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Design of simvastatin-loaded polymeric microbubbles targeted to atherosclerotic plaque as ultrasound contrast agents for vascular imaging and drug delivery

Xiangyu Zhang,^a^ Kai Yue Zhao,^a^ Jun Wang,^b^ Shujie Bai,^a^ Shuqing Jiao,^a^ Jie Zhang,^a^ Lian Yu,^*^a

^a^College of Pharmacy, Jiamusi University, Jiamusi 154007, China
^b^College of Material Science and Chemical Engineering, Harbin Engineering University, Harbin 150001, China

**Abstract:** Targeting ultrasound contrast agents (UCAs) offer further opportunity to enhance the capabilities of diagnostic ultrasound imaging and controlled drug delivery to pathological tissue. In this study, Simvastatin (Sim) loaded polymeric microbubbles (MBs) as UCAs were developed and targeted to inflamed vascular endothelium to aid in the diagnosis and treatment of atherosclerosis. The polymeric shelled MBs were used to enable localized targeting on inflamed vascular endothelium by modifying the surface of these MBs with specific antibodies. The morphology, size distribution, chemical composition and antibodies conjugation efficiency of the MBs were evaluated. The gas-filled drug delivery platform described promises faster drug release, when subjected to ultrasound, compared to the drug release without ultrasound. In vivo ultrasound contrast imaging confirmed that these polymeric MBs could provide a stable acoustic enhancement and effectively identify atherosclerotic areas of plaque within the ventral aorta of rabbits. The results demonstrated that the elaboration of MBs has the potential to exhibit excellent ultrasound contrast-enhancing capabilities and provide a sufficient amount of drug to target disease sites.

**Keywords** ultrasound contrast agents, vascular imaging, targeted antibodies, drug delivery, atherosclerotic plaque
1. Introduction

The desire to combine simultaneous theranostics in the practice of medicine has led to the design of multifunctional materials with enhanced diagnostic and therapeutic functionalities.\textsuperscript{1-4} This integration of diagnostic imaging capability with therapy is important in meeting the challenge of disease specificity and adaptation. Polymeric microbubbles (MBs) as ultrasound contrast agents (UCAs) are ideal theranostic agents for improving the resolution and sensitivity of clinical US imaging.\textsuperscript{5-7} They consist of gaseous bubbles encapsulated within an outer shell, oscillating in an acoustic field, and effectively reflecting the ultrasound signal. Gas-filled UCAs have also attracted researchers to investigate ultrasound-triggered drug release, involving non-invasive imaging of most tissues at low concentrations and release of loaded therapeutic drugs with the pressure of an ultrasound wave.\textsuperscript{8-10}

Atherosclerosis, a chronic and highly variable vascular endothelium disease, could remain asymptomatic for decades before grave consequences emerge.\textsuperscript{11,12} Because endothelial dysfunction is associated with vascular disease progression, a technique that assesses the inflammatory status of the vascular endothelium could facilitate therapeutic monitoring of the disease activity of atherosclerosis. Nevertheless, current quantifiable methods to evaluate vascular endothelial function in vivo are limited to indirect assessment or invasive procedures. Moreover, the limitations of conventional inflammation therapy include a lack of drug specificity to the inflamed site and insufficient local drug concentration to target the inflammation. This results in poor control over drug release.

Ultrasound has long been utilized for vascular and cardiac imaging, and UCAs which acoustically activate MBs as red blood cell tracers, have been promoted to ultrasonically image vascular conditions.\textsuperscript{13-15} Recent reports have shown that drug-
loaded targeted UCAs adhere to dysfunctional vascular endothelium, permitting ultrasonic detection of inflamed blood vessels, and directly deliver sufficient anti-atherosclerotic drugs to the targeted disease site. Simvastatin (Sim) is a hypolipidemic drug used to control hypercholesterolemia, and then treat atherosclerosis. The beneficial effects of Sim on clinical events of vasomotor function, inflamed responses and plaque stability were confirmed. We found that targeted UCAs combined with ultrasound-triggered drug release technology might serve as a method to effectively diagnose and treat atherosclerosis.

