

Synthesis, characterization and *in vitro* and *in vivo* investigation of C₃F₈-filled poly(lactic-co-glycolic acid) nanoparticles as an ultrasound contrast agent

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Abstract. The present study aimed to prepare perfluoropropane (C₃F₈)-filled poly(lactic-co-glycolic acid) (PLGA) nanoparticles and investigate the feasibility of using PLGA nanoparticles as an ultrasound contrast agent. The PLGA nanoscale ultrasound contrast agent was prepared using a modified double-emulsion solvent evaporation method. Camphor in the form of a sublimable porogen was added to render the nanoparticles hollow and enable C₃F₈ gas introduction. Various physicochemical properties of PLGA nanoparticles, including morphology, size and dispersion, were analyzed by electron microscopy and dynamic laser scattering. *In vitro* ultrasound imaging of C₃F₈-filled PLGA nanoparticles was also investigated under various imaging conditions. Further *in vivo* ultrasound imaging was conducted on male rats following intratesticular injection of PLGA nanoparticles. C₃F₈-filled PLGA nanoparticles with a mean diameter of 152.0±58.08 nm were obtained. Electron microscopy revealed spherical-shaped nanoparticles with smooth surfaces, a capsular morphology and a large hollow within. *In vitro* ultrasound imaging of hollow PLGA nanoparticles indicated marked signal enhancement. Local intensity of the acoustical signal continued to increase during PLGA-nanoparticle injection into the testicle and the ability of hollow PLGA nanoparticles to enhance ultrasound imaging *in vivo* was demonstrated. The enhancement image of testicular tissue following injection with C₃F₈-filled PLGA

nanoparticles was sustained for a minimum of five minutes. In conclusion, the hollow C₃F₈-filled PLGA nanoparticles were demonstrated to have potential for applications as a novel ultrasound contrast agent.

Introduction

At present, ultrasound contrast agents (UCAs) are frequently used in order to expand the scope of ultrasound diagnostics, in particular for tumors (1-3). The materials used in the fabrication of UCAs are lipids, proteins, carbohydrates and polymers. The polymers, including poly(lactic-co-glycolic acid) (PLGA), have been extensively studied in recent years (4,5). PLGA was selected for the present study due to its good biodegradability and biocompatibility as reported in previous studies, which is also the reason why the Food and Drug Administration approved its usage in sutures and drug delivery devices (4).

PLGA microcapsules are the most common type of UCA available at present. The polymeric shell improves the stability of the capsules, compared to that of those stabilized by a monomolecular layer of surfactant (5,6). Furthermore, PLGA microcapsules contain gas, which increases their scattering power and leads to a high echogenicity due to the high compressibility and low density of the gases (5,7). However, the large scale of PLGA microcapsules limits their applications. Sun *et al* (8) reported that the superparamagnetic PLGA-iron oxide microcapsules for dual-modality ultrasound/magnetic resonance imaging had an average diameter of 885.6 nm. The endothelial gap of a tumor was found to be ~400-600 nm, which makes it difficult for microcapsules to penetrate the vasculature and detect tumors (7). To overcome this limitation, studies have focused on developing PLGA capsules on a nanoscale (9,10). Néstor *et al* (9) prepared air-filled PLGA nanocapsules with a mean diameter of 370±96 nm and evaluated their echogenic power and stability *in vitro*. Kohl *et al* (10) prepared and evaluated multifunctional PLGA nanoparticles for photoacoustic imaging. PLGA nanobubbles, of mean diameter 268 nm, were developed by Xu *et al* (11) for cancer targeting and imaging. In these previous studies, the imaging effects of PLGA nanoparticles were examined *in vitro* and the results suggested that the PLGA shell may

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improve nanocapsule stability. However, there were almost no systematic studies investigating the *in vitro* imaging capacity of PLGA nanoparticles under varying conditions. Furthermore, to the best of our knowledge, few studies have been conducted to investigate the properties of PLGA nanoparticles in *in vivo* ultrasound imaging (7,9-11).

The present study was therefore designed to: *i*) Prepare perfluoropropane (C₃F₈)-filled nanoparticles with a biodegradable polymeric shell composed of PLGA and *ii*) investigate the feasibility of using PLGA nanoparticles to enhance ultrasound imaging *in vitro* and *in vivo*.

