

Phase-shift, targeted nanoparticles for ultrasound molecular imaging by low intensity focused ultrasound irradiation

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http://dx.doi.org/10.2147/IJN.S166200

Purpose: Ultrasound (US) molecular imaging provides a non-invasive way to visualize tumor tissues at molecular and cell levels and could improve diagnosis. One problem of using US molecular imaging is microbubbles challenges, including instability, short circulation time, and poor loading capacity and penetrability. It is urgent to design new acoustic contrast agents and new imaging methods to facilitate tumor targeted imaging. In this study, phase-shift poly lactic-co-glycolic acid (PLGA) nanoparticles modified with folate as an efficient US molecular probe were designed and the long-term targeted imaging was achieved by low-intensity focused US (LIFU) irradiation.

Methods: A new 5-step method and purification procedure was carried out to obtain uniform folic acid polyethylene glycol PLGA (PLGA-PEG-FA), the structure of which was confirmed by ¹H nuclear magnetic resonance spectroscopy and thin-layer chromatography. Perflunonene (PFP) was wrapped in PLGA-PEG-FA by a double emulsion solvent evaporation method to obtain PFP/PLGA-PEG-FA nanoparticles. The targeted ability of the resulting nanoparticles was tested in vivo and in vitro. LIFU irradiation can irritate nanoparticle phase-shift to enhance tumor imaging both in vivo and in vitro.

Results: PLGA-PEG-FA was a light yellow powder with a final purity of at least 98%, the structure of which was confirmed by ¹H nuclear magnetic resonance spectroscopy and thin-layer chromatography. Highly dispersed PFP/PLGA-PEG-FA nanoparticles with spherical morphology have an average diameter of 280.9±33.5 nm, PFP load efficiency of 59.4%±7.1%, and shells, thickness of 28±8.63 nm. The nanoparticles can specifically bind to cells expressing high folate receptor both in vivo and in vitro. Ultrasonic imaging was significantly enhanced in vitro and in vivo by LIFU irradiation. The retention time was significantly prolonged in vivo.

Conclusion: Phase-shift PFP/PLGA-PEG-FA nanoparticles induced by LIFU can significantly enhance ultrasonic imaging, specifically targeting tumors expressing folate receptor. As a potential targeting acoustic molecular probe, PFP/PLGA-PEG-FA nanoparticles can be used to achieve targeted localization imaging.

Keywords: folic acid, targeted, phase-shift, nanoparticles, acoustic contrast agent

Introduction

Ultrasound (US) molecular imaging provides a non-invasive way to visualize tumor tissues at molecular and cell levels and could improve diagnosis and treatment. US molecular probes can serve as contrast agents for tumor imaging as well as drug vehicles for target therapy. Commonly used US molecular probes, such as targeted microbubbles and perfluorocarbon emulsions are limited by a conflict between US visualization and particle penetration. Targeted microbubbles challenges include

instability, short circulation time, and poor loading capacity and penetrability, while perfluorocarbon nanoemulsions that effectively penetrate tumor tissue are limited by poor acoustic echogenicity.¹

In recent years, phase-changeable perfluorocarbon nanoemulsions have been developed as novel US molecular probes or contrast agents for possible clinical translation. These phase-changes contrast agents based on an US-triggered phase transition has provided medical researchers with a “middle-ground” between the inert liquid emulsion and gas-based microbubbles contrast agent platforms.² We previously developed perfluorohexane (PFH)@poly lactic-co-glycolic acid (PLGA)/Fe₃O₄ nanocapsules in which phase transition could be induced to form microbubbles and demonstrated that they could serve as contrast agents to enhance US and magnetic resonance imaging (MRI).³ Furthermore, they have synergistic tumor ablation effects when used to enhance near infrared-induced photothermal therapy and high-intensity focused US (HIFU) irradiation, offering a dual-modal, imaging-guided protocol for tumor therapy.

Typical US molecular probes consist of a biodegradable shell, an acoustic core, and ligands on or within the shell targeting to specific sites.⁴ PLGA possesses characteristics of favorable biocompatibility, controllable degradation, and low toxicity,⁵ and has been widely used to form the shells of various nanoparticles. Liquid PFH can be converted to gas when the temperature is close to its boiling point (b.p.), thereby generating acoustic bubbles. This typical phase-shift material is often encapsulated into nanocapsules. Phase-changeable polymeric and lipid nanoparticles encapsulating PFH have been produced by many groups.^{6–8} They can pass through the vascular endothelium gap and penetrate tumor tissue where they are retained due to enhanced permeability and retention effect. However, because of the relatively high boiling temperature of PFH (58°C–60°C), the phase transition of these nanoparticles requires heat or HIFU exposure, which could cause damage to normal tissue.^{9,10}

In the present work, we prepared phase-shift PLGA nanoparticles encapsulating perflubron (PFP) with a low b.p. (29°C) as a novel US molecular probe that requires phase transition by low-intensity focused US (LIFU). We added a folate-targeting group to the nanoparticle surface to improve penetrating and targeting abilities. It has been reported that folate receptors (FRs) are overexpressed on the surface of many epithelium-derived malignant tumor cells with good tumor tissue specificity. In contrast to monoclonal antibodies, folic acid (FA) has a low molecular weight and does not induce human immunogenicity, which allows it to penetrate

tissue and target tumors.^{11,12} Here, we show that phase-shift PLGA nanoparticles modified with folate are an efficient US molecular probe. Their basic characteristics were determined, and the targeting effect and phase transition for US imaging were investigated in vitro and in vivo (Figure 1). Collectively, our findings suggest they could be useful for non-invasive tumor imaging and therapy.

Materials and methods

PLGA-polyethylene glycol (PEG)-FA preparation and structure formation

A 5-step method was performed to prepare FA-targeted PEG PLGA (PLGA-PEG-FA) (Figure 2).

Step 1: NH₂-PEG-NH₂ (MW = 3,500, JenKun, Beijing, China) and Di-Boc (Boc, TCI, Tokyo, Japan) reactions were performed for NH₂-PEG-BOC, which was purified with reverse-phase chromatography (purification equipment [NP7030C; Hubei Science & Technology, Huaian, China]). Step 2: FA (TCI) was mixed with N-hydroxysuccinimide (NHS, TCI) to obtain FA-NHS. Step 3: NH₂-PEG-BOC and NH₂-FA were mixed, and the products were purified by ion chromatography and reverse-phase chromatography to obtain uniform FA-PEG-NH₂. Step 4: PLGA (PLGA-COOH, 50:50, MW = 25,000, Daigang, Jinan, China) was reacted with NHS to obtain PLGA-NHS. Step 5: PLGA-NHS and FA-PEG-NH₂ were dissolved, and the mixture was then subjected to dialysis with a molecular weight cutoff of 10 kDa prior to ion chromatography purification and lyophilization to obtain PLGA-PEG-FA.

For ¹H nuclear magnetic resonance spectroscopy (¹H-NMR) analysis (AV-500, Bruker, Billerica, MA, USA), 20 mg PLGA-PEG-FA was dissolved in dimethyl sulfoxide-d₆, with tetramethylsilane as an internal standard.

Thin-layer chromatography (TLC) detection of PEG groups in PLGA-PEG-FA: 10 mg each of PEG, PLGA, PLGA-NHS, PLGA-PEG-FA, FA-PEG-NH₂, and FA were dissolved in DMSO, each with a concentration of 1 mg/mL. Each solution was applied on a thin-layer plate (Jiangyou, Yantai, China), sprayed with iodoacetic acid, and stained.