The goal of this work is to design and develop Sim-loaded targeted UCAs with specific antibodies, based on polymeric MBs, to facilitate diagnosis and therapy of atherosclerosis. A gas-filled polymeric matrix, using biocompatible poly (lactic-co-glycolic acid) (PLGA), was prepared by an emulsion solvent evaporation process. Anti-ICAM-1 was chosen as cell surface proteins which can be adhesion to inflamed vascular endothelium. We expected that this targeted UCAs would facilitate site-specific recognition of atherosclerotic plaque and make an appropriate loading method for future drug delivery studies.

2. Experimental Section

2.1 Materials

Poly-D,L-lactide-co-glycolide (PLGA) (MW=10 KDa) with carboxyl end groups was supplied by Shan Dong Institution of Medical Instrument, China. Rabbit anti-ICAM-1/FITC was supplied by Biosynthesis Biotechnology Co., Ltd, China. N-hydroxysuccinimide (NHS), 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 2-morpholinoethanesulfonic acid (MES) were supplied by Aladdin Industrial Co., China. Lipophilic drug simvastatin was supplied by the General Pharmaceutical Factory of Harbin Pharmaceutical Group, China.
Dichloromethane (DCM) was supplied by Tianjin Regent, China. Polyvinyl alcohol (PVA) and other inorganic reagents were supplied by Tianjin Kermel Chemical Reagents Development Center, China. The New Zealand white rabbits were supplied by Affiliated Hospital of Jiamusi University, China. The ultrapure deionized water used in all experiments was purified by a Milli-Q Plus water purification system. All chemicals were of analytical grade and were utilized without further purification.

2.2 Preparation of Sim-loaded polymeric MBs

Sim-loaded polymeric MBs were fabricated using the emulsion solvent evaporation procedure based on a water-in-oil-in-water (W/O/W) emulsion method. First, PLGA (0.5g) was dissolved in 10 mL of DCM to create a homogenous solution and different amounts of Sim (drug: polymer, 40-100, wt %) were added to the polymer solution. After mutual saturation of the organic phase, 3 mL ammonium carbonate (4%, w/v) was added to the mixture. It was then sonicated at 20 kHz, using an ultrasonic cell disruption system (BILON96-II, Shanghai, China), in an ice bath, with 100W of applied power for 5 min at 3 s on and 1 s off, in nitrogen atmosphere. The obtained droplet phase W/O emulsion was then poured into 100 mL of 5% PVA solutions and ultrasonically homogenized for 5 min. After homogenization, the resulting (W/O/W) emulsion was added to 100 mL of 2% isopropyl alcohol. The solution was continuously stirred for 3 h at room temperature to evaporate the DCM. The precipitate was washed with deionized water and centrifuged twice. The dry powder sample of the MBs was collected by lyophilization for 48 h in order to analyze its further characteristics. MBs fabricated with different amounts of Sim were labeled S1, S2, S3 and S4 respectively. Finally, blank MBs were also prepared without Sim according to a process similar to the fabrication of Sim-loaded polymeric MBs.

2.3 Preparation of FITC-labeled targeted MBs
The FITC-labeled targeted MBs (S-MBs) were fabricated via the carbodiimide covalent method.\textsuperscript{22} Firstly, the MBs were dissolved in a MES buffer solution (0.1mol/L, pH 5.5). Secondly, EDC (0.1g) and NHS (0.2g) as coupling agents were added to the MES buffer solution to activate the carboxyl group of the S-MBs, and the mixture was magnetically stirred at 4°C for 30 min. Thirdly, the activated S-MBs were centrifuged with MES buffer solution (0.1mol/L, pH 5.5) to remove unreacted NHS and EDC, and then dissolved in a MES buffer solution (0.1mol/L, pH 8). Subsequently, Anti-ICAM-1/FITC was poured into the solution and the mixture was magnetically stirred, at 4°C, overnight. The excess antibodies from the solution were purified by centrifugal washing.

