Preparation and characterization of PLA ultrasound contrast agents by combining an ultrasound method and a Shirasu Porous Glass (SPG) membrane emulsification technique.

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***Abstract*—The ultrasound contrast agent based on a poly lactic acid (PLA) was prepared by combining an ultrasound method and a Shirasu Porous Glass (SPG) membrane emulsification technique. An aqueous phase containing ammonium bicarbonate was used as the internal water phase (W_1), and PLA and Span 80 were dissolved in a solvent of dichloromethane (DCM), which was used as the oil phase (O). These two solutions were probe sonicated to form a W_1/O primary emulstion. The primary emulsion was permeated through the uniform pores (1.1 μm) of an SPG membrane into the external water phase (W_2) by the pressure of nitrogen gas to form the uiniform $W_1/O/W_2$ droplets. After DCM was evaporated, the hardened PLA microcapsules were further formulated into a lyophilized powder containing decafluorobutane gas. SEM image demonstrated that the PLA microcapsules were sphere-shaped and internally hollow with an average diameter ranging from 1.99 to 3.58 μm. In vitro, the PLA contrast agents showed high acoustic enhancement properties, the enhancement increased nonlinearly with dose, and the minimal loss (less than 5 dB) of signal was observed over 20 min of analysis at 37 ºC, the maximum acoustic enhancement was 45 dB, which significantly higher ($p<0.01$) compared to a value of 28 dB for those prepared by a conventional solvent evaporation method. In conclusion, the hollow PLA microcapsules prepared by the novel method have the characteristics desirable for an intravenously administered ultrasound contrast agents.**

Keywords-SPG membrane emulsification; Poly(lactide); Ultrasound contrast agent

I. INTRODUCTION

Ultrasound contrast agents (UCA) can provide enhanced scattering signatures to distinguish the acoustic scattering signatures of blood from the surrounding tissues [1, 2]. To become a viable UCA, certain properties are desirable [3, 4], these include being nontoxic, easy intravenously administration, small enough to traverse the capillaries, stable enough to provide enhancement over multiple cardiac cycles, and higher acoustically responsive. This property allows UCA to act as blood pool markers with a behavior similar to that of red blood cells.

Poly lactic acid (PLA) is a popular biodegrade-able and bioconsistent polymer that has been approved by the FDA for use in sutures, microcapsule, micobubble and others in intravenous injection. PLA can slowly be degraded by non-enzymatihydrolysis into lactic acids which is a natural body metabolite and can be degraded by enzymes to carbon dioxide and aqueous. Polymer (e.g PLA or PLGA)-based UCA showed more stability in vivo, high natural resonance frequency, and acquired ideal scattering intensity under high emitted frequency [5, 6]. Nevertheless, the polymer-made UCA has relatively hard shell, a few of big size of UCA may alter the blood flow and impede the capillary circulation.

From an acoustic perspective, the average size of UCA should be as large as possible, as larger microbubbles generate more intense ultrasound signals [7]. However, most of UCA should be no larger than the size of a red blood cell (7-8 μm in diameter). Several techniques, such as double emulsion combining with solvent evaporation, ultrasonication and spray drying are used to produce polymer-based UCA, the size distribution of UCA obtained is relatively broad, which is unfavorable for intravenously administration. Therefore, developing a method which can provide the uniform diameter of UCA composed of biodegradable polymers with better acoustic enhancement properties is highly appreciative.

The SPG membrane emulsification technique is a promising technique first proposed by Nakashima [8] to prepare uniform sized emulsion and later developed by Ma et al. [9, 10] to prepare uniform microspheres by polymerizing uniform monomer droplets. However, the SPG membrane emulsification technique has not been used to prepare PLA ultrasound contrast agents.

II. MATERIALS AND METHOD

A. Materials

PLA (Mw = 10,000) was obtained from the Institute of Medical Instrument (Shandong, China). Decafluorobutane (DFB) gas was provided by the Linggas. LTD (Beijing, China). Dichloromethane (DCM), polysorbate 80, ammonium

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bicarbonate, Poly(vinyl alcohol) (PVA, degree of polymerization 1700, degree of hydrolysis 88.5%) used as a stabilizer in the external water phase were purchased from Xiamen Chemical Reagents Company (Xiamen, China). All other reagents and solvents were of analytical grade.