PFP/PLGA-PEG-FA nanoparticle preparation and characterization

PFP/PLGA-PEG-FA nanoparticles were prepared with a double emulsion solvent evaporation method. Briefly, 25 mg PLGA was dissolved in 3 mL dichloromethane (DCM) and added to 200 µL PFP (Sigma-Aldrich Chemical Co., St Louis, MO, USA), the resulting emulsion was obtained using an ultrasonic probe (VCX-130, Sonics & Materials Inc,

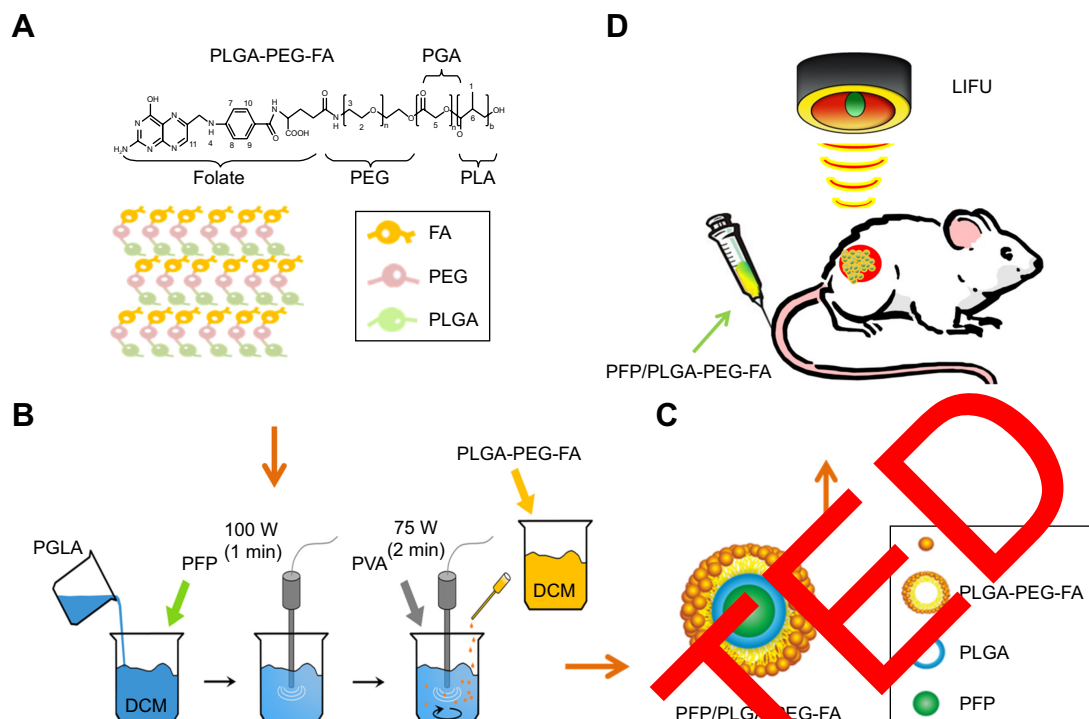


Figure 1 Illustration on the whole experiment.

Notes: (A) Illustration of the structure of PLGA-PEG-FA. (B) Illustration of PFP/PLGA-PEG-FA nanoparticle preparation. (C) The structure of PFP/PLGA-PEG-FA nanoparticle. (D) LIFU irradiation after injections in tumor-bearing mice.

Abbreviations: DCM, dichloromethane; FA, folic acid; LIFU, low-intensity focused ultrasound; PEG, polyethylene glycol; PFP, perflenapent; PLGA, poly lactic-co-glycolic acid.

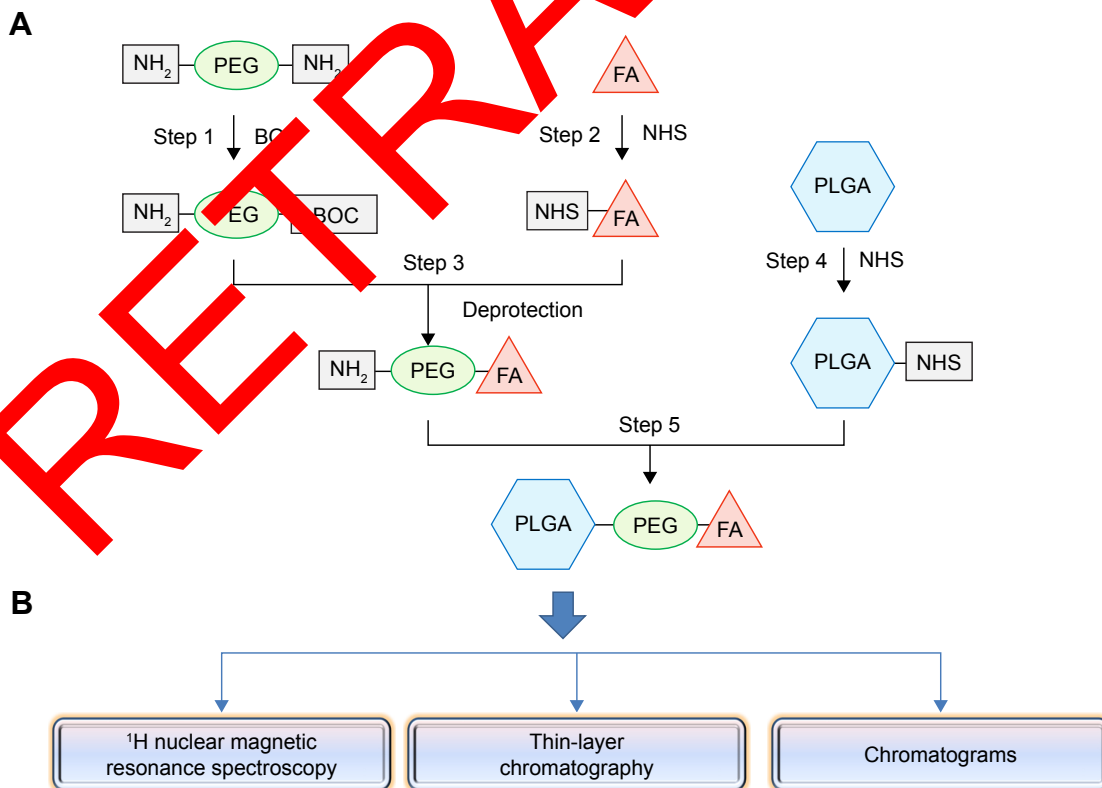


Figure 2 (A) Schematic illustration of the 5-step synthesis method. (B) Confirmation and purification of PLGA-PEG-FA.

Abbreviations: BOC, di-tert-butyl dicarbonate; FA, folic acid; PEG, polyethylene glycol; PLGA, poly lactic-co-glycolic acid; NHS, N-hydroxysuccinimide.

Newtown, CT, USA) at a constant power output of 100 W for 1 min in a 4°C saline bath. Next, 75 mg PLGA-PEG-FA was dissolved in 2 mL DCM and added into the aforementioned emulsion, followed by shaking for 2.5 min. Next, 3 mL 5% poly (vinyl alcohol) (MW=25,000, Sigma) solution was slowly added with continuous agitation, followed incubation in a saline bath at 4°C with an ultrasonic probe (75 W, 2 min). Agitation was continued until the DCM completely evaporated. The precipitate was collected after centrifugation and washed 3 times, re-suspended with double distilled water to a nanoparticle concentration of 3.6×10^{15} particles/mL, and divided into 1-mL aliquots that were lyophilized and stored in the dark at -20°C.

To determine PFP load of PFP/PLGA-PEG-FA nanoparticles, we followed the same procedures which are mentioned above to prepare the nanoparticles. After these nanoparticles were collected, DCM was applied to dissolve PFP/PLGA-PEG-FA nanoparticles. Because PLGA-PEG-FA dissolved in DCM but PFP did not, they were separated by centrifugation at low temperature and then were determined. PFP load efficiency was calculated according to the obtained volume from centrifugation and the added volume during the preparation.

The PFP/PLGA-PEG-FA nanoparticles were observed by scanning electron microscopy (SEM; Hitachi S-3400, Tokyo, Japan) and transmission electron microscopy (TEM; Hitachi H-7600, Japan). The size distribution and zeta potential of PFP/PLGA-PEG-FA nanoparticles were determined by dynamic light scattering with a Malvern Zetasizer Nano ZS unit (Malvern Instruments, Westborough, MA, USA). The pH value was measured, and nanoparticle US images were recorded by 5–12 MHz probe of US machine (Philips IU 22, Amsterdam, the Netherlands) with the parameter setting: 11 MHz, power 85%, Gain 68, Mechanical Index 0.47, Depth 4 cm, Focus Depth 3–4 cm.

Target specificity of PFP/PLGA-PEG-FA nanoparticles

In vitro target specificity

SKOV3 cells expressing high levels of FR and WISH cells not expressing FR were selected to assess target specificity. Using PLGA-PEG-FA-fluorescein isothiocyanate (FITC) and PLGA-PEG-FITC as shell materials, respectively, FITC-labeled nanoparticles (PFP/PLGA-PEG-FA-FITC and PFP/PLGA-PEG-FITC) were prepared according to the method described previously.