Materials and methods

Materials. Poly(D,L-lactic-co-glycolic acid) (PLGA; 50:50; molecular weight, 40,000) was purchased from Jinan Daigang Biomaterial Co., Ltd (Jinan, China). Polyvinyl alcohol (PVA; 88% mole hydrolyzed) and (D+)-camphor were purchased from Aladdin Chemistry Co., Ltd (Shanghai, China). Methylene chloride, isopropanol, mannitol and phosphotungstic acid were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Deionized water used in all experiments was purified using a Milli-Q Plus 185 water purification system (Millipore, Bedford, MA, USA) with resistivity >18 MΩ cm. All chemical reagents were of at least analytical grade and were used directly without further purification.

Animals. A total of ten male Wistar rats (three months old; mean weight, 150 g) were purchased from the Shanghai Laboratory Animal Center (Shanghai Laboratory Animal Co., Ltd, Shanghai, China) and housed in pathogen-free facilities with a 12-hour light/dark cycle (6am-6pm) and provided with rodent diet and tap water *ad libitum*. Animal studies were conducted according to the guidelines of the Renji Hospital, School of Medicine, Shanghai Jiao Tong University. Animal procedures were approved by the Institutional Animal Care and Use Committee, which is certified by the Shanghai Association for Accreditation of Laboratory Animal Care (Shanghai, China).

Preparation of C₃F₈-filled PLGA nanoparticles. PLGA hollow nanoparticles were prepared based on a modified double-emulsion solvent evaporation method (12). For the first emulsion, 125 mg PLGA and 12.5 mg camphor were dissolved in 5 ml methylene chloride. The polymer was fully dissolved prior to the addition of 1 ml PVA (3%, w/v). The mixture was subsequently emulsified in an ice bath using a Ultrasonic processing 04717 (Cole-Parmer, Vernon Hills, IL, USA) at 130 W for 180 sec, in pulse mode with sonication turned off for 2 sec and on for 4 sec to prevent heat stacking. For the second emulsion, the emulsified solution was added to 20 ml PVA (3%, w/v) and sonicated for 180 sec at 4 sec on, 2 sec off. Following the double emulsion, the resulting water-in-oil-in-water emulsion was added to 100 ml isopropyl alcohol (5%, v/v) and continually stirred for 1 h to evaporate any organic solvent. Following evaporation, samples were collected using centrifugation (17,800 x g for 10 min) and washed three times with 5 ml deionized water. The precipitation was subsequently flash frozen and lyophilized for 48 h.

As the mixture underwent the freeze-drying process, camphor sublimed out of the particles, leaving a void in their place. This hollow core was filled with C₃F₈ gas (Renjieling Optical Instrument Co, Ltd., Shanghai, China) when later exposed to C₃F₈ pressure.

Characterization of C₃F₈-filled PLGA nanoparticles. The surface morphology of the nanoparticles was investigated using an S-4800 field emission scanning electron microscope (FE-SEM; Hitachi, Tokyo, Japan). The PLGA nanoparticle samples were dispersed in deionized water, spread over a piece of aluminum foil and dried at room temperature. The samples were subsequently sputter coated with a layer of gold using a fine coat ion sputter (JFC-1100; JEOL, Ltd, Tokyo, Japan) prior to FE-SEM imaging.

Transmission electron microscopy was performed using a JEOL JEM-2100 low- to high-resolution transmission electron microscope (HR-TEM; Hitachi). The PLGA nanoparticle suspension was dropped onto a formvar film-coated copper grid for 2 min, excess solution was removed using filter paper and phosphotungstic acid solution (1%, w/v) was used to stain the sample for 2 min (13). Finally, the grids were air-dried prior to observation.

The samples were dispersed in deionized water to obtain a uniform suspension. Dynamic laser scattering (DLS; Zetasizer Nano ZS model ZEN3690; Malvern Instruments, Ltd, Malvern, UK) was used to evaluate the mean size and size-distribution of the PLGA nanoparticles dispersed in deionized water. The laser light wavelength was 633 nm and measurements were obtained at a 90° fixed-angle for 180 sec at 25°C. Measurements were made in triplicate for all batches prepared.

