR FT-IR) spectra of the samples were measured on an EQUINOXSS/HYPERION 2000 FTIR spectrometer (Bruker, Germany). D_2O was utilized as solvent to verify the formation of micelle by ^1H NMR analysis using Bruker DMX 500 NMR. The magnetic properties of MZF and MZF-HA were also recorded by a vibration sample magnetometer (Lake Shore 7410, USA) at 25°C. MRI experiment of MZF-HA *in vitro* were performed on a clinical 3.0 TMR scanner (GE Discovery MR750).

3. Cell culture

A549（human non-small cell lung cancer cell line） and Beas-2B cells [normal human lung (bronchial) epithelium) were purchased from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. A549 cells were cultured in F-12K medium (Invitrogen, 21127-022) and Beas-2B cells were cultured in Dulbecco's Modified

Eagle's medium (DMEM, Hyclone). Both of the medium were supplemented with 10% fetal bovine serum (Gibco), penicillin G (50 U/mL) and streptomycin (50 μg/mL, Gibco). All cells were kept in a humidified incubator at 37 °C with 5% CO_2.

4. CD44 Detection in A549 cells by immunohistostaining

The CD44 expression on A549 cells was detected by immunohistostaining. A549 cells were inoculated in a 12-well plate on cell slides at a density of 3×10^4 cells/well and incubated for 24 h. After that, 4% paraformaldehyde in PBS with 4 °C precooling was used for fixing sections on slides for 20 min. After washing three times for 5 min with PBS, the cells were incubated in PBS containing 1% BSA and 0.3% Triton for 60 min. CD44 primary antibody (15675-1-AP, Proteintech) was diluted in PBS containing 5% donkey serum and then incubated with cells for 60 min at RT. After removing the excess primary antibody, cells were washed three times for 5 min with PBS. Secondary antibody (goat anti-rabbit IgG coupled to DyLight 594, Invitrogen) was added into the cells. After incubating for 1 hour at RT, cells were washed three times for 5 min with PBS to remove the excess secondary antibody. The nuclei were stained with DAPI (Invitrogen) for 15 min at RT and washed 6 times for 10 min with PBS to remove the excess reagents. Prepared cell slides were observed under a fluorescence microscope, where the dimming parameter of DAPI is excitation light wavelength/emission light wavelength: 346 nm/454 nm, and the dimming parameter of DyLight 594 is excitation light wavelength/emission Light wavelength: 592 nm/617 nm.

5. Fluorescence confocal experiment for cell intake of MZF-NH_2 and MZF-HA

MZF-NH_2 and MZF-HA were required to be labelled with FITC in advance. Briefly, a solution of FITC (1 mg) dissolved in DMSO (1 ml) was added into MZF-NH_2 or MZF-HA (2mg/ml, 10 ml), stirring in the dark at room temperature for 24 hours. The reaction mixture was dialyzed against PBS for another 24 hours to remove the excess DMSO and FITC completely. Subsequently, A549 or Beas-2b cells at a density of 3×10^4 cells were incubated with 60 μg/mL FITC labeled MZF-NH_2 or MZF-HA in complete DMEM. After incubation for 4 h, the cells were washed with PBS for 3 times to remove the nanoparticles not up-taken by the cells. The wells without any of nanoparticles were used as a control. Then the cells were fixed by1 ml 4% paraformaldehyde solution for 20 min and stained by DAPI. The confocal microscopy images of two kind of cells were obtained by a fluorescence microscope (Olympus BX5, Pooher, Japan).

6. Prussian blue staining experiment for cell intake of MZF-NH_2 and MZF-HA

A549 or Beas-2b cells at a density of 3×10^4 cells/well were seeded on the 12-well plate and incubated for 24 h. After removing the medium, 60 μg/mL MZF-NH_2 or MZF-HA in complete DMEM was added and incubated at 37 °C for 24 h. The cells were washed with PBS for three times and then were fixed by 1 ml 4% paraformaldehyde solution which was precooled at 4 °C for 20 min. 1 ml of a mixture of 2% Prussian blue and 4% hydrochloric acid in a 1: 1 ratio of PBS were added to each well, and incubated at 37 °C for 30 minutes in the dark until a dark blue complex is visible under the microscope. After washing for 3 times with PBS for 5 min each, nuclear red staining solution were added and lightly stained the nucleus for 5 min. The samples were washed 3 times with PBS for 5 min each time, and finally sealed with neutral resin. The wells without MZF-NH_2 or MZF-HA were used

as a control. Then the cells were counterstained with nuclear fast red for 5 min. Prussian blue staining images were taken on optical microscope (Olympus BX5, Pooher, Japan).