SKOV3 cells were cultured in Roswell Park Memorial Institute-1640 medium supplemented with 10% fetal bovine serum, 100 U/mL of penicillin, and 100 U/mL of

Table 1 PFP/PLGA-PEG-FA nanoparticle binding with SKOV3 cells (n=3)

PFP/PLGA-PEG-FA concentrations ($\times 10^4$ particles/mL)	Positive cells (%)
0 (64×10^4 particles/mL PFP/PLGA-PEG-FITC)	0.27 $\pm$ 0.12
1	8.13 $\pm$ 1.90
2	15.88 $\pm$ 2.22* $\ddagger$
4	45.1 $\pm$ 5.44* $\ddagger$
8	83.45 $\pm$ 4.60 $\ddagger$
16	97.42 $\pm$ 1.56 $\ddagger$
32	99.51 $\pm$ 0.51 $\ddagger$
64	99.90 $\pm$ 0.17 $\ddagger$

Notes: *Compared with the control group, $P < 0.05$; $\ddagger$ Comparison between two groups, $P < 0.05$; $\ddagger$ Comparison between two groups, $P < 0.01$; $\ddagger$ Comparison with control group (PFP/PLGA-PEG-FITC), $P < 0.01$.

Abbreviations: FA, folic acid; FITC, fluorescein isothiocyanate; PEG, polyethylene glycol; PFP, perfluent; PLGA, poly lactic-co-glycolic acid.

streptomycin. The cells were maintained in an incubator with a humidified atmosphere containing 5% CO₂ at 37°C. Cells in an exponentially growth phase were used for the experiment. All cell culture reagents were purchased from Invitrogen (Carlsbad, CA, USA).

SKOV3 cells were seeded in culture dishes, and stable monolayer was formed 24 h post-seeding. The cells were collected and re-suspended to 10^6 particles/mL. SKOV3 cells were divided into Group A and B. For Group A: 200 μ L of PFP/PLGA-PEG-FA-FITC nanoparticles was added to the cells at the concentrations described in Table 1 (64×10^4 particles/mL PFP/PLGA-PEG-FITC as a control). For Group B (antagonized group), 10 μ L of FA was added to the cells with the concentrations listed in Table 2. Cells were incubated at 37°C for 30 min followed by the addition of 200 μ L 64×10^4 particles/mL PFP/PLGA-PEG-FA-FITC nanoparticles.

For WISH cells, the concentration of PFP/PLGA-PEG-FA-FITC nanoparticles was the same as in Group A of

Table 2 Different concentrations of PFP/PLGA-PEG-FA nanoparticles bind with WISH cells (n=3)

PFP/PLGA-PEG-FA concentrations ($\times 10^4$ particles/mL)	Positive cells (%)
0 (64×10^4 particles/mL PFP/PLGA-PEG-FITC)	0.20 $\pm$ 0.07
1	0.52 $\pm$ 0.14
2	0.54 $\pm$ 0.35
4	0.31 $\pm$ 0.13
8	0.60 $\pm$ 0.29
16	0.83 $\pm$ 0.40
32	0.60 $\pm$ 0.21
64	0.73 $\pm$ 0.16

Abbreviations: FA, folic acid; FITC, fluorescein isothiocyanate; PEG, polyethylene glycol; PFP, perfluent; PLGA, poly lactic-co-glycolic acid.

SKOV3 cells (Table 3). Same experimental procedure was used in the aforementioned 3 groups following PFP/PLGA-PEG-FA-FITC nanoparticle treatment, and PBS was used as a negative control.

Different concentrations of these nanoparticles were incubated with SKOV3 or WISH cells at 37°C for 30 min. Then all the cells in each group were washed with PBS 3 times, centrifuged to pellet, and re-suspended for flow cytometer analysis to check the numbers of positive cells. Tubes were wrapped with foil to protect from light whenever possible.

In vivo target specificity

According to the method aforementioned, 1,1'-diiodo-3,3,3',3'-tetramethylindocarbocyanine (DiI) was added at the same time as PFP to obtain DiI/PFP/PLGA-PEG-FA and DiI/PFP/PLGA-PEG nanoparticles at a concentration of 3.6×10^{15} particles/mL. All of our experimental protocols were approved by the Institutional Animal Care and Use Committee, Chongqing Medical University. The SKOV3 tumor-bearing nude mice model was described previously.¹³ Female immunodeficient BALB/c nude mice (6–8 weeks old, 18–24 g) were housed between 19°C and 22°C and humidity conditions. To induce solid tumors, 3×10^6 cells suspended in 100 μ L PBS (pH = 7.4) were subcutaneously injected into the right flank of mouse. Nine mice bearing xenograft SKOV3 tumors (10 mm in diameter) were randomly divided into 3 groups, including targeted group (0.2 mL of DiI/PFP/PLGA-PEG-FA injected via tail vein), non-targeted group (equivalent DiI/PFP/PLGA-PEG was injected), and antagonized group (injected with 20 μ g/100 μ L FA first, then with DiI/PFP/PLGA-PEG-FA 2×10^9 particles/mL for 0.2 mL). Tumor tissues were collected and fixed in 4%

paraformaldehyde. Slides were prepared for fluorescent microscope (Olympus CKX41, Tokyo, Japan).

LIFU-induced phase-shift for US imaging

In vitro LIFU-induced phase-shift

Different concentrations of PFP/PLGA-PEG-FA nanoparticles were phase-shift induced by LIFU irritation. The LIFU device was specifically designed and developed in our laboratory (LM.SC051 ACA; Institute of Ultrasound Imaging of Chongqing Medical Sciences, Chongqing, China) with a driving frequency of 1.0 MHz, focal length of 1.5 cm, and focus area of 0.4 cm². The acoustic intensity in a focal spot is 1.2 W/cm². The parameter duty cycle was 10% and pulse repetition frequency was 5 kHz. The PFP/PLGA-PEG-FA nanoparticles with various concentration were placed in a gel mold (3% agarose in distilled water). LIFU probe was placed perpendicular to the surface of the gel mold. Images of nanoparticles were obtained by US.

In vivo LIFU-induced phase-shift

Three concentrations of PFP/PLGA-PEG-FA nanoparticles were used to induce phase-shift by LIFU irritation. Mice were housed as aforementioned. Twelve mice bearing xenograft SKOV3 tumors (10 mm in diameter) were randomly divided into 4 groups: 3.6×10^{15} particles/mL as high concentration group, 3.6×10^{13} particles/mL as medium concentration group, 3.6×10^{11} particles/mL as low concentration group, and H₂O/PLGA-PEG-FA nanoparticles as control. Point two of a millilitre of each nanoparticle was injected via tail vein. Five minutes after nanoparticle injection, tumors were irritated by LIFU with acoustic intensity of 1.20 W/cm² for 2 min. US images were taken in fundamental and harmonic modes.

In vivo enhancement for US molecular imaging at different time

Six mice bearing xenograft SKOV3 tumors (10 mm in diameter) were randomly divided into 2 groups (n=3), and 0.2 mL injections were given via the tail vein. One group was injected with 3.6×10^{15} particles/mL PFP/PLGA-PEG-FA nanoparticles, and the other with SonoVue (Bracco International B.V., Milan, Italy) as a control. Then the tumors were irradiated by LIFU (1.20 W/cm², 2 min) at 5 and 20 min, and 2 and 4 h after the injection. Finally, US images were observed in fundamental and harmonic modes.

Image analysis and statistical method

All images were analyzed by ImageJ (NIH, Bethesda, MD, USA). All data were presented as mean $\pm$ SD. Differences

Table 3 Different FA concentrations compete with PFP/PLGA-PEG-FA binding of SKOV3 cells (n=3)*

FA concentration (μ M)	Positive cells (%)
0	99.77 $\pm$ 0.40
25	47.60 $\pm$ 2.98 ^{§§}
50	14.52 $\pm$ 5.19 ^{§§}
100	5.67 $\pm$ 1.99 ^{§§}
200	0.39 $\pm$ 0.19 [‡]
400	0.68 $\pm$ 0.16 [‡]
800	0.24 $\pm$ 0.06 [‡]

Notes: *SKOV3 cells were treated with 10 μ L of FA with different concentrations, followed by incubation in nitrogen dioxide for 30 min at 37°C, 40 μ L of 64×10^4 particles/mL of PFP/PLGA-PEG-FA was then added and incubated in nitrogen dioxide at 37°C for another 30 min. [‡]Comparison to control group (without FA), $P < 0.01$; [§]Comparison between 2 groups, $P < 0.05$; ^{§§}Comparison between 2 groups, $P < 0.01$.