***In vitro* ultrasound imaging of C₃F₈-filled PLGA nanoparticles.** A preliminary evaluation of the ultrasound contrast behavior of PLGA nanoparticles was performed. Various concentrations of PLGA nanoparticles in deionized water were imaged in a tank filled with degassed and deionized water at ~37°C. Images of degassed and deionized water were acquired to serve as background controls. The mechanical index (MI) was 0.06. The samples were scanned using an ultrasonic diagnostic instrument (MyLab Twice; Esaote SpA, Genova, Italy) in conventional B mode. The center frequencies of the transducers were 13 and 22 MHz, respectively.

***In vivo* ultrasound imaging of C₃F₈-filled PLGA nanoparticles.** *In vivo* investigation was performed on healthy male rats, randomly divided into two groups (n=5). The rats were anesthetized with an intraperitoneal injection of 10% chloral hydrate, and then fixed on a board. The PLGA nanoparticles were suspended in deionized water at a concentration of 2 mg/ml and 200 μl PLGA nanoparticle solution was injected through a 1-ml syringe into the right testicle. The testicles of the rats were scanned by the MyLab Twice scanner (Esaote SpA) prior to and following the administration of PLGA nanoparticles. A total of 200 ml deionized water was also injected into the control group.

Statistical analysis. Values are presented as the mean ± standard deviation. The nanoparticles were analyzed using DLS (14,15).

Results

Preparation and characterization of C₃F₈-filled PLGA nanoparticles. The capsular structure of C₃F₈-filled nanocapsules was evaluated by electron microscopy. The examination of C₃F₈-filled nanocapsules by SEM revealed spherical particles with smooth surfaces (Fig. 1). TEM revealed the typical core-shell structure of C₃F₈-filled nanocapsules with a thin shell, where the shell appeared darker and the gas core appeared gray due to the differences in electron density (Fig. 2). As indicated in Fig. 3, the average size of nanoparticles prepared in the present study was 152.0±58.08 nm and the polydispersity index was 0.221, revealing uniform size and good dispersion of the PLGA nanoparticles.

In vitro imaging. The capability of C₃F₈-filled PLGA nanoparticles as a contrast agent for ultrasound imaging was assessed *in vitro* using the MyLab Twice scanner system at various concentrations and frequencies and at MI 0.06. Three concentrations of C₃F₈-filled PLGA nanoparticles (3, 2 and 1 mg/ml) were evaluated in the first experiment. As shown in Fig. 4, there was almost no visible difference between 3 and 2 mg/ml at 22 or 13 MHz. However, the echo signal of 1 mg/ml C₃F₈-filled PLGA nanoparticles was a little weaker than those of 2 and 3 mg/ml nanoparticles. For the prepared C₃F₈-filled PLGA nanoparticles, the optimal ultrasound imaging concentration was 2 mg/ml. Accordingly, 2 mg/ml was selected as the maximum concentration in the subsequent study of the imaging effects of PLGA nanoparticles.

As indicated in Fig. 5, the ultrasound signals gradually decreased with decreasing concentrations of PLGA nanoparticles at 13 and 22 MHz. The signal intensity was strongest at 2 mg/ml and remained relatively strong at the low concentration of 0.125 mg/ml. These *in vitro* results demonstrated that the PLGA nanoparticles were able to be used as the contrast agents for efficient ultrasound imaging. Identical concentrations of PLGA nanoparticles imaged more effectively at 22 MHz compared with imaging at 13 MHz. Therefore, the optimal ultrasound imaging conditions were 2 mg/ml nanoparticles at a frequency of 22 MHz.

In addition, the length of time during which the signal enhancement was produced by different concentrations of PLGA nanoparticles at 22 MHz was sustained was investigated. As indicated in Fig. 6, the signal intensity of 2 mg/ml PLGA nanoparticles remained strong until 60 sec, there was slight decrease at 120 sec and signal enhancement could still be detected at 180 sec. At 0.125 mg/ml the signal intensity gradually decreased with the extension of time. The results indicated that the PLGA nanoparticles were able to image at low concentrations for at least two minutes and that the higher the concentration of PLGA nanoparticles was, the longer the potential imaging time persisted.