7. Cytotoxicity *in vitro* analysis

The cytotoxicity of MZF-NH_2 and MZF-HA were assessed in A549 and Beas-2b cells by CCK-8 reaction. Briefly, A549 or Beas-2b cells were seeded in a 96-well plate and placed in a 37°C, 5% CO_2 cell incubator for 24 hours to adhere the cells. Dilute the magnetic fluid (MZF-NH_2 and MZF-HA) in PBS to different concentrations with complete culture medium, and after adding 100 μL of the above material-containing medium, the cells were placed at 37°C to incubate for another 24 hours. 10 μL of pre-mixed CCK-8 and 100 μL of DMEM were cultured with medium above for 1-2 hours. Detection was then performed on a microplate reader (Biotek Epoch, USA) with a detection wavelength of 450 nm. All experiments were carried out in triplicate.

References

1. Qu, Y.; Li, J.B.; Ren, J.; Leng, J.; Lin, C.; Shi, D. Enhanced Magnetic Fluid Hyperthermia by Micellar Magnetic Nanoclusters Composed of $Mn_xZn_{1-x}Fe_2O_4$ Nanoparticles for Induced Tumor Cell Apoptosis. *ACS Appl. Mater. Interfaces* **2014**, *6*, 16867-16879.
2. Yang, R. M.; Fu, C. P.; Li, N. N.; Wang, L.; Xu, X. D.; Yang, D. Y.; Fang J Z; Jiang, X.Q.; Zhang, L. M. Glycosaminoglycan-Targeted Iron Oxide Nanoparticles for Magnetic Resonance Imaging of Liver Carcinoma. *Materials Science and Engineering: C* **2014**, *45*, 556-563.

3. Lee, T.; Lim, E. K.; Lee, J.; Kang, B.; Choi, J.; Park, H. S.; Suh, J. S.; Huh, Y. M.; Haam, S. Efficient CD44-Targeted Magnetic Resonance Imaging (MRI) of Breast Cancer Cells Using Hyaluronic Acid (HA)-Modified $MnFe_2O_4$ Nanocrystals. *Nanoscale Research Letters* **2013**, *8*, 149.

Supplementary Figures and Tables

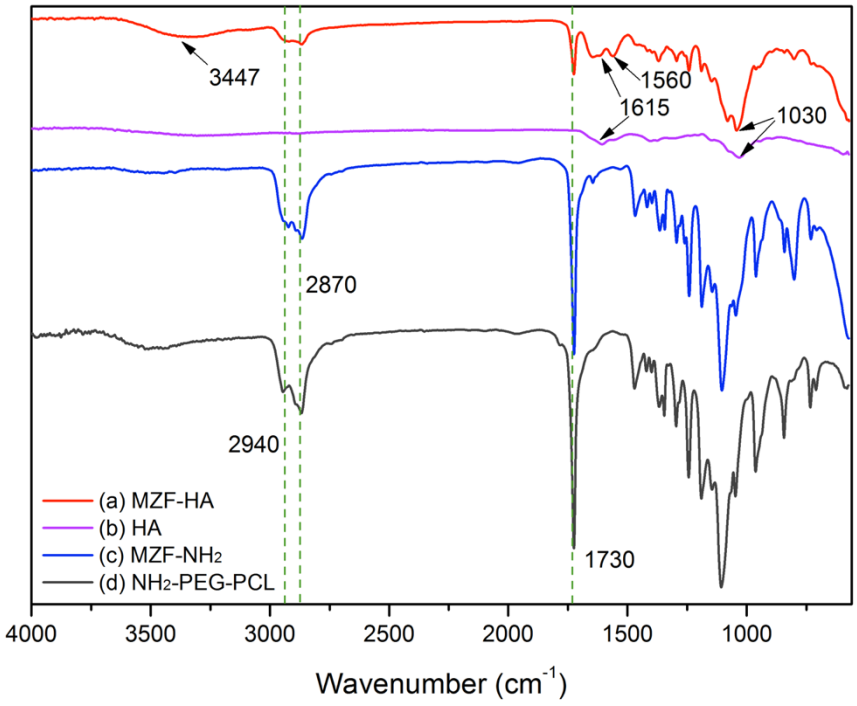

Figure S1. FT-IR spectra of (a) MZF-HA, (b) HA, (c) MZF-NH_2 and (d) NH_2-PEG-PCL.

