*Supporting Information for*

# Multifunctional L-arginine-based Magnetic nanoparticles for Multiple-synergistic Tumor Therapy

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## 1. Materials and methods

### 1.1. Evaluation of LA-loaded $Fe_3O_4$@PLGA/LA NPs

High-phase liquid chromatography (HPLC) (LC-20AD, Japan) was used to determine the LA encapsulation and loading efficiency in $Fe_3O_4$@PLGA/LA NPs. The HPLC conditions are listed as follows. Column: XB-C18 4.6 ×150 mm; flow rate: 1.0 mL/min; temperature: 30 °C; wavelength: 210 nm; buffer solution: sodium heptane sulfonate and $KH_2PO_4$ (pH = 2.0); mobile phase: buffer solution vs acetonitrile (80:20). The calibration curve of LA was obtained with LA solutions of different concentrations. Then, the LA loading efficiency and encapsulation were calculated by the following equations:

Loading efficiency (%) = (total LA - unbound LA)/total LA × 100%;

Encapsulation (%) = (total LA - unbound LA)/total lipid nanoparticles × 100%.

### 1.2. The HIFU-mediated NO release

Griess assay was employed to monitor NO release after triggering by HIFU[1]. First, different concentrations of LA/PLGA NPs and $Fe_3O_4$@PLGA/LA NPs in PBS were exposed to HIFU irradiation (150 W, 5 s) (Chongqing Haifu Technology, China). Then, 0.1 mL of each above solution was mixed with excessive $H_2O_2$ (50 μM) respectively in a 96-well cell-culture plate. Finally, the Griess assay kit was used to test NO release.

### 1.3. Evaluation of $Fe_3O_4$-encapsualted $Fe_3O_4$@PLGA/LA NPs

$Fe_3O_4$ encapsulation efficiency in $Fe_3O_4$@PLGA/LA NPs was determined using Inductively Coupled Plasma (ICP) (HORIBA JOBIN YVON, model: Activa). The hysteresis curve of $Fe_3O_4$/PLGA NPs was tested by a vibrating sample magnetometer (VSM, Lake Shore 7410-S). The encapsulation efficiency of $Fe_3O_4$ was calculated using the equation: Encapsulation (%) = ($Fe_3O_4$ in the NPs) / (the total added $Fe_3O_4$) × 100 %.

### 1.4. Hemolysis test

Different concentrations of $Fe_3O_4$@PLGA/LA NPs (0.1, 0.5, 2.0, 5.0, 10, and 25 mg/mL) were mixed with blood samples collected from female BALB/c mice at 37 °C for 4h. The hemolysis rate was measured using 0.1mL supernatants after a rotation at 1200 rpm for 8 min, the absorbance was measured at 541 nm. RBCs in deionized

water and PBS were acted as the positive and negative control group respectively. The hemolysis rate was calculated by the equation:

Hemolysis rate (%) = $(OD_{541sample}-OD_{541negative})/(OD_{541positive}-OD_{541negative})$ ×100%

1.5. *In vivo* pharmacokinetic evaluation of $Fe_3O_4$@PLGA/LA NPs

MDA-MB-231 tumor-bearing nude mice were intravenously injected with 0.2 mL (5 mg/mL) $Fe_3O_4$@PLGA/LA NPs. At 2, 24, and 48 h post injection, nude mice were sacrificed. Major organs (heart, liver, spleen, lung, and kidneys) and tumors were dissected, weighted, and homogenized. The biodistributions in different organs and tumors were calculated as Fe percentage of injected dose per gram of tissues.

Female Kunming mice (n = 3) were intravenously injected with 0.2 mL (5 mg/mL) $Fe_3O_4$@PLGA/LA NPs when they were 4 weeks old. At 2, 8, 15, 30 min, 1, 2, 4, 8, and 24 h, 50 μl blood were drawn by nicking the tail vein. The *in vivo* circulating half-life of Fe3O4@PLGA/LA NPs is calculated by a double-compartment pharmacokinetic model.

1.6. Synergistic effect of $Fe_3O_4$@PLGA/LA NPs by HIFU therapy

A Model-JC200 Focused Ultrasound Tumor Therapeutic system (Chongqing Haifu Medical Technology Co., Ltd., Chongqing, China) was used for all HIFU experiments. The focal length, diameter, and operating frequency were 100-250 mm, 100-300 mm, and 0.5-2 MHz, respectively.