In vivo imaging. In order to evaluate the acoustic behavior of C₃F₈-filled PLGA nanoparticles *in vivo*, testicular ultrasound imaging was performed using the B-Scan imaging mode at a frequency of 22 MHz and an MI of 0.06. Two groups of five male rats were anesthetized intraperitoneally with 10% chloral hydrate prior to agent injection. The transducer was placed on the testicle for real-time monitoring and 200 µl PLGA nanoparticles dispersed

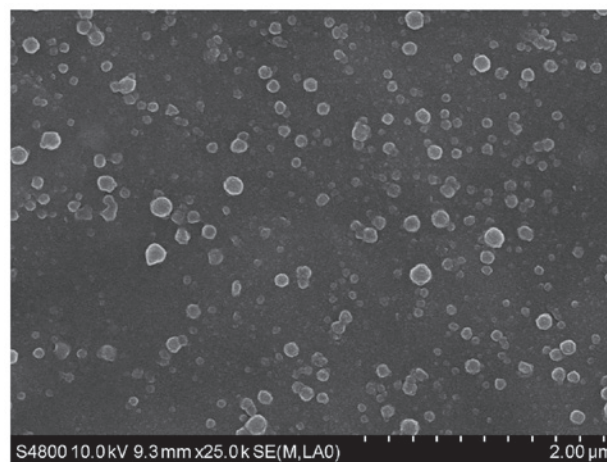


Figure 1. Scanning electron microscope image of C₃F₈-filled poly(lactic-co-glycolic acid) nanoparticles.

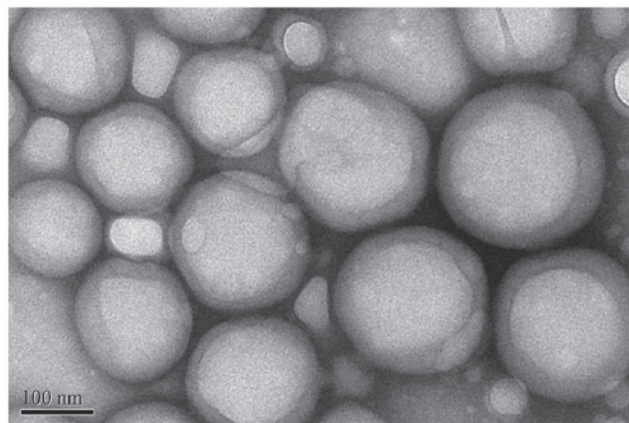


Figure 2. Transmission electron microscope image of poly(lactic-co-glycolic acid) nanoparticles showing the typical capsular structure, where the polymeric shell appears darker.

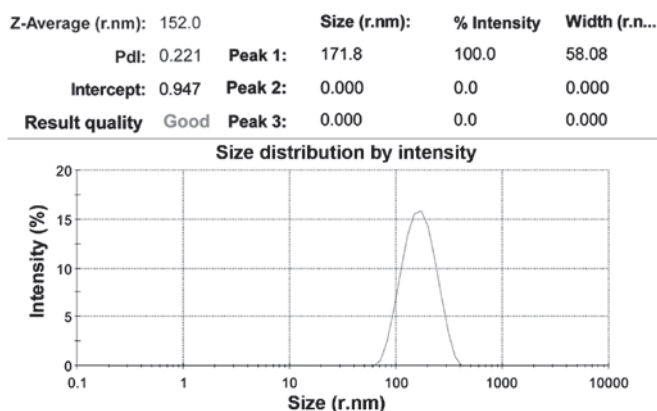


Figure 3. Average size and distribution of poly(lactic-co-glycolic acid) nanoparticles measured by dynamic laser scattering. Pdl, polydispersity index.

in deionized water were intra-testicularly injected. Ultrasound imaging was recorded during the injection. Fig. 7A and B indicated that the local intensity of the acoustic signal continued to increase during PLGA nanoparticle-injection into the testicle and the ability of hollow PLGA nanoparticles to enhance ultra-

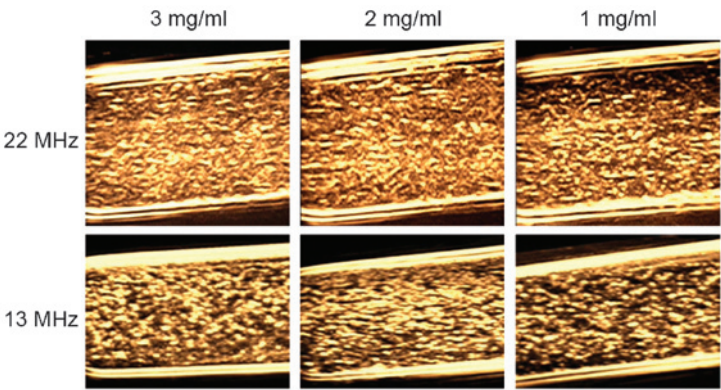


Figure 4. *In vitro* ultrasound images of various concentrations of poly(lactic-co-glycolic acid) nanoparticles at 13 and 22 MHz.