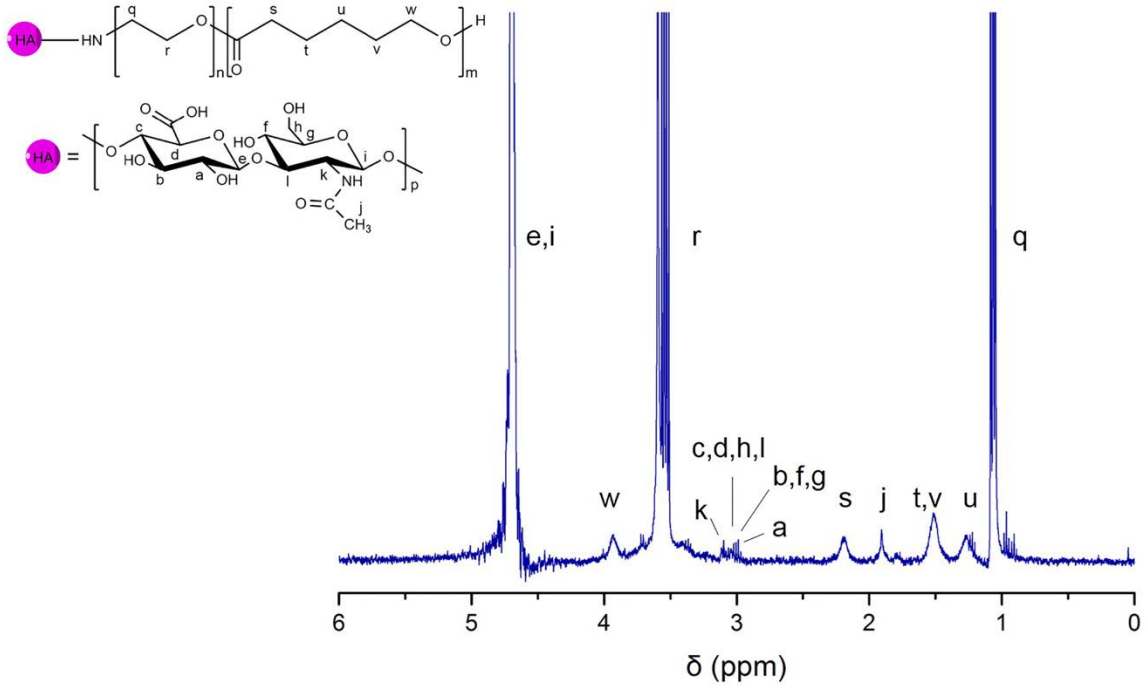

Figure S2. ^{1}H NMR spectra of HA-PEG-PCL.

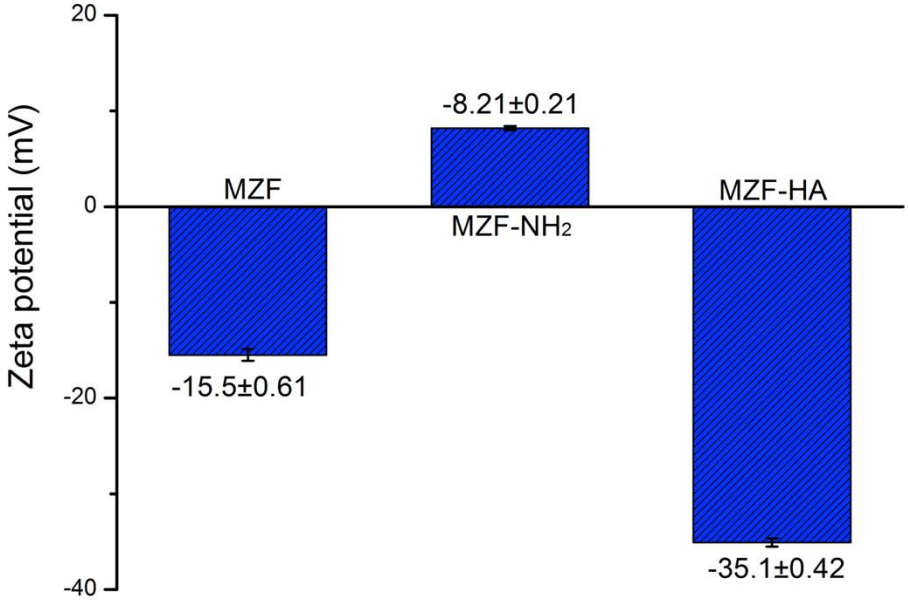

Figure S3. Zeta potential of MZF, MZF-NH_2 and MZF-HA in aqueous dispersions.