Abbreviations: FA, folic acid; PEG, polyethylene glycol; PFP, perfluent; PLGA, poly lactic-co-glycolic acid.

were considered statistically significant if $P < 0.05$. The data were analyzed by Statistical Package for the Social Sciences software (SPSS; IBM, Armonk, NY, USA). One-way analysis of variance was used among multiple groups, and statistical analysis was performed between 2 groups if $P < 0.05$. The least significant difference test was used for comparison between 2 groups if equal variances were assumed, and Dunnett T3 test was used if equal variances were not assumed.

Results

Preparation and structure confirmation of PLGA-PEG-FA

After several purifications, we produced a light yellow powder with a final purity of at least 98%. All the characteristic peaks were verified by $^1\text{H-NMR}$.

TLC results showed coloration in lanes 4 and 5, indicating that PEG groups were contained in PLGA-PEG-FA and FA-PEG- NH_2 (Figure 3).

PFP/PLGA-PEG-FA nanoparticle preparation and characterization

The nanoparticles were a white emulsion and separated if left to stand. PFP load efficiency is obtained by demulsification. For the added volume during the preparation of 200 μL , the resulting PFP load efficiency was $59.4\% \pm 7.1\%$.

The diameter measured as a single peak with a mean of 280.9 ± 33.5 nm, the surface was relatively charged with an electric potential of -56.9 ± 14.8 mV, and the pH value was 7.2 ± 0.6 . Both SEM and TEM revealed smooth and uniform spherical morphology. SEM showed PFP/PLGA-PEG-FA

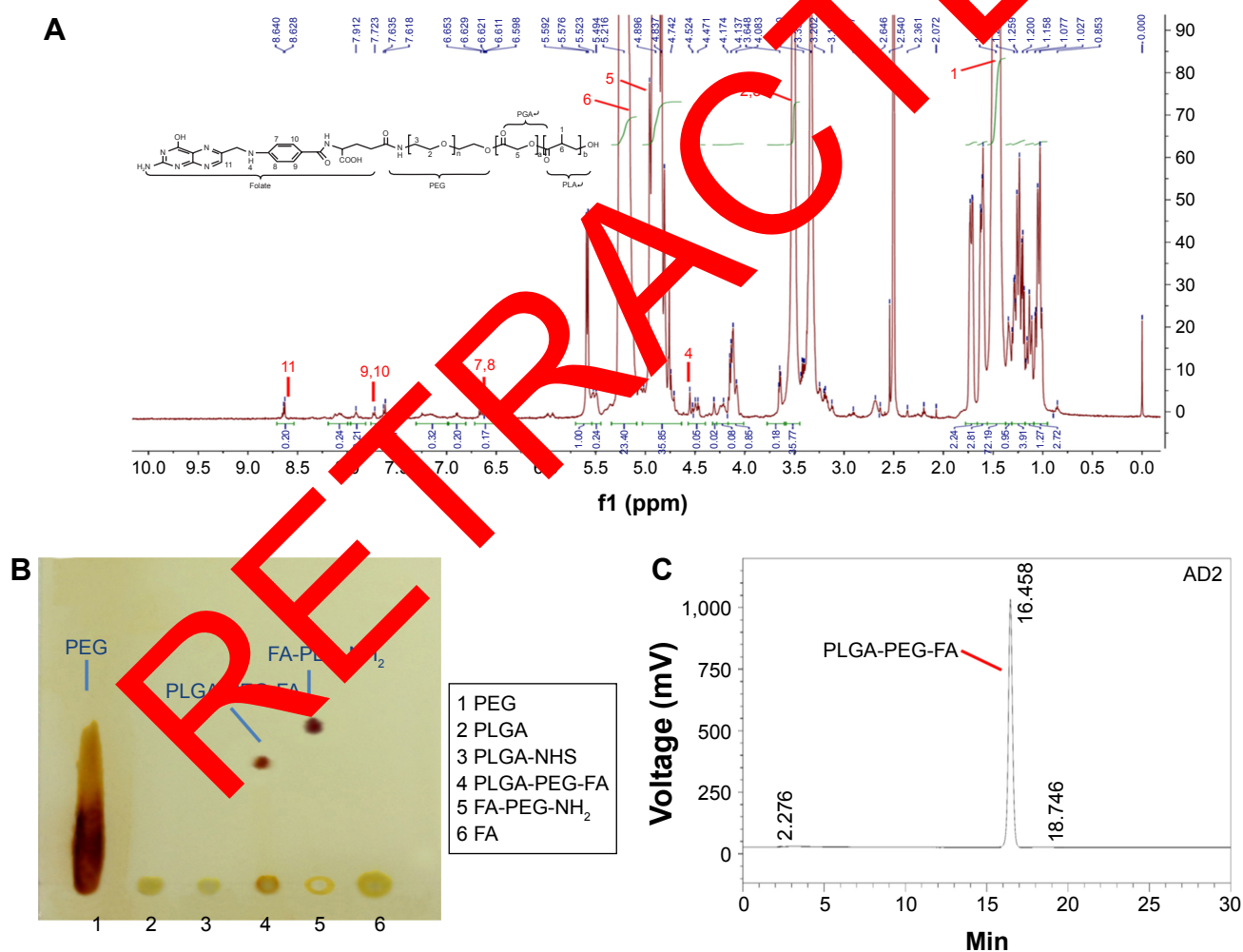


Figure 3 PLGA-PEG-FA structure and purification.

Notes: (A) PLGA-PEG-FA characterization by $^1\text{H-NMR}$. (B) TLC of PLGA-PEG-FA. (C) Chromatograms demonstrating PLGA-PEG-FA purity.

Abbreviations: FA, folic acid; $^1\text{H-NMR}$, ^1H nuclear magnetic resonance spectroscopy; PEG, polyethylene glycol; NHS, N-hydroxysuccinimide; PLGA, poly lactic-co-glycolic acid; TLC, thin-layer chromatography.

nanoparticles texture clearly, the inside core was PFP, with high electronic density, and appears black whereas the polymeric shell seemed lighter. The nanoparticles polymer shell thickness was 28 ± 8.63 nm and could be measured by TEM. US showed hyperechogenicity of dense, evenly distributed dots (Figure 4).

Target specificity of PFP/PLGA-PEG-FA nanoparticles

In vitro target specificity

After incubation with different concentrations of PFP/PLGA-PEG-FA-FITC nanoparticles, the amount of FITC-positive SKOV3 cells, which highly express FR, was increased in a dose-dependent manner in the range of $1-8 \times 10^4$ particles/mL concentration ($P < 0.05$ or $P < 0.01$, Table 1). The number of positive SKOV3 cells did not increase following treatment with concentrations of nanoparticles at and above 8×10^4 particles/mL ($P > 0.05$, Table 1). Binding between PFP/PLGA-PEG-FA-FITC nanoparticles and FR followed an S-shape curve (Figure 5), which is consistent with the receptor-ligand interaction pattern. No positive cells were detected in the negative control group (PFP/PLGA-PEG-FITC) and in WISH cells that do not express FR, following

incubation with different concentrations of PFP/PLGA-PEG-FA-FITC nanoparticles ($P > 0.05$, Table 2). The result showed that compared with the control group, the positive cells rate were not statistically significant.

Different concentrations of FA were incubated with SKOV3 cells, which were then treated with the same concentration of PFP/PLGA-PEG-FA-FITC nanoparticles. In this case, the percentage of FITC-positive cells was negatively correlated with the FA concentration ($P < 0.01$), and positive cells appeared to be undetectable under 200 $\mu\text{g/mL}$ FA (Table 3).

The results which are mentioned above demonstrated that PFP/PLGA-PEG-FA nanoparticles can specifically bind to FR in vitro; FA can block those combinations.