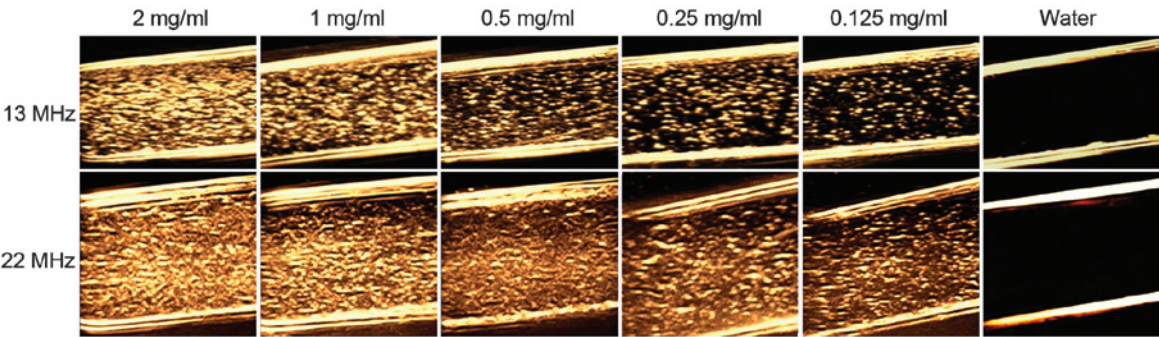


Figure 5. *In vitro* ultrasound images of various concentrations of poly(lactic-co-glycolic acid) nanoparticles at 13 and 22 MHz.

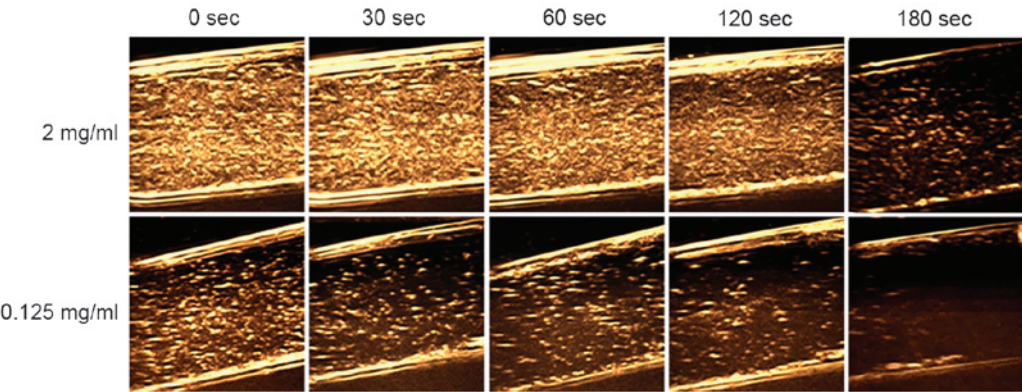


Figure 6. *In vitro* ultrasound images of poly(lactic-co-glycolic acid) nanoparticles at various imaging times. Frequency, 22 MHz.

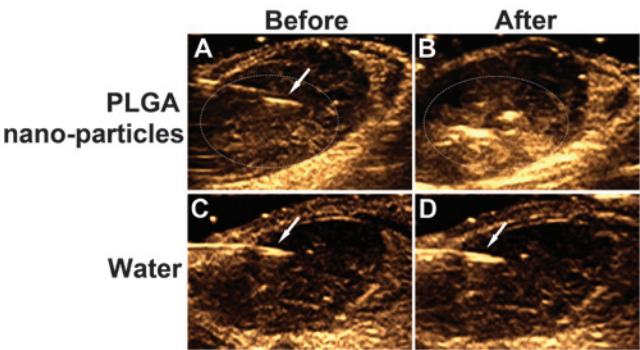


Figure 7. *In vivo* ultrasound images of PLGA nanoparticles. (A and B) Local testicular tissue and local enhancement prior to and following nanoparticle injection into the rat testicle are represented by a white circle. (C and D) Imaging behavior of degassed and deionized water injected into the testis in the control group. Syringe needles are marked with white arrows. PLGA, poly(lactic-co-glycolic acid).

sound imaging *in vivo* was demonstrated. The results revealed that the enhancement image of testicular tissue following injection of hollow PLGA nanoparticles was able to be sustained for ≥ 5 min. Fig. 7C and D demonstrated the imaging behavior of deionized water injected into the testicle of rats in the control group. There was almost no echo difference detected prior to and following injection of degassed and deionized water.