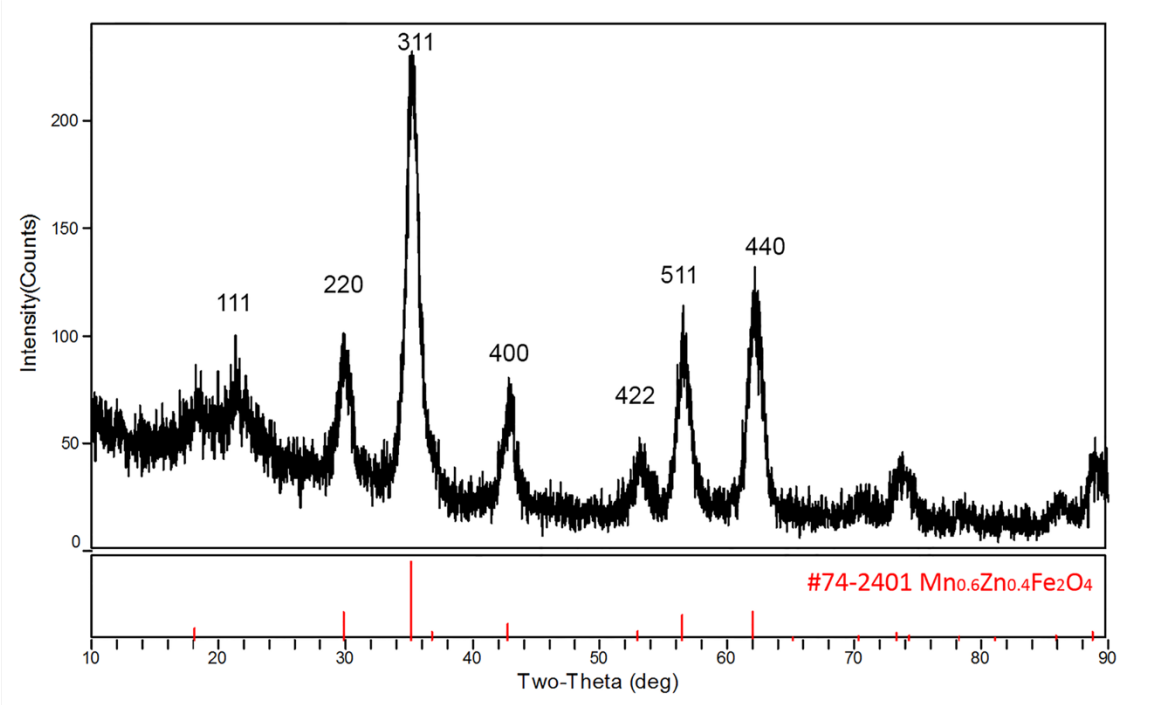

Figure S4. XRD pattern of MZF.

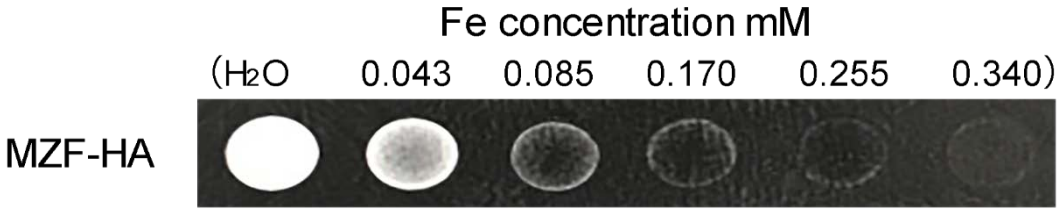

Figure S5. MR images of MZF-HA at different concentrations of iron ions.