In vivo target specificity

DiI is a red fluorescence dye. In targeted group, the mean fluorescence intensity was significantly higher than those in other groups ($P < 0.01$, Table S1), which indicated that DiI/PFP/PLGA-PEG-FA nanoparticles bound to FRs. No difference was found in the fluorescence intensity between antagonized group and non-targeting group ($P > 0.05$, Table S1), which confirmed the targeting mechanism via folate (Figure 6).

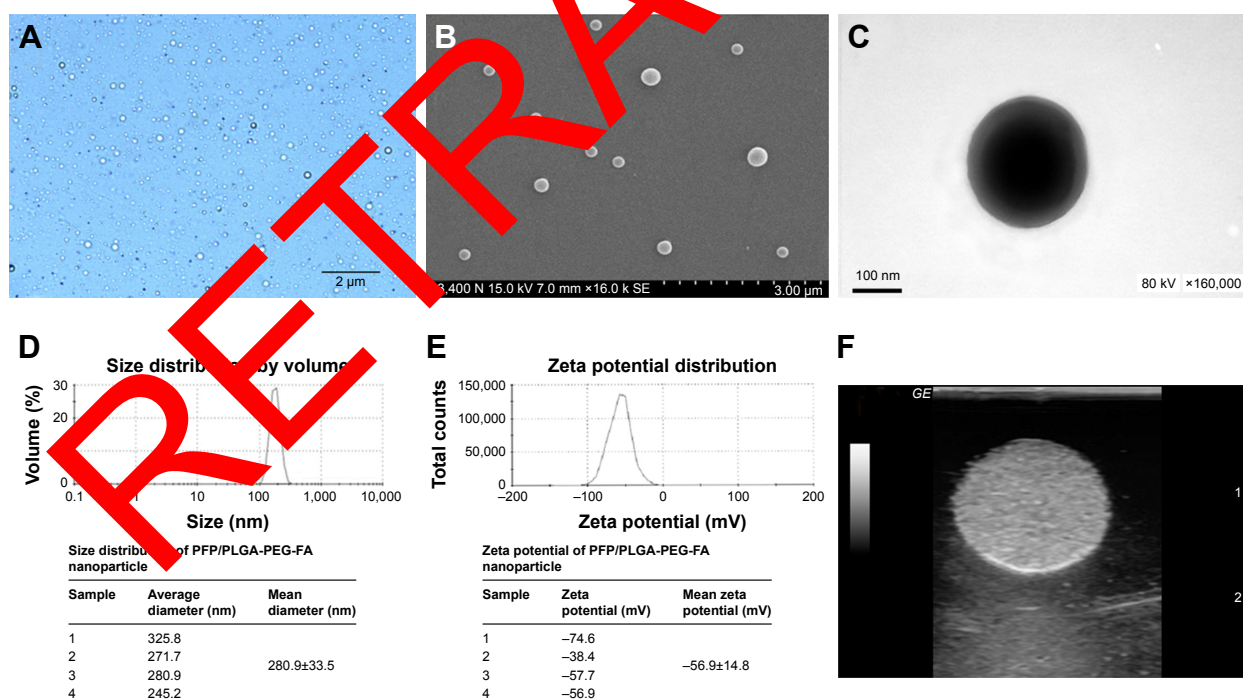


Figure 4 PFP/PLGA-PEG-FA nanoparticle characterizations.

Notes: (A) Bright field optical microscopy. (B and C) SEM and TEM image of the nanoparticles. (D and E) Size distribution and zeta potential of the nanoparticles. (F) In vitro US image of the nanoparticles.

Abbreviations: FA, folic acid; PEG, polyethylene glycol; PFP, perfluent; PLGA, poly lactic-co-glycolic acid; SEM, scanning electron microscopy; TEM, transmission electron microscopy; US, ultrasound.

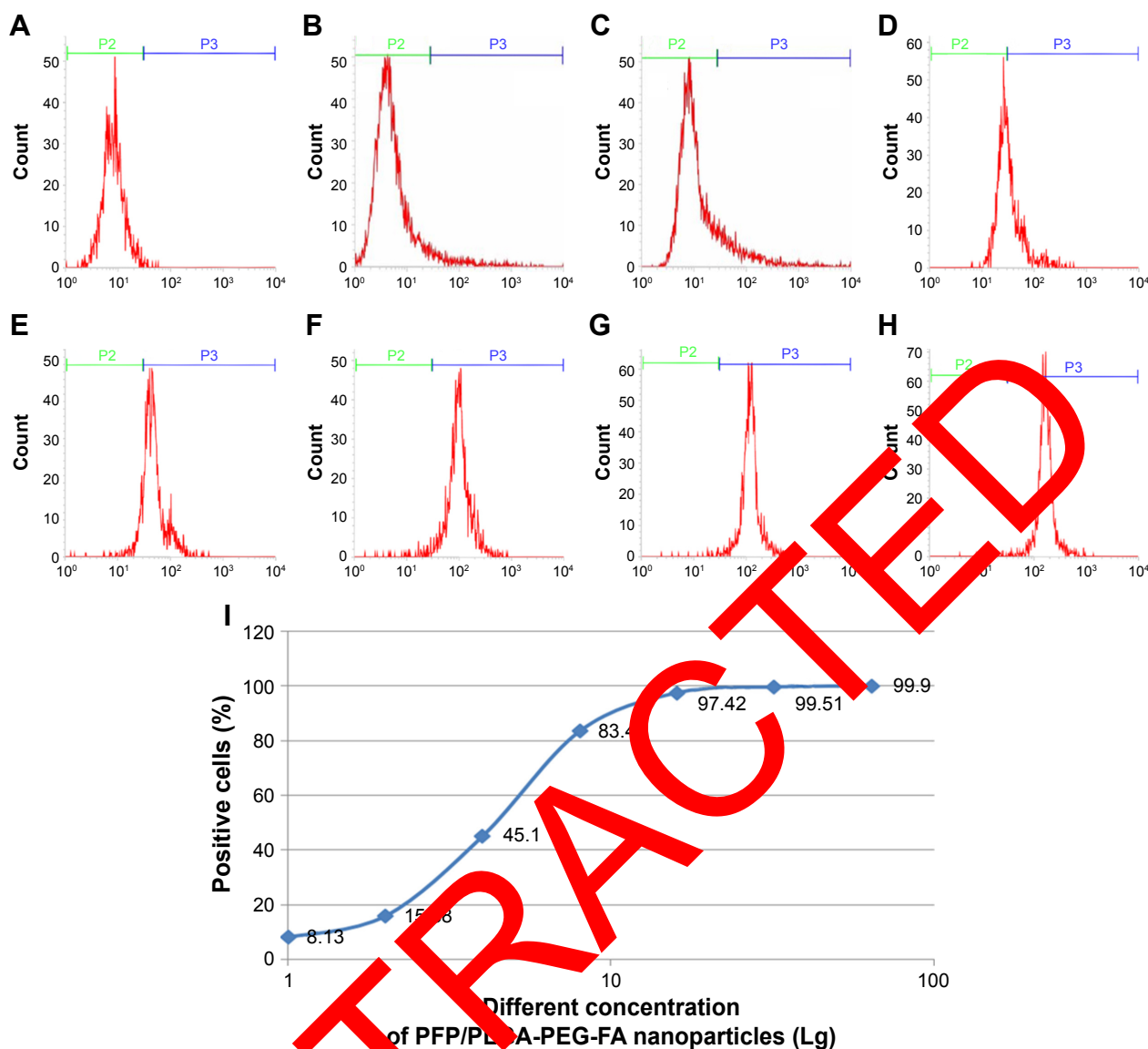


Figure 5 Flow cytometry results showing SKOV3 cell binding to different PFP/PLGA-PEG-FA concentrations.

Notes: (A) PFP/PLGA-PEG (64×10^4 particles/mL). (B–H) PFP/PLGA-PEG-FA concentrations ($\times 10^4$ particles/mL) are 1, 2, 4, 8, 16, 32, and 64. (I) Binding curve (P2 indicates negative cell % in A–H. P3 indicates positive cell % in A–H).

Abbreviations: FA, folic acid; PEG, polyethylene glycol; PFP, perfluent; PLGA, poly lactic-co-glycolic acid.