Discussion

Various nano-scale UCAs, including solid nanoparticles, liquid-core nanoagents and gaseous-core nanoagents have been reported (16-19). Although the feasibility of the use of solid nanoparticles to enhance high-frequency ultrasound B-mode images was reported by Liu *et al* (16), the biodegradation and biocompatibility of solid nanoparticles have remained major issues. Liquid-core nanoagents have also been evaluated as UCAs (18); however, drawbacks include poor echogenicity due to low compressibility and the use of high frequencies of insonification. Gas-filled nanobubbles were developed to further enhance the ultrasound signal (9). A biodegradable polymer shell was more rigid than surfactants and lipids and was easily modified with various ligands to achieve active targeting to specific tissues and cells (18,19). Therefore, the present study aimed to synthesize a novel C_3F_8 -filled polymer UCA using PLGA as the shell.

The micro-scale method of using a porogen to create voids for accommodating gas in the particles was adapted for use in the present study to produce hollow nanocapsules. Camphor and other substances that are able to sublime or decompose rapidly without the generation of toxic reactions have the ability to form hollows within particles. Kwon and Wheatley (20) produced gas-loaded PLA nanoparticles for use as UCAs by using camphor. Néstor *et al* (9) prepared air-filled PLGA nanocapsules using ammonium bicarbonate as sublimable porogens. The PLGA nanoparticles used in the present study were prepared using camphor as the sublimable porogen, which produced a large hollow in the particles. The hollows were subsequently filled with C_3F_8 gas, producing higher echo reflection (5).

In the present study, methylene chloride was used as a solvent, with high-power emulsification and high-speed centrifugation, to produce PLGA nanoparticles markedly smaller than the microbubbles prepared using the traditional double-emulsion method (21,22). The average diameter of the nanoparticles fabricated was 152.0 ± 58.08 nm, markedly smaller than the endothelial gap. In theory, particles < 400 - 600 nm are able to penetrate the endothelial gap of tumor feeding vessels (23). It was therefore possible for the PLGA nanoparticles prepared in the present study to image the target tissue outside the vascular structures, overcoming the drawbacks of conventional ultrasound contrast agents that are only able to image within the blood pool.

The preliminary *in vitro* studies performed confirmed the imaging effects of various concentrations of PLGA nanoparticles at 22 and 13 MHz. The results indicated that the identical concentration of PLGA nanoparticles imaged better at a frequency of 22 MHz than at 13 MHz, which indicated that the PLGA nanoparticles were more suitable for use in high frequencies of insonification. Furthermore, low concentrations

(0.125 mg/ml) of PLGA nanoparticles were able to image for at the 120th sec, suggesting higher stability compared with that of UCAs made of lipid or protein. Therefore, there was also sufficient time for the PLGA nanoparticles to penetrate the endothelial cell gap into the tumor tissue and detect tumors efficiently when circulating in blood (24).

The PLGA nanoparticles were also imaged effectively in the rat testis compared with imaging using degassed water. This result indicated that the prepared C_3F_8 -filled PLGA nanoparticles had potential to be used as a novel UCA that may be modified for use in *in vivo* targeted molecular imaging (22,25,26).

In conclusion, C_3F_8 -filled PLGA nanoparticles for efficient ultrasound imaging were generated via a modified double-emulsion solvent evaporation method. Results of *in vitro* and preliminary *in vivo* studies demonstrated that C_3F_8 -filled PLGA nanoparticles exhibited effective ultrasound imaging capabilities. Therefore, these prepared C_3F_8 -filled PLGA nanoparticles have potential applications as a novel UCA. Further study is required in order to investigate the imaging capacity of functionalized PLGA nanoparticles in tumor models. Further study is required in order to investigate the imaging capacity of functionalized PLGA nanoparticles in tumor models, and much more quantitative analysis is needed.

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