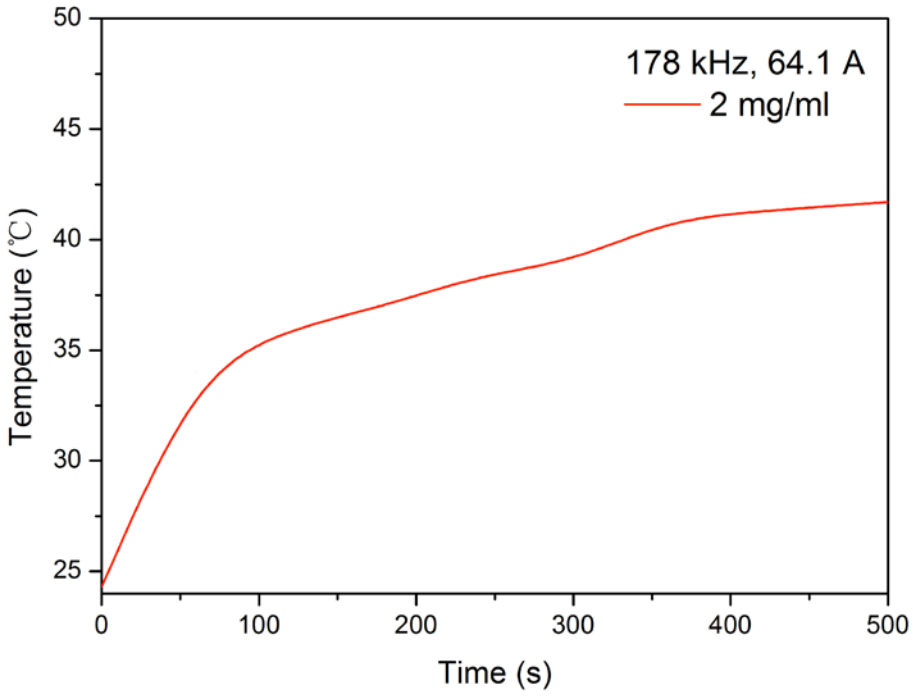

Figure S6. Time-temperature curves of MZF-HA under AMF (178 kHz, 64.1 A).

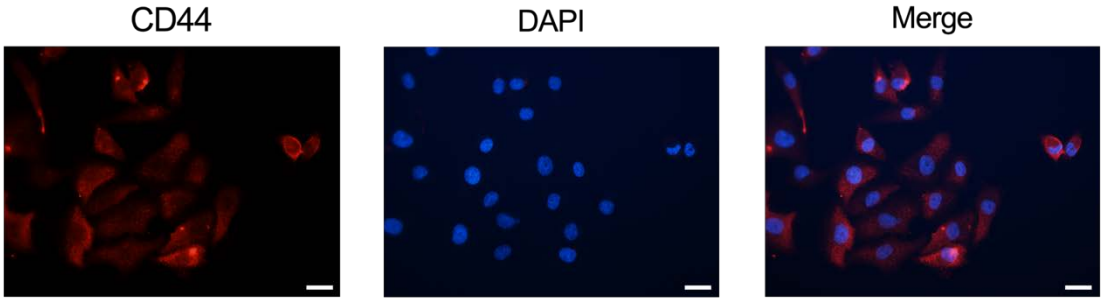

Figure S7. Immunofluorescence microscopy image of A549 cells stained by CD44 primary antibody and goat anti-rabbit IgG coupled to DyLight 594. Scale bar: 20 μm.