We used fresh *ex vivo* bovine livers to evaluate in vitro HIFU ablation efficacy, the detailed operational methods were according to Tang et al [2]. In brief, 0.2 mL solutions of 5 mg/mL (PBS, $Fe_3O_4$/PLGA, LA/PLGA, and $Fe_3O_4$@PLGA/LA NPs) mixed with $H_2O_2$ (50 μM) were injected into the bovine livers respectively. Immediately after that, the injection site was treated with HIFU ablation at different power (120 W, 150 W, and 180 W) for 5 s. The gray value and coagulative necrosis volume of the ablation area were quantitatively analyzed.

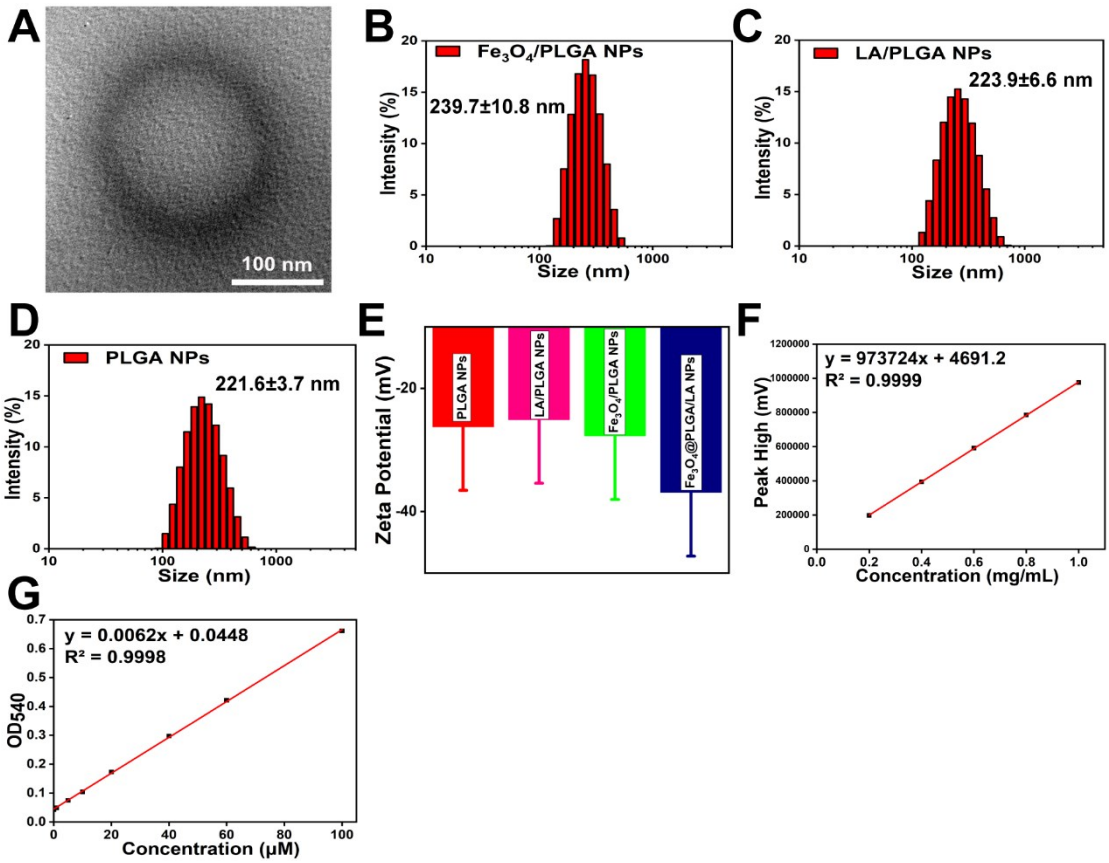


**Figure S1. (A)** The TEM of LA/PLGA NPs. DLS-based size distribution of **(B)** $Fe_3O_4$/PLGA NPs, **(C)**LA/PLGA NPs and **(D)** pure PLGA NPs. **(E)** Zeta potential of $Fe_3O_4$@PLGA/LA NPs, $Fe_3O_4$/PLGA NPs, LA/PLGA NPs, and pure PLGA NPs. **(F)** Standard curve of LA as a function of mass concentration via the HPLC method. **(G)** Standard curve of NO via Griess assay.