B. Preparation of PLA ultrasound contrast agents

Fast mini Kit (KH-125) for emulsification was purchased from SPG Technology Co.,TLD. A tubular membrane with an average pore size of 1.1 μm was used. Preparation process was as follow: 5 mL of inner aqueous phase containing 0.125 g ammonium bicarbonate (w_1) was injected into 50 mL DCM oil phase in which was dissolved 1.25 g PLA10000 and 0.5 mL Span 80, then this solution was probe sonicated at 80 W for 10s and repeated 5 times to form a w_1/o primary emulsion. An aqueous phase (500 mL) containing 1% PVA (w/v) and 0.5% (w/v) SDS was used as the external water phase (w_2). The w_1/o emulsion was permeated through the uniform pores of an SPG membrane into the external water phase by the pressure of nitrogen gas to form the uniform-sized droplets. Then, DCM was evaporated at room temperature for 24 h under a gentle stirring at a rate of 200 rpm. After DCM was evaporated, the hardened PLA microcapsules were collected by centrifugation and washed with distilled water three times and the purified PLA microcapsules were further processed to form PLA ultrasound contrast agents by dispersing the microcapsules in aqueous vehicle followed by deaggregation, sieving, filling, freezing, and lyophilization. An aqueous vehicle was prepared containing mannitol (28 g/L water) and polysorbate 80 (0.82 g/L water). The purified PLA microcapsules were dispersed in the vehicle at a concentration of 19.6 mg/mL. The dispersion was deaggregated using a stainless steel blender at rate of 1000 rmp, sieved through disposable infusion apparatus. The sieved dispersion was filled into vials (10 mL fill in 20 mL vials), partially stoppered, and frozen in a -80 ºC freezer. Following freezing, the vials were lyophilized in vacuum refrigerant drier (Labconco Free Zone). At the end of lyophilization, the chamber was isolated, and DFB gas was backfilled into the vials prior to completely stoppering.

PLA contrast agents (the finished dosage form of vials containing PLA microcapsules, DFB, mannitol and polysorbate 80) were stored at 2-8 ºC. For reconstitution, 10 mL of sterile water was added into a vial of the agent, and the vial was shaken.

To compare the results, those obtained by an ultrasound method, the microcapsules were also prepared by the solvent evaporation method. The procedure for PLA microcapsules by the solvent evaporation method was similar to the process mentioned above except that the w_1/o emulsion droplets were poured into an aqueous phase (500 mL) containing 1% PVA and 0.5% SDS and homogenized at a rate of 1000 rpm.

To observe the cross-section of PLA microcapsule, the $w_1/o/w_2$ the double emulsion droplets yielded by the homogenizer were further stirred by a high shear mixer with whirling blades at rate of 10000 rpm.

C. Scanning electron microscopy (SEM) of PLA microcapsule

SEM was used to examine the surface morphology and cross sections of the microcapsules. The dried microcapsules were mounted on metal stubs with double-sided electrical tape. They were gold coated under reduced pressure with a sputter coater before being viewed under the SEM at 20 kV.

D. Determination of size distribution

Zetasizer (Nano-ZS, Malven) was used to measure the size distribution of PLA microcapsules.