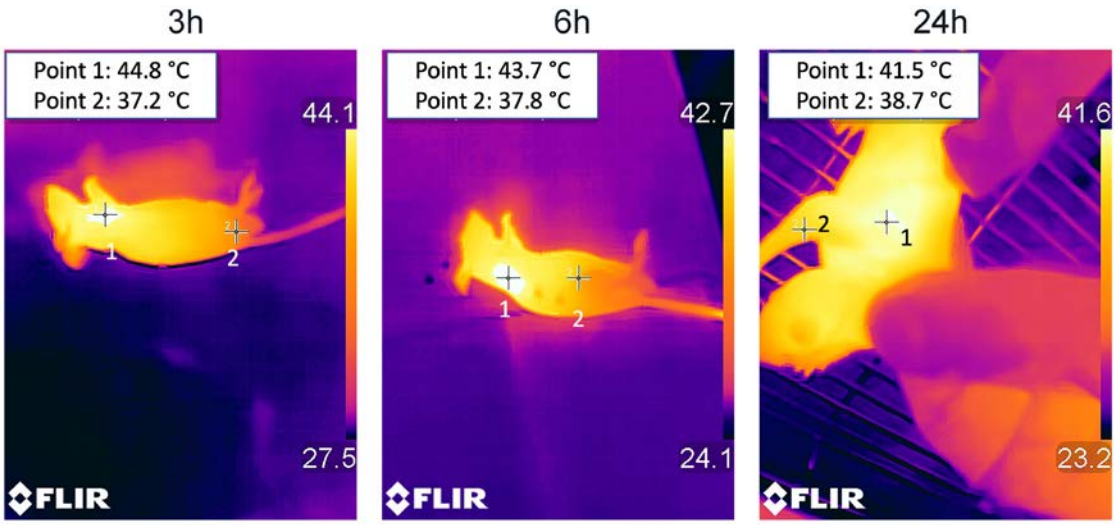

Figure S8. Thermal image of mice tumor under AMF after different injection time (3 h, 6 h and 24 h).

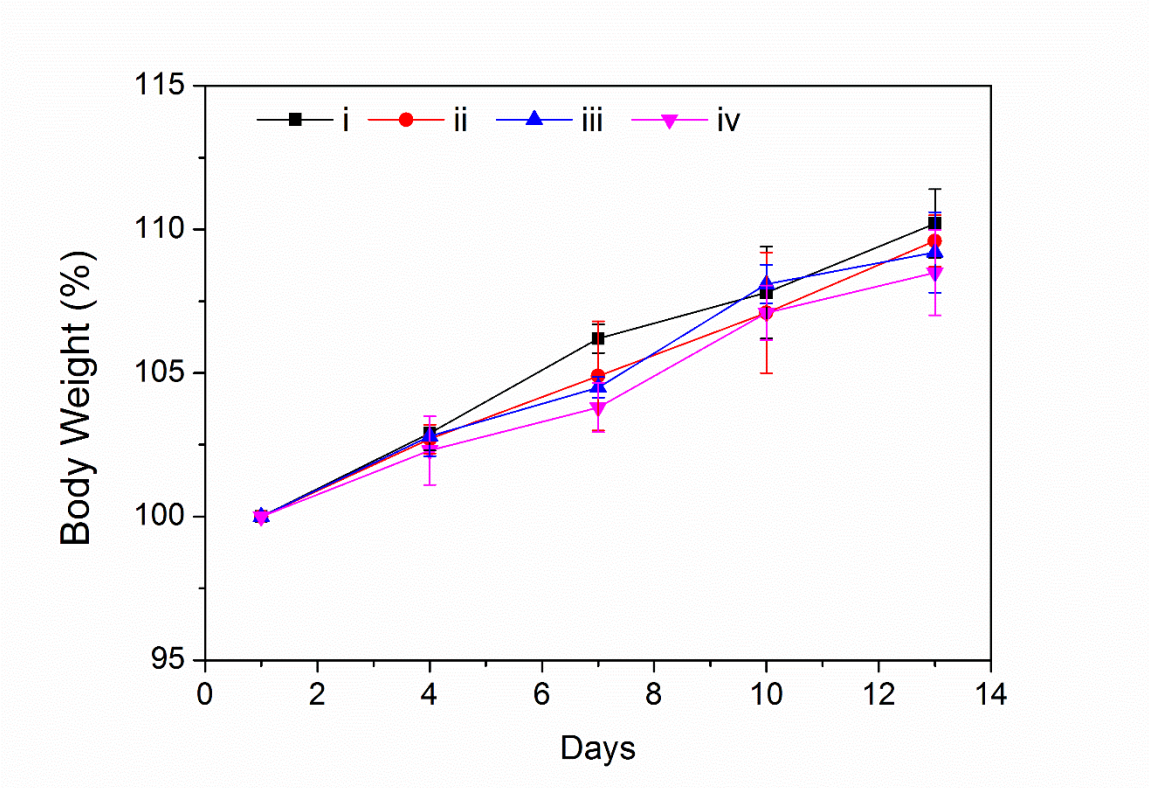

Figure S9. Body weight changing rate of various treatment group: (i) control group: PBS

injection via tail vein, (ii) HT group: treated with MZF-HA and AMF, (iii) RT group: treated with RT alone, (iv) HT plus RT group: treated MZF-HA, AMF plus RT. The treatment was performed on Day 1 and Day 4. Error bars represented standard deviation.

Table S1 $MGV_{(Tumor)}/MGV_{(Muscle)}$ values before and after the injection of MZF-HA after different time periods in MRI.