### 2.4 Determination of drug content and encapsulation efficiency

The amounts of Sim absorbed and encapsulated were determined by dissolving S-MBs in DCM and measuring fluorescence. An exact weighed amount of freeze-dried S-MBs (10mg) was introduced into a glass tube and dissolved in DCM (1 mL). Then, 10 mL of phosphate buffer solution (PBS pH 7.4) was added and the system was magnetically stirred for 2 h. After the evaporation of DCM, the system was centrifuged (6000 rpm for 10 min) to separate the aqueous solution. The Sim concentration in PBS was determined by UV/Vis spectroscopy at 242 nm from a standard calibration curve. Drug content and encapsulation efficiency were calculated using following equations, respectively. All analyses were performed out in triplicate.

\[
\text{Drug content} = \frac{\text{weight of encapsulated drug in MBs}}{\text{weight of MBs}} \times 100\%
\]

\[
\text{Encapsulation efficiency} = \frac{\text{weight of encapsulated drug in MBs}}{\text{weight of initial loading of drug}} \times 100\%
\]

### 2.5 Microbubbles characterization
Morphology of MBs was observed using a light microscope (LM, Leica DM, 4500P) and a scanning electron microscopy (SEM, HITACHIS-400, Japan). The average size and size distribution were determined by dynamic light scattering (DLS, NICOP 380ZLS). Ultraviolet-visible (UV-vis) absorption spectra of the product dispersed in ethanol were recorded on a Shimadzu UV-1601 ultraviolet-visible spectrophotometer.

2.6 Antibodies conjugation efficiency measurements

The conjugation efficiency of the antibodies on the surfaces of the S-MBs was determined by fluorescence measurement of FITC-labeled antibodies. The fluorescent images were captured with a cooling CCD (DP72, Olympus, Tokyo, Japan) equipped with an inverted microscope (IX71, Olympus). The conjugation efficiency of the antibodies during each flow cytometry test (FACScan, BD Biosciences, CA. USA) was approximately $10^6$ MBs/ mL, which was determined using a hemocytometer. The flow chamber was placed in an inverted position on a microscope (Axioskop2-FS, Carl Zeiss Inc, Thornburg, NY).

2.7 Determination of ultrasound-triggered drug release

Ultrasound-triggered drug release was measured for each sample as follows: the S-MBs (100mg) were immersed with 5mL PBS in the dialysis tube (MWCO 3k) which allowed the micromolecules to pass through while holding back the macromolecules (molecular weight $>$3000), 95mL PBS was added and stirred into the 500mL beaker and the immersing temperature was kept at 37°C. After immediate sampling, the solution was insonated using a pulse repetition frequency of 40 Hz and peak positive pressure amplitude of 450W. At a given time, 3mL of the solution was removed for measurement of released Sim into the solution. An equal volume of PBS was immediately added to keep the volume constant. Immediately after measuring, samples were centrifuged at 7500 g for 5 min to remove the polymer and the
fluorescence of the supernatant was read. The Sim concentration in PBS was determined by UV/Vis spectroscopy (at 242 nm) from a standard calibration curve, and expressed as a percent of the total. After 30 min of continuous insonation, in vitro, Sim release enhancement became undetectable, indicating complete S-MBs destruction. Moreover, the drug release study of S-MBs exposed to ultrasound and uninsonated controls was examined in vitro. The experiment was performed according to the before-mentioned procedure with no insonation for 600 min.

2.8 In vivo acoustic imaging
To evaluate ultrasound imaging and the acoustic behavior of the S-MBs, in vivo ventral aorta imaging was performed in a fat plaque model implanted in rabbits. Each rabbit was anesthetized with an injection of 3% pentobarbital sodium through the marginal ear vein. The rabbits were placed on a warm blanket to keep body temperature within normal range. Pulsed Doppler US imaging of the ventral aorta was performed using a Color Doppler Ultrasound Diagnostic System (iU22, Philips Ultrasound Inc, USA). The MBs were intravenously injected into the left ear vein at a concentration of 0.1 mL kg$^{-1}$ through a catheter.

3 Results and discussion
3.1 The mechanism of the fabrication of S-MBs
S-MBs were built on an emulsion solvent evaporation method. Fig. 1 explains a schematic process for the fabrication of S-MBs. First, the PLGA/DCM/Sim mixture was emulsified into a nonsolvent phase (PVA) solution. After ultrasound emulsification, a gas-