The results showed that PFP/PLGA-PEG-FA nanoparticles can specifically bind with FRET only in vitro, but also in vivo.

LIFU-induced phase-shift for US imaging

In vitro LIFU-induced phase-shift

Different concentrations of PFP/PLGA-PEG-FA nanoparticles were used to induce phase-shift by LIFU irradiation (Figure 7A). The results showed that nanoparticles could still be converted into gas bubbles at concentration as low as 3.6×10^3 particles/mL. However, the enhancement effect for US was positively correlated with the nanoparticle concentration after LIFU induction ($P < 0.05$ or $P < 0.01$,

Table S2). The nanoparticles at higher concentration produced better enhancement for US imaging (Figure 7B and D).

In vivo LIFU-induced phase-shift

Three concentrations of PFP/PLGA-PEG-FA nanoparticles were used to induce phase-shift by LIFU irradiation. When tumors were irradiated with LIFU (1.20 W/cm^2 , 2 min), the irradiated area exhibited enhanced echoes in harmonic mode. The results from 3 groups for enhancement of US imaging have significant difference compared with the control group. The enhancement effect is positively correlated to the concentration ($P < 0.01$, Table S3), with the group with

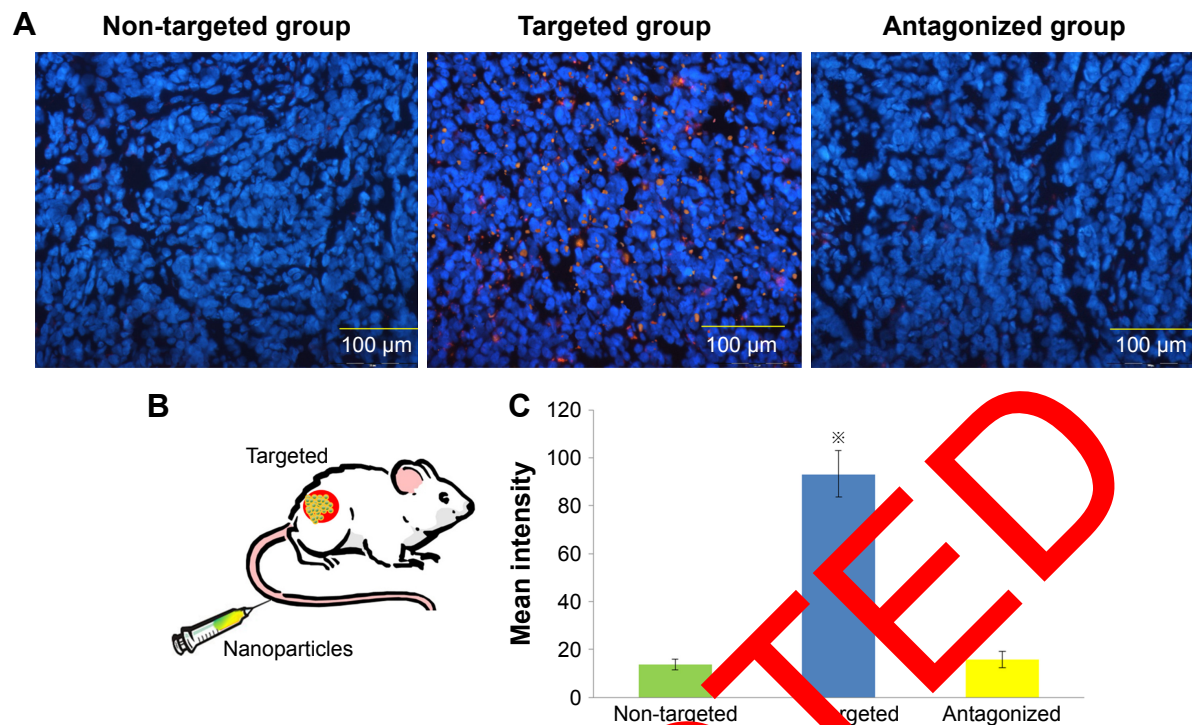


Figure 6 In vivo target specificity fluorescence images of tumor tissue.

Notes: (A) Fluorescence images of non-targeted group, targeted group and antagonized group. (B) Illustration of the injection method. (C) Relationships of mean intensities among the three groups. “*” Comparison to non-targeted group, $P < 0.01$.

a high concentration having the best enhancement effect. No significant signal was observed. The tumor image did not change after LIFU irradiation (Figure 8).

In vivo enhancement for US molecular imaging at different times

Before and after PFP/PLGA-PEG-FA nanoparticle injection, there were no remarkable changes in tumor imaging for either mode. When the tumor was irradiated with LIFU (1.20 W/cm^2 , 2 min), the irradiated area exhibited slightly enhanced echoes in fundamental mode but distinct enhancement in harmonic mode. The irradiated area showed scattered dots that completely disappeared after ~5 min. The tumor was again irradiated with LIFU 20 min after injection and exhibited slightly enhanced echoes in fundamental mode and remarkably enhanced images in harmonic mode. These signals also disappeared completely about 5 min later. Two hours after injection, the tumor was irradiated by LIFU for a third time, and slightly enhanced images were noted. On the final LIFU application 4 h after injection, no enhancement was observed. ($P < 0.01$, Table S4). For control group, 10 s after SonoVue injection into the tail vein of tumor-bearing nude mice, imaging in harmonic mode revealed inhomogeneous high enhancement with some unenhanced areas. After 2 min, the contrast agent had almost washed out, and no

Discussion

Precise diagnosis and treatment planning require visualizing tumors at molecular and cellular levels. US molecular imaging technology provides a non-invasive method to identify malignant tumors at an early stage and quantitatively evaluate their development and evolution. Nanoscaled US contrast agents can penetrate tumor tissues and accumulate in the local area. In this work, phase-shift, folate-targeted PLGA nanoparticles were developed as a novel US molecular probe for tumor imaging. The PFP/PLGA-PEG-FA nanoparticles had a mean diameter of $280.9 \pm 33.5 \text{ nm}$, allowing them to extravasate into tumor tissue with defective vasculature, including enlarged endothelial gaps. In addition, the small molecule folate was bound to the nanoparticle surface to improve penetrability compared with monoclonal antibodies, which are commonly used to prepare US molecular probes. This natural ligand also allows nanoparticles to specifically accumulate near tumors. To improve nanoparticle quality and targeting efficiency, the shell material PLGA-PEG-FA was synthesized, purified, and confirmed. The most important step is obtaining uniform PLGA-PEG-FA, which we