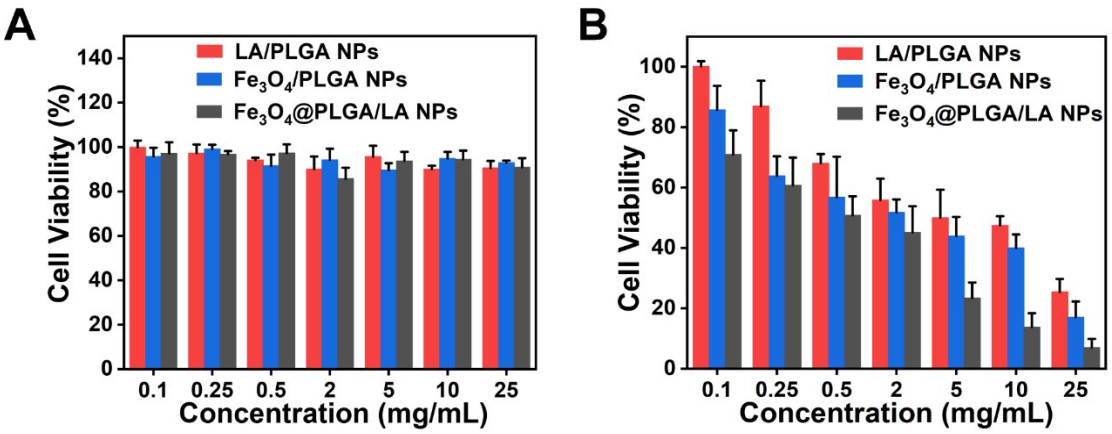


**Figure S2. (A)** 4T1 cell viability after incubation with different NPs for 24 h (n = 6). **(B)** 4T1 cell viability after incubation with different NPs triggered by HIFU for 4 h (n = 6).

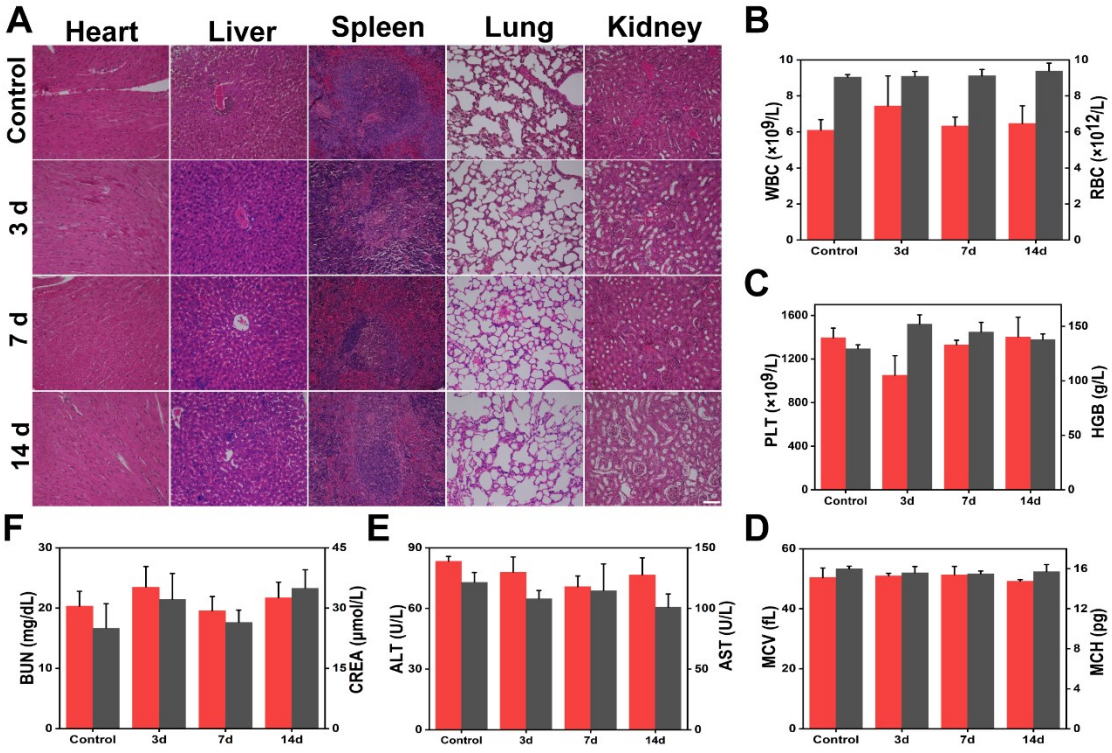


**Figure S3.** In vivo biosafety assay of $Fe_3O_4$@PLGA/LA NPs. **(A)** H&E staining in major organs (heart, liver, spleen, lung, and kidney) of control group and the experimental groups 3 ,7, 14 days post intravenous injection of $Fe_3O_4$@PLGA/LA NPs. The scale bar is 50 μm. **(B-F)** Hematological assay of BALB/c mice of control group and the experimental groups at the corresponding time point.