E. In vitro acoustic test

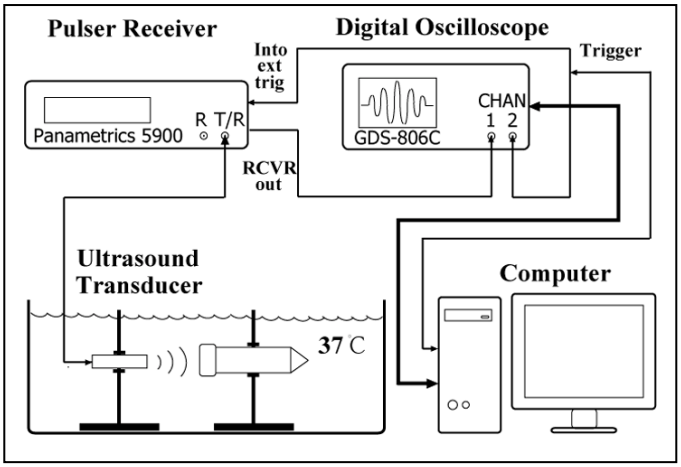

Figure 1. In vitro acoustic test setup

An in vitro acoustic test was performed by exposing a sample of the PLA contrast agents to a focused, pulsed ultrasound beam, using a system as shown in Fig.1. The time-response curves were constructed to measure the echogenicity of the microcapsules. A pulser-receiver shock-excited a focused ultrasound transducer (2.25 MHz, Panametrics, PR5800, Watertown, MA) sending a pulse of ultrasound into a suspension of microcapsules, which were contained in a cylindrical sample chamber (50 mL). The custom-made sample chamber was made from one of 50-mL plastic centrifuge tubes with open mouth sealed with polyester film and served as an acoustic window. The chamber was situated in a temperature controlled deionized water bath adjusted to 37ºC, and the transducer was submerged in the bath and focused through an acoustic window of the chamber. The backscattered signal was received by the same transducer, and the returned signal was then amplified by the pulser-receiver unit. The amplified signal was passed to a digital oscilloscope (GDS-806C, Instek, Taiwan, China) for digitization at 100 MSa/s. The operation of the digital oscilloscope and ultrasonic pulser-receiver were controlled and analyzed by a PC using Lab View (National Instruments Corp.,Austrin, TX). The digitized signal was

further processed using the method reported by J.A. Straub et al. [11]

The water tank was equipped with a stable mounting for the cylindrical sample chamber. The agents were diluted in different ratio (1:200, 1:500 and 1:1000) with phosphate buffered saline (PBS) pH 7.4, the concentration of agents evaluated as 0.098, 0.0392, 0.0196 mg/mL, respectively. In comparison, the agent prepared by un ultrasound method was diluted (1:200) with PBS, the concentration of the agent was 0.098 mg/mL.

The acoustic enhancement was measured immediately after the agents were diluted in different concentration, time was recorded as zero minutes, and the acoustic enhancement was measured at selected time points over 20 minutes. The time-response curve values are based on an average of three different sample preparations. The reference (PBS) was taken as an average of three values.

F. Statistical analysis

The results were expressed as mean ± S.E.M and Student's t-test were used to compare duration of enhancement and parameters derived from the time-intensity curves for three PLA contrast agents. Differences between agents were considered significant when $P < 0.05$.

III. RESULTS

A. Physical Characterization of PLA contrast agent

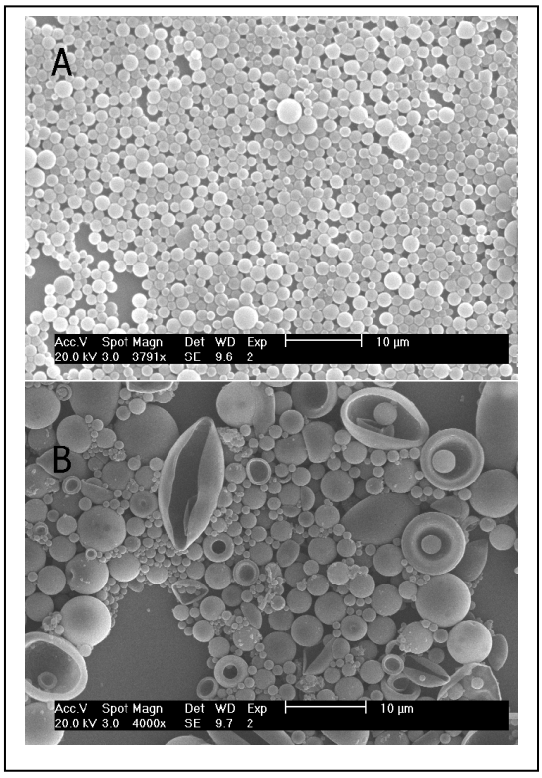

Figure 2. SME image of surface and cross-sections of representative PLA microcapsules

The SEM photographs of surface and cross section of PLA microcapsules are shown in Fig.2, which demonstrates that the microcapsules were essentially spherical in shape and reveal internally hollow microcapsules instead of internally porous microcapsules with multiple internal voids.