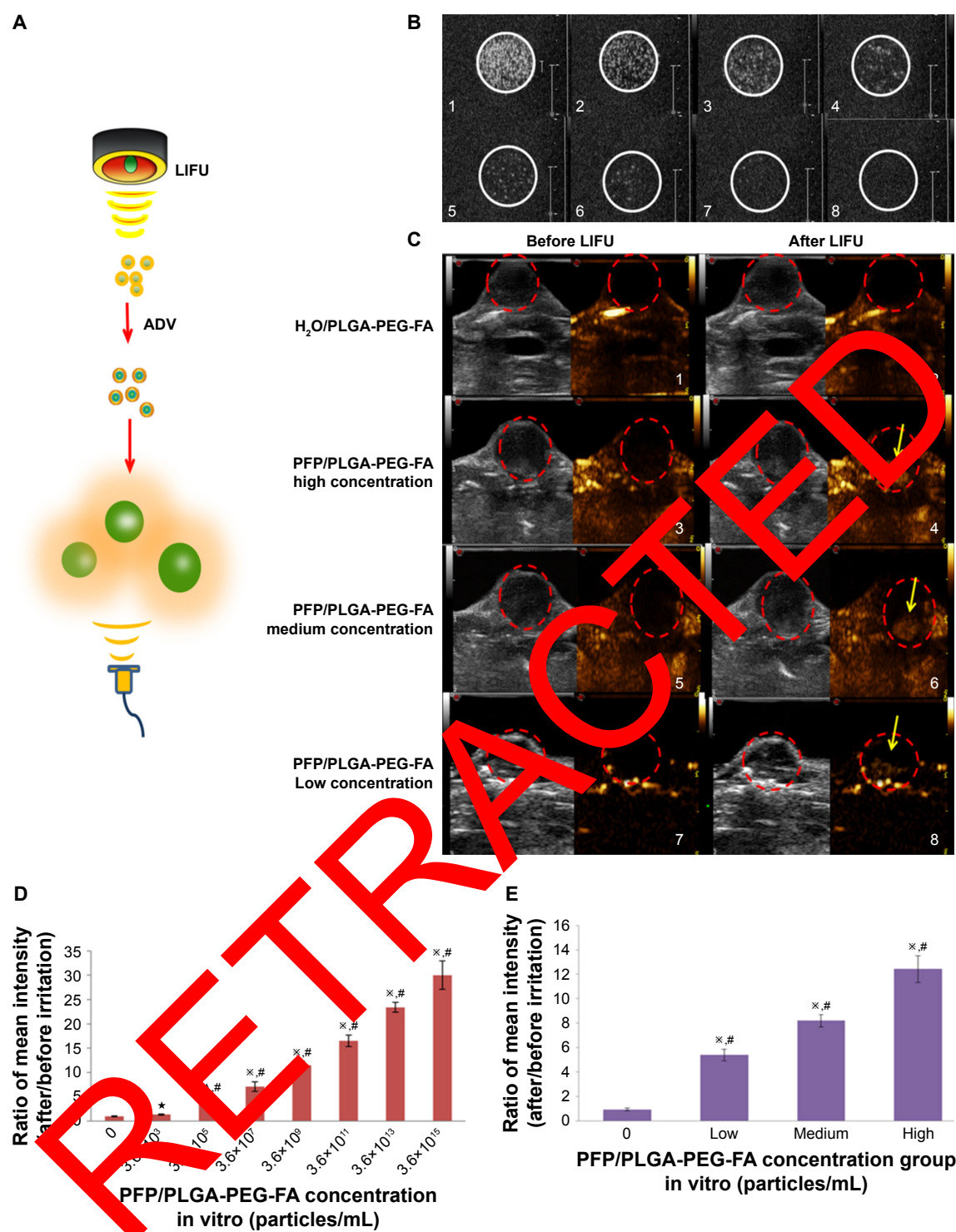


Figure 7 In vitro and in vivo phase-shift US images of PFP/PLGA-PEG-FA nanoparticle with different concentrations (particles/mL).

Notes: (A) Illustration of phase shift irradiated by US-induction. (B) In vitro phase-shift US images in harmonic mode; 1–7: concentrations of PFP/PLGA-PEG-FA nanoparticle 3.6×10^3 , 3.6×10^5 , 3.6×10^7 , 3.6×10^9 , 3.6×10^{11} , 3.6×10^{13} , 3.6×10^{15} particles/mL; 8: H_2O /PLGA-PEG-FA (negative control). (C) In vivo phase-shift US images; 1 and 2: H_2O /PLGA-PEG-FA (negative control); 3 and 4: high concentration of 3.6×10^{15} particles/mL of PFP/PLGA-PEG-FA; 5 and 6: medium concentration of 3.6×10^{13} particles/mL of PFP/PLGA-PEG-FA; 7 and 8: low concentration of 3.6×10^{11} particles/mL of PFP/PLGA-PEG-FA (fundamental and harmonic mode images were presented on the left and right of each panel, respectively; red circles indicate tumor tissues; yellow arrows show the enhanced areas). (D) Relationship between PFP/PLGA-PEG-FA concentration and nanoparticle phase-shift induced by LIFU irradiation in vitro. “*” Comparison with the control group, $P < 0.01$. “Δ” Comparison with the control group, $P < 0.05$. “★” Comparison between the 2 groups, $P < 0.05$. “#” Comparison between the 2 groups, $P < 0.01$. (E) Relationship between PFP/PLGA-PEG-FA concentration and nanoparticle phase-shift induced by LIFU irradiation in vivo. “*” Comparison to the control group, $P < 0.01$. “#” Comparison between the 2 groups, $P < 0.01$.

Abbreviations: ADV, acoustic droplet vaporization; FA, folic acid; LIFU, low-intensity focused US; PEG, polyethylene glycol; PFP, perflubron; PLGA, poly lactic-co-glycolic acid; US, ultrasound.

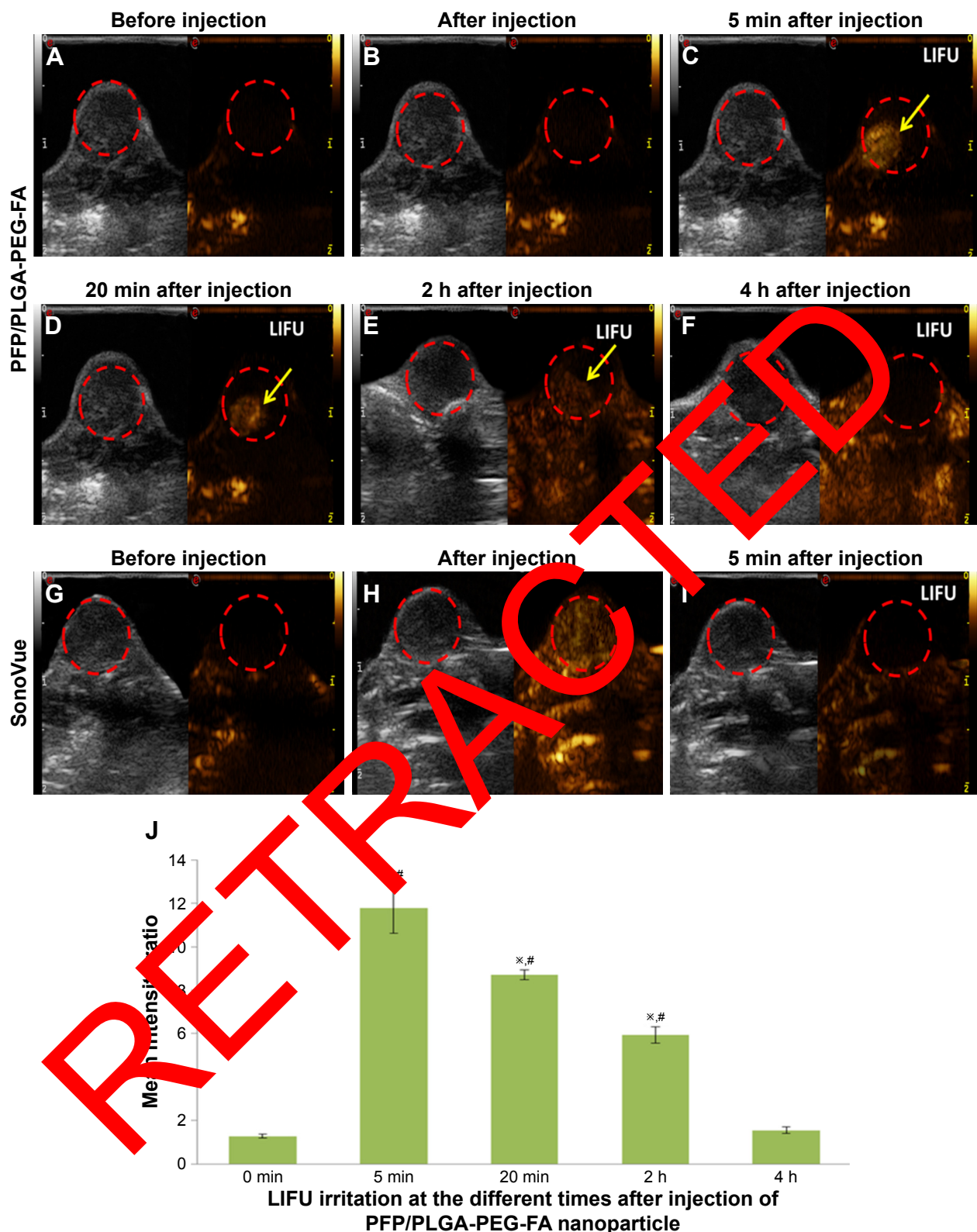


Figure 8 Following PFP/PLGA-PEG-FA nanoparticle and SonoVue injections into tumor-bearing mice, ultrasonic images were taken before and after LIFU irradiation.