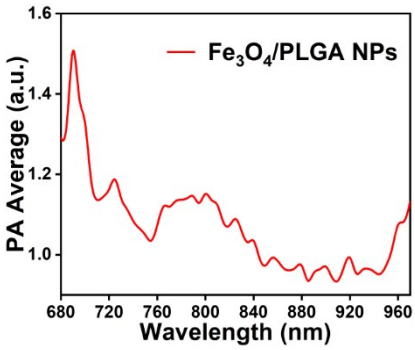


**Figure S4.** PA spectrum of $Fe_3O_4$/PLGA NPs (5 mg/mL) from 680 nm to 970 nm.

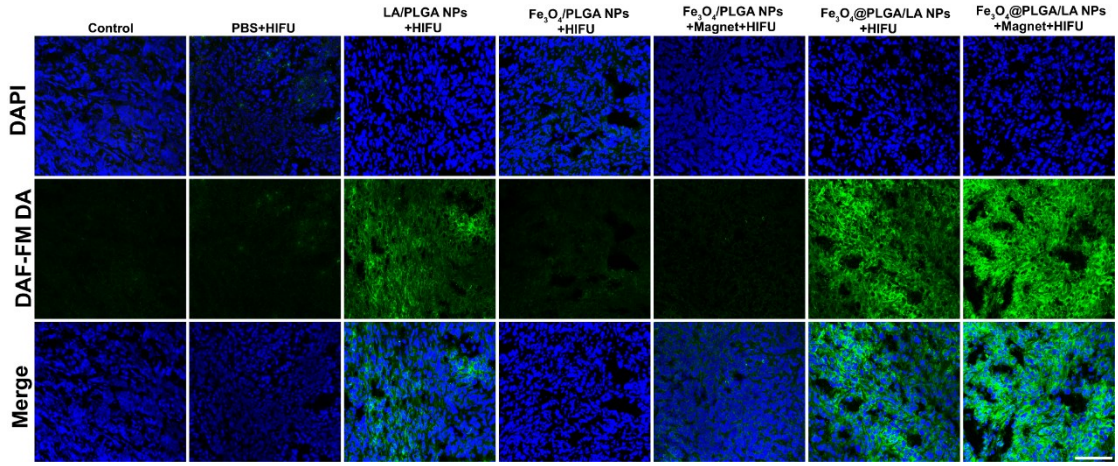


**Figure S5.** Ultrathin section of tumor tissues at 24 h post-HIFU therapy with the indicated formulations detect by CLSM. NO was stained green with DCFH-DA, nuclei were stained blue with DAPI. The scale bar is 50 μm.

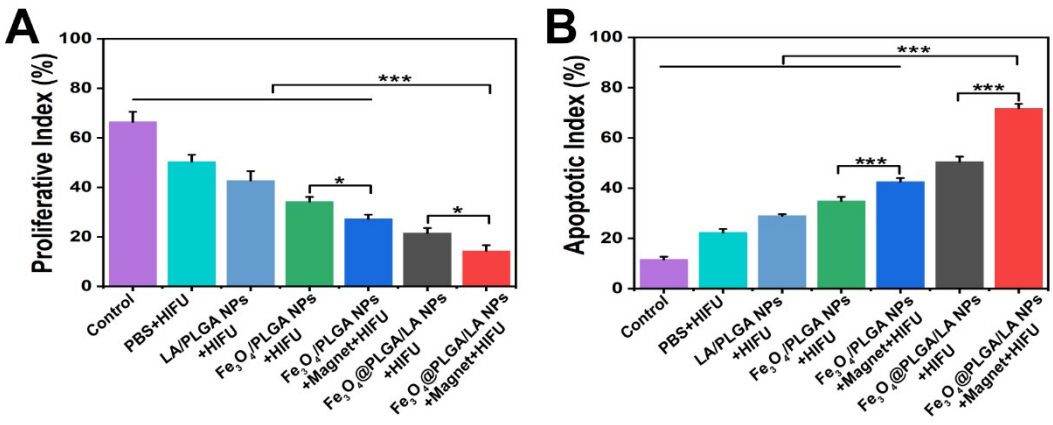


**Figure S6.** Quantitative analysis of **(A)** proliferative index (n = 6, *p < 0.05, ***p < 0.001) and **(B)** apoptotic index (n = 6, ***p < 0.001) by Image-J software from immunohistochemical images.

## References


1. R. Li, H. Qi, Y. Ma, Y. Deng, S. Liu, Y. Jie, J. Jing, J. He, X. Zhang, L. Wheatley, C. Huang, X. Sheng, M. Zhang and L. Yin, *Nature communications*, 2020, **11**, 3207.
2. H. Tang, Y. Guo, L. Peng, H. Fang, Z. Wang, Y. Zheng, H. Ran and Y. Chen, *ACS applied materials & interfaces*, 2018, **10**, 15428-15441.