A particle size distribution of PLA contrast agents obtained by combining an ultrasound method and a SPG membrane emulsification technique ranged from 1.99 to 3.58 µm. While those prepared by the solvent evaporation method presented very broad size distribution and ranged from 955 nm to 6.44 µm.

B. Functional Characterization of PLA contrast agent

The relative acoustic backscatter data at 2.25 MHz in vitro are shown in Fig. 3. The PLA contrast agents display enhancements of up to 26 dB, 37 dB and 45 dB with dose of 0.0196, 0.0392, 0.098 mg/mL, respectively, the enhancement increased nonlinearly with dose increase. While the PLA contrast agents obtained by the ultrasound method had maximum acoustic enhancement of 28 dB, being significantly lower than that of PLA contrast agents prepared by the novel method at same dose of 0.098 mg/mL. The acoustic backscatter for PLA contrast agents at different of concentrations were all stable and minimal loss of signal (less than 5 dB) was observed over the 20 min duration of analysis at 37 ºC, much longer than would be needed for diagnostic imaging.

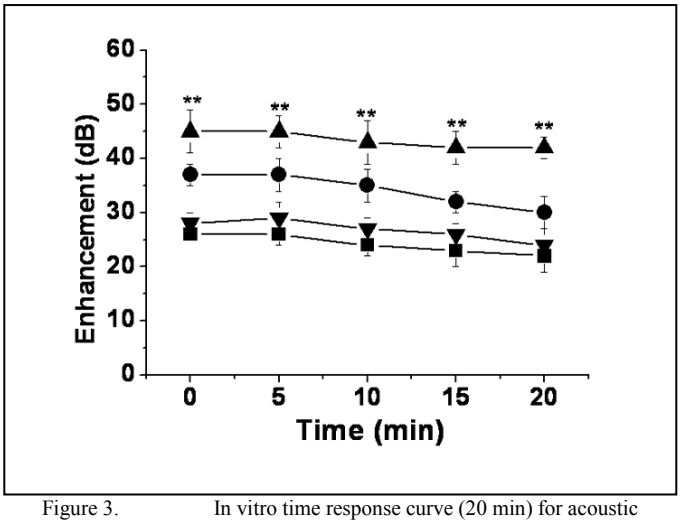

Figure 3. In vitro time response curve (20 min) for acoustic enhancement of different concentration of PLA contrast agents obtained by combining un ultrasound method and a SPG membrane emulsification technique: (■) 0.0196 mg/mL, (●) 0.0392 mg/mL, (▲) 0.098 mg/mL; PLA contrast agent prepared by un ultrasound method: (▼) 0.098 mg/mL, used as comparison agents, results are mean ± S.E.M. of three samples. **p< 0.01 Vs comparison agents

IV. DISCUSSION AND CONCLUSION

The most important to the function of an ultrasound contrast agent in vivo is the intensity of the backscattered

acoustic signal, which is used to create the ultrasound image. In vitro acoustic analysis of PLA contrast agents was performed at 2.25 MHz, as this is in the range of acoustic frequencies used for standard cardiac imaging. The results indicate that the PLA contrast agents obtained by the novel method showed the maximum acoustic enhancement of 45 dB, which significantly higher ($p < 0.01$) compared to a value of 28 dB for those prepared by the conventional method for same dose of suspending medium. As backscatter is reported in dB (a log-based scale), the data indicate that the integrated backscattered power is more than two orders of magnitude higher for PLA contrast agents than that obtained by the conventional method within the start of the analysis. This phenomenon may be due to the fact that higher uniform size of PLA microcapsules generates more intense ultrasound signals.

In conclusion, the hollow PLA microcapsules prepared by the novel method have the characterisitics desirable for an intravenously administered ultrasound contrast agents.

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