Notes: (A and B) Before and after injection of nanoparticles. (C–F) LIFU irradiation at 5 min, 20 min, 2 h, and 4 h after injection. (G and H) Before and after SonoVue injection. (I) LIFU irradiation at 5 min post-injection, (fundamental and harmonic mode images are on the left and right of each panel, respectively; red circles indicate tumors; yellow arrows show the enhanced areas). (J) PFP/PLGA-PEG-FA nanoparticle enhanced US image in vivo by LIFU irradiation at different time points. “*” Comparison after/before injection, $P < 0.01$. “#” Comparison among the 3 groups, $P < 0.01$.

Abbreviations: FA, folic acid; LIFU, low-intensity focused US; PEG, polyethylene glycol; PFP, Perflenapent; PLGA, poly lactic-co-glycolic acid; US, ultrasound.

achieved with a 5-step synthesis method. These laid a good foundation for the preparation of targeting nanoparticles. Furthermore, ion chromatography and reverse-phase chromatography were employed to ensure high purity PLGA-PEG-FA.

PFP load is $59.4\% \pm 7.1\%$ in our study. Since the particles are prepared by sonication, the energy input is very high, which will have a substantial impact on the final product.¹⁴ This was the main reason that parts of PFP were lost during this procedure.

PFP/PLGA-PEG-FA nanoparticle targeting requires that folate ligands are exposed on the exterior surface. We developed a new double-emulsion method based on that described by Esmaili et al¹⁵ and Chen et al.¹⁶ Our in vitro results showed that PFP/PLGA-PEG-FA nanoparticles targeted to SKOV3 cells with high FR expression. The conjugation occurred in a dose-dependent way and was competitively inhibited by the FA addition, indicating specific binding between nanoparticles and FRs. Furthermore, the in vivo results confirmed red fluorescence accumulation in tumor tissue that was not observed in the non-targeted and antagonized groups, indicating high specificity of PFP/PLGA-PEG-FA nanoparticles.¹⁷

A number of studies have demonstrated that US can induce liquid-gas phase transformation to generate perfluorocarbon bubbles for enhancing US imaging.^{14,18,19} The mechanisms of acoustic droplet vaporization mainly include US-inherent biological effects, such as cavitation and mechanical effects, and thermal effects, which trigger the phase transition of lipid perfluorocarbon encapsulated in the nanoparticle core, thus producing gas bubbles.^{20–23} Commonly used PFH is converted into gas under HIFU, while the liquid-gas phase shift in PFP can be induced by LIFU due to its much lower b.p.^{24,25} In the present work, PFP was encapsulated by biodegradable FA-modified PLGA materials to form phase-shift nanoparticles that could be transformed to gas bubbles to enhance US molecular imaging with LIFU.

The LIFU device was specially designed and developed in our laboratory with a driving frequency of 1.0 MHz, focal length of 1.5 cm, and focus area of 0.4 cm². The acoustic intensity in a focal spot of 1.2 W/cm² was applied for 2 min to induce phase transition. Our in vitro and in vivo results showed that PFP/PLGA-PEG-FA nanoparticles enhanced US imaging in tumor tissue following LIFU irradiation. Notably, enhancement was still possible 2 h after nanoparticle administration, demonstrating long retention time in tumor areas. More importantly, localized imaging was accomplished. Owing to the good penetrability of US and favorable focusing

effect of LIFU, deeper sites can be viewed, but enhancement was not found in non-irradiated areas, indicating that gas bubbles were generated by LIFU-induced phase shift to enhance tumor imaging.

Conclusion

Here, we employed new methods to synthesize highly pure shell materials and to prepare the nanoparticles with the targeting ligand facing outward. Then, phase-shifted and folate-targeted PLGA nanoparticles encapsulating liquid perfluorocarbon with a low b.p. were successfully developed. Both in vitro and in vivo experiments suggested that the nanoparticles possessed favorable characteristics for use as a contrast agent, and their targeting ability and specificity to tumor tissue accumulation were verified. More important, phase shift was induced in the nanoparticles by LIFU irradiation for US imaging, indicating that the nanoparticles could serve as molecular probes for targeted tumor imaging. Combining folate-targeted PLGA nanoparticles with LIFU application provided a novel diagnostic protocol for tumors.

Acknowledgments

The authors are grateful to Chao Yu for technical support with flow cytometry and to Donghan Yang for providing helpful suggestions. This work was supported by the National Natural Science Foundation of China (Grant Nos 81227801, 81601513, 81630047, 31630026, 81371578, 81571688, 81371718), National Distinguished Young Scholars (Grant No 81425014), National Research Program of China (973 Program, Grant No 2011CB707905), Grant of Chongqing Medical University, China (Grant No CXTDG 201602007), and General Program of Health and Family Planning Commission of Chongqing, China (Grant No 2010-2-115).

Disclosure

The authors report no conflicts of interest in this work.

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Supplementary materials

Table S1 PFP/PLGA-PEG-FA nanoparticles target efficiency in vivo fluorescence images (n=3)

Group	Mean intensity
Non-targeted group (DiI/PFP/PLGA-PEG-FA)	13.73±2.20
Targeted group (DiI/PFP/PLGA-PEG)	93.12±9.46 ^a
Antagonized group (FA + DiI/PFP/PLGA-PEG-FA)	15.76±3.46

Notes: ^aComparison to non-targeted group. $P<0.01$.

Abbreviations: FA, folic acid; PEG, polyethylene glycol; PFP, perflenapent; PLGA, poly lactic-co-glycolic acid.

Table S2 Relationship between PFP/PLGA-PEG-FA concentration and nanoparticle phase-shift induced by LIFU irritation (measured by in vitro US image; n=3)

PFP/PLGA-PEG-FA concentration (particles/mL)	Ratio of mean intensity (after/before irritation)
0 (control group with only H ₂ O/PLGA-PEG-FA)	0.99±0.10
3.6×10 ³	1.35±0.12 [*]
3.6×10 ⁵	4.12±0.65 ^{*,‡,§}
3.6×10 ⁷	7.10±1.00 ^{‡,§}
3.6×10 ⁹	11.48±0.83 ^{‡,§}
3.6×10 ¹¹	16.52±1.18 ^{‡,§}
3.6×10 ¹³	23.40±1.00 ^{‡,§}
3.6×10 ¹⁵	30.02±2.92 ^{‡,§}

Notes: ^{*}Comparison between the 2 groups, $P<0.05$; [‡]Comparison to the control group. $P<0.05$; [§]Comparison between the 2 groups. $P<0.01$; [‡]Comparison to the control group. $P<0.01$.

Abbreviations: FA, folic acid; LIFU, low-intensity focused US; PEG, polyethylene glycol; PFP, perflenapent; PLGA, poly lactic-co-glycolic acid.

Table S3 Relationship between PFP/PLGA-PEG-FA concentration and nanoparticle phase-shift induced by LIFU irritation (measured by in vivo US image; n=3)

PFP/PLGA-PEG-FA concentration group (particles/mL)	Ratio of mean intensity (after/before irritation)
0 (control group with only H ₂ O/PLGA-PEG-FA)	0.95±0.11
3.6×10 ¹¹ (low)	5.40±0.48 ^{*,‡}
3.6×10 ¹³ (medium)	8.21±0.51 ^{*,‡}
3.6×10 ¹⁵ (high)	12.42±1.10 ^{*,‡}

Notes: ^{*}Comparison to the control group. $P<0.01$; [‡]Comparison between the 2 groups. $P<0.01$.

Abbreviations: FA, folic acid; LIFU, low-intensity focused US; PEG, polyethylene glycol; PFP, Perflenapent; PLGA, poly lactic-co-glycolic acid; US, ultrasound.

Table S4 PFP/PLGA-PEG-FA nanoparticle enhanced US image in vivo by LIFU irritation at the different times (n=3)

LIFU irritation at different time after injection	Mean intensity ratio
0 (control group with no LIFU irritation)	1.28±0.09
5 min	11.77±1.14 ^{*,‡}
20 min	8.71±0.22 ^{*,‡}
2 h	5.94±0.39 ^{*,‡}
4 h	1.55±0.15

Notes: ^{*}Comparison after/before injection. $P<0.01$. [‡]Comparison among the 3 groups. $P<0.01$.

Abbreviations: FA, folic acid; LIFU, low-intensity focused US; PEG, polyethylene glycol; PFP, perflenapent; PLGA, poly lactic-co-glycolic acid.

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