Before	24h	48h
1.8946	1.7096	1.9489
1.9072	1.4492	1.7282
1.8939	1.7980	2.2840
2.0410	1.2727	1.6037
1.9258	1.3301	1.9421
1.9558	1.4510	1.4061
2.0769	1.2441	1.7319
2.1240	1.3486	1.5897
2.1532	1.4233	1.4527
1.6667	1.7450	1.5640

1. For each baseline and post injection T_2 -weighted image slice, a region of interest (ROI) was manually placed around the tumor edge and muscle edge on each slice, and the division of $MGV_{(Tumor)}/MGV_{(Muscle)}$ were calculated based on 5 slices for each mouse at 3 different time points.
2. Statistical analysis: Statistical differences were assessed in Student's t-test for comparison between two groups. $P<0.05$ represented statistically significant.

Before vs. 24h: P<0.0001; Before vs. 48h: P=0. 0.0228; 24h vs. 48h: P= 0.0311

Table S2 Percent of Hypoxyprobe positive tumor cells per field in hypoxia immunohistochemistry (IHC) analysis.

	1	2	3	4	5
Control 1	10.14%	9.27%	11.40%	3.72%	9.69%
Control 2	2.98%	1.21%	1.12%	0.91%	1.94%
Control 3	7.41%	3.76%	1.18%	0.35%	5.32%
HT 1*	1.32%	0.83%	1.42%	0.41%	0.16%
HT 2	0.12%	2.71%	0.08%	0.17%	1.92%
HT 3	0.20%	0.81%	1.19%	1.16%	0.42%

*HT: heat therapy group

1. Quantification of hypoxia areas were performed with ImageJ (1.8.0_112, National Institutes of Health), by analyzing five random views under 5x field for each tumor section. Threshold was set at a fixed value (0,148) for all images. Data in the table showed the percent of Hypoxyprobe positive tumor cells in 5 random fields tumor section of each mouse.

2. Statistical analysis: Statistical differences were assessed in Student's t-test for comparison between two groups. P<0.05 represented statistically significant.

Control vs. HT: P= 0.0008

Table S3 Change of absolute tumor volume(cm^3).

	Day 1	Day 4	Day 7	Day 10	Day 13

a1	0.196	0.184	0.196	0.239	0.288
a2	0.040	0.044	0.048	0.052	0.071
a3	0.075	0.098	0.106	0.135	0.135
b1	0.075	0.063	0.063	0.040	0.040
b2	0.063	0.069	0.063	0.063	0.051
b3	0.051	0.051	0.036	0.046	0.046
c1	0.100	0.094	0.094	0.088	0.088
c2	0.036	0.032	0.025	0.014	0.006
c3	0.063	0.063	0.051	0.051	0.046
d1	0.051	0.051	0.040	0.036	0.025
d2	0.036	0.036	0.032	0.036	0.036
d3	0.091	0.075	0.061	0.051	0.028

Table S4 Change of relative tumor volume (%), compared to the tumor volume of Day 1 of each mouse.

	Day 1	Day 4	Day 7	Day 10	Day 13
a1	100.00	93.75	100.00	121.97	146.94
a2	100.00	110.00	120.00	130.00	177.19
a3	100.00	131.08	141.17	180.00	180.00
b1	100.00	83.33	83.33	53.33	53.33
b2	100.00	110.00	100.00	100.00	81.00
b3	100.00	100.00	71.11	90.00	90.00

c1	100.00	93.75	93.75	87.50	87.50
c2	100.00	88.89	68.06	37.50	16.67
c3	100.00	100.00	81.00	81.00	72.90
d1	100.00	100.00	79.01	71.11	48.40
d2	100.00	100.00	88.89	100.00	100.00
d3	100.00	82.64	66.94	55.79	30.37

1. Groups were divided as follows: (i) control group: PBS injection via tail vein, (ii) HT group: treated with MZF-HA and AMF, (iii) RT group: treated with RT alone, (iv) HT plus RT group: treated with MZF-HA and AMF plus RT.

2. The tumor volumes were calculated using the formula: $V = ab^2/2$, where a is the longest and b is the shortest diameter of the tumor. V/V0 was defined as relative tumor volume ratio.

3. Statistical analysis: Statistical differences were assessed in Student's t-test for comparison the relative tumor volume ratio at Day 13 between two groups. $P<0.05$ represented statistically significant.

Group (a) vs. Group (b): P= 0.0037; Group (a) vs. Group (c): P= 0.0105; Group (a) vs. Group (d): P= 0.0098