Bio-Medical Materials and Engineering 26 (2015) S911–S916
DOI 10.3233/BME-151384
IOS Press

Novel DiR and SPIO nanoparticles embedded PEG-PLGA nanobubbles as a multimodal imaging contrast agent

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Abstract. Fluorescence dye DiR and superparamagnetic iron oxide nanoparticles (SPIONs) embedded in PEG-PLGA nanobubbles (DiR-SPIO-NBs) were produced using double emulsion method on a membrane of Shirasu porous glass (SPG). The nanobubbles encapsulated with DiR and SPIONs had a liquid core (perfluoropentane) and a PEG-PLGA shell. DiR-SPIO-NBs showed biocompatibility based on MTT cytotoxicity and hemolysis studies. The PFP encapsulated in the nanobubbles experienced phase transition under ultrasonic irradation. Nanobubbles dispersed well in saline over 3 months, and the relaxivity was 127.9 $mM^{-1}s^{-1}$, suggesting that it could be used as a contrast agent in MRI. The MR and fluorescence images *in vivo* demonstrated that the signal intensity in the spleen and liver was significantly enhanced with the treatment of nanobubbles. In addition, results of ultrasound images suggested that the nanobubbles had persistent contrast ability. In conclusion, nanobubbles could be utilized as an US/MRI/fluorescence contrast agent.

Keywords: Nanobubbles, ultrasound-triggered phase transition, multimodal contrast agent, ultrasound imaging, magnetic resonance imaging, fluorescence imaging

1. Introduction

Medical imaging technologies such as ultrasound (US) imaging, magnetic resonance imaging (MRI) and fluorescence imaging, were applied to diagnosing cancer. However, each of these technologies has certain advantages and disadvantages. Recently, multimodal imaging has attracted the interest of researchers, due to its ability to make up for the shortcomings of a single-imaging mode [1, 2]. In previous studies, microbubbles encapsulated with fluorescent dyes or superparamagnetic iron oxide (SPIO) have been used as US/fluorescence or MRI/US contrast agents [3, 4]. The diameters of the microbubbles are usually several microns (2~8 μm), causing them to be trapped in the blood pool and

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thus imposing severe limitations on ultrasonic imaging and drug/gene [5]. In recent years, nanoscale bubbles (nanobubbles) have been the subject of research regarding extravascular ultrasonic imaging and drug/gene delivery [6, 7]. Nanobubbles effectively accumulate in the tumor interstitium due to the enhanced permeability and retention (EPR) effect, naturally enabling them to serve as promising contrast agents for tumor ultrasonic imaging and treatment [8].

In previous studies, the preparation methods of nanobubbles are similar to those of microbubbles. These similar techniques include the mechanical stirring method, the ultrasonic treatment method, the freeze-drying method, the film hydration method, and the double emulsion-solvent evaporation method. These procedures are not well-controlled by the particles size. Furthermore, these methods use large amounts of organic solvents or strong mechanical stirring force, resulting in the encapsulated biomolecules losing their bioactivities [9].

In this study, uniform-sized poly (ethylene glycol)–poly (lactide-co-glycolide) (PEG-PLGA) nanobubbles encapsulated with DiR (1,1′-dioctadecyltetramethyl indotricarbocyanine ioide) and SPIO nanoparticles (DiR-SPIO-NBs) were produced using double emulsion method. The physi-chemical characteristics, the ultrasound-triggered phase transition, the biocompatibility and the utilization of nanobubbles as an US/MRI /fluorescence contrast agent were studied.

2. Materials and methods

2.1. Materials

PEG-PLGA (LA:GA=50:50 and molecular weight of 35,000 Da) was obtained from Shangdong Institute of Medical Instrument (Shangdong, China). PVA-217 (hydrolysis degree of 88.5%) was purchased from Kuraray (Tokyo, Japan). 1, 1′-dioctadecyltetramethyl indotricarbocyanine ioide (DiR) was obtained from Biotium, Inc (Hayward, CA, USA). Superparamagnetic iron oxide (SPIO) nanoparticles (10 nm) were synthesized by our laboratory as reported by Xu, et al. [10]. Perfluoropentane was purchased from Sigma (Sigma-Aldrich, USA). SPG membrane (the diameter of 0.6 μm) was purchased from Kiyomoto Iron Works Co. (Sadowara, Japan).

2.2. Preparation of DiR-SPIO-NBs

The premix membrane emulsification and solvent evaporation method was used to prepare DiR-SPIO-NBs. DiR (1 mg), PEG-PLGA (0.1 g) and SPIO (50 mg) were first dissolved in the THF solution (5 mL). PFP (1 mL) was added to the solution, followed by a high shearing at 10000 rpm for about 4 min using homogenizer (Shanghai, China) under the low temperature in darkness to form O_1/O_2 primary emulsion. 35 mL saline and 0.3% PVA solution (w/v) were poured into the primary emulsion and stirred mechanically for about 2 min to form $O_1/O_2/W$ double emulsion. The $O_1/O_2/W$ double emulsion passed through SPG membrane equipment five times with 106 psi of nitrogen pressure at room temperature. Organic solvents were removed by evaporation, and the nanobubbles were formed after solidification. The nanobubble dispersion was centrifuged (10000 rpm, 10 min) to obtain nanobubble precipitates, which were then dispersed in a normal saline solution. The samples were then stored at 4℃ for further study.

2.3. Characterization of DiR-SPIO-NBs

The hydrodynamic size and the polydispersity index (PDI) of DiR-SPIO-NBs were measured on a Nano-ZS90 zetasizer (Malvern, UK) at 25℃ using He-Ne laser of 633 nm. To determine the zeta potential, the nanobubbles were diluted with ultrapure water until the conductivity of the dilute suspension was 40-50 μS/cm. The morphology of DiR-SPIO-NBs was examined by TEM (Tecnai G2 20, FEI, USA).

In the stability experiment, DiR-SPIO-NBs were stored at 4°C for 3 months. Changes in particle size and zeta potential were examined during the storage period (0, 0.5, 1, and 3 months).

2.4. Imaging study in vitro

2.4.1. MR imaging

To assess the T_2 relaxation times, the nanobubbles were diluted with saline until the concentrations were 0.32%, 0.48%, 0.64% and 0.80% (m/m). Samples were examined on a 3.0-T whole-body MR scanner (MAGNETOM Trio, A Tim System 3T, Siemens, Munich, Germany) with an 8-channel wrist joint coil. The parameters were optimized as follows: field of view=120mm, base resolution=384×384, slice thickness=1.5 mm, multiple echo times=20, 40, 60, 80, 100, 120, 140 ms, repetition time 2000 ms, and scan time about 13-14 min. T_2 relaxation rates were plotted against iron concentrations in the dilutions. Relaxivity was determined through a linear fitting model.

2.4.2. US imaging

The nanobubbles solution was added to the latex finger cot, positioned in a degassed water bath. The ultrasound contrast results were obtained from a B-mode clinical ultrasound imaging system (Philips Medical Systems, USA). To collect ultrasonic images, the 10 MHz probe of a clinical ultrasound imaging system (L12-1) was immersed in the liquid. All contrast images were recorded in the B-mode, with frame rate of 37 Hz and Mechanical Index (MI) of 0.07.

2.5. In vivo imaging study

2.5.1. In vivo MRI

Rabbits afflicted with in situ VX2 liver cancer were anaesthetized with 10% of chloral hydrate (0.4 mL /100 g), then placed in the magnetic resonance imaging system at the prone position, and scanned before and after injection of nanobubbles (1 mL) through the ear vein. The scanning parameters were as follows: Field of view=200 mm, base resolution=320×320, slice thickness=4 mm, echo time =87 ms, repetition time =1000 ms, and scanning time=39 s.

2.5.2. In vivo US imaging

The anaesthetized rabbits were injected with 1 mL of the nanobubbles through the ear vein. The probe (10 MHz) was used to obtain the tumor imaging. Sonovue microbubbles (Bracco, Italy), a commercial ultrasonic contrast agent, are used as control. The rabbits were sacrificed after completion of the experiments, and perfused with saline followed by 4% paraformaldehyde. The liver was removed and the tumor was separated, both of these were studied using an IVIS Lumina XR system (Caliper Life Sciences, USA). The ex vivo fluorescent images of the organs were acquired and analyzed. Finally, the organs were treated with hematoxylineeosin (H & E) staining.

2.5.3. In vivo fluorescence imaging

The anesthetized BALB/c nude mice with HepG2 tumor were injected with a dose of 0.2 mL

DiR-SPIO-NBs. The mice were then placed into the IVIS Lumina XR system in the ventral positions. The fluorescence images were acquired at 10 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h, 11 h and 31 h after injection, respectively. The parameters as follows: exposure time =auto, f/stop = 2, binning = 4, and FOV= 10 cm. After fluorescence imaging experiments, the major organs were separated, which would be analyzed by the IVIS Lumina XR system.

3. Results and discussion

3.1. Characterization of DiR-SPIO-NBs

The size/size distribution and TEM image of DiR-SPIO-NBs were shown in Figure 1. DiR-SPIO-NBs has a uniform size of 302.7 ± 6.4 nm with PDI of 0.124 ± 0.032, and zeta potential of -8.75 ± 0.26 mV. TEM image indicated that the DiR-SPIO-NBs were core-shell spherical nanoparticles. The lipophilic SPIO and DiR were encapsulated in the polymer shell, while the PFP was in the core of the nanobubbles. The particle size and zeta potential measurements revealed that DiR-SPIO-NBs remained stable for over 3 months when stored in an aqueous solution at 4°C. DiR-SPIO-NBs showed good biocompatibility based on MTT cytotoxicity of HepG2 or HL-7702 cells and hemolysis studies.

A novel method using premix membrane emulsification and solvent evaporation was developed for the preparation of nanobubbles and other nanocarriers [11]. Unlike the conventional methods, which usually result in instability and uneven nanocarrier size [12], the nanobubbles prepared in this study are stable and uniform.

3.2. In vitro imaging study

MRI *in vitro* of nanobubbles was studied using a 3T MRI instrument. MR images of various iron concentrations were obtained with a T_2-weighted sequence. The DiR-SPIO-NBs could reduce T_2 significantly. Also, the T_2 relaxation corresponded with the iron concentration. These suggested that the nanobubbles could be used as a T_2 contrast agent.

At room temperature, the PFP maintained its liquid state because its boiling point is 29℃. The transition from liquid to gas occurred when the nanobubbles were activated by the ultrasound at 25℃. The results showed that the DiR-SPIO-NBs exhibited phase transition behavior in response to the ultrasonic wave. Many bright spots appeared in the latex finger cot, which reflected the enhancement

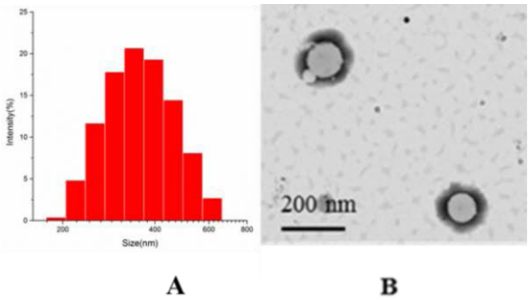

Fig. 1. The size distribution and morphology of DiR-SPIO-NBs. (A) Size distribution; (B) TEM image.

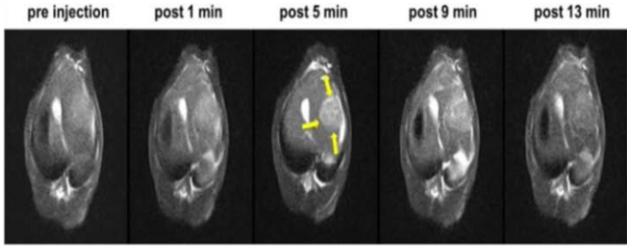

Fig. 2. T2-weighted MR images of rabbit liver bearing VX2 tumor with injection of DiR-SPIO-NBs.

of echogenic movement signals. Naturally, this observation confirmed that the nanobubbles possessed acoustical activity [8].

3.3. In vivo imaging study

3.3.1. In vivo MRI

To evaluate whether or not the nanobubbles could act as an MRI contrast agent, rabbit liver were examined using a 3T MRI instrument with T_2 sequence. As shown in Figure 2, before the nanobubble injection, the liver tumor boundaries were indistinct, while after injection, the boundaries gradually clear as time goes on. The signal strength reached its maximum at 5 min, after, it began to decrease.

3.3.2. In vivo ultrasound imaging

The liver tumors could be recognized as low echoic mass. As shown in Figure 3, before the injection of the DiR-SPIO-NBs, the delineation and the boundaries were unclear, since the lesion was almost isoechoic with the normal tissue. After the injection, the entire liver and tumor rim were enhanced, the entirety of the tumors was enhanced within 5 min. The signals did not decrease significantly in the tumor within 17 min. The H&E staining result confirmed that the tumor cells were of a large volume, and an irregular shape, exhibiting a hypertrophy of the nucleus and visible nuclear division.

3.3.3. In vivo fluorescence imaging

The fluorescence images in tumor-bearing nude mice were shown in Figure 4. After the injection of the DiR-SPIO-NBs, the tissue distribution of the fluorescence *in vivo* was obtained. At 10 min after injection, the signals were strongest, and continued to be for more than 11 h. The fluorescence intensity ex vivo at 11 h after injection showed that higher quantities of DiR accumulated in the liver, spleen, and tumor more so than in other organs.

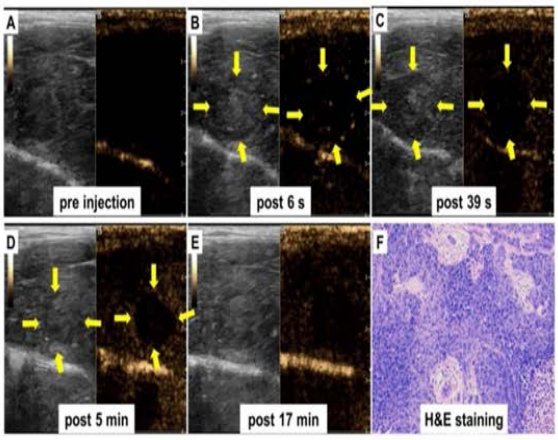

Fig. 3. Ultrasound images of rabbits liver bearing VX2 tumor before and after injection of DiR-SPIO-NBs. (A) - (E) Ultrasound images. (F) H&E staining of tumor cell (200 ×).

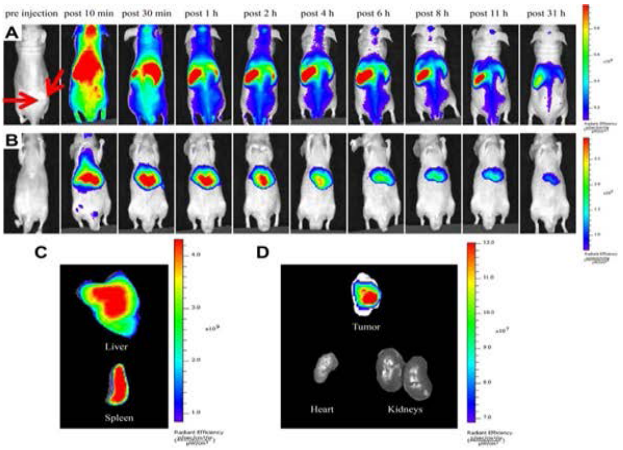

Fig. 4. Fluorescence imaging of nude mice bearing HepG2 tumor with injection of DiR-SPIO-NBs. (A) Dorsal views; (B) Ventral views; (C) Ex vivo images of liver and spleen; (D) Ex vivo images of tumor, heart and kidneys.

4. Conclusion

Uniform-sized and stable DiR and SPIO nanoparticles embedded in PEG-PLGA nanobubbles were successfully prepared with the premix membrane emulsification and solvent evaporation method. The results from the *in vitro* and *in vivo* MRI, ultrasound images, and fluorescence images revealed that the nanobubbles had strong, long-lasting tumor-targeting and imaging contrast enhanced abilities. In conclusion, the results suggest that nanobubbles could be utilized as an US/MRI /fluorescence contrast agent for diagnosing cancer.

Acknowledgment

This work was financially supported by National Science Foundation of China (NSFC, 31170960, 31470968) and National High Technology Research and Development Program of China (863 Program, 2012AA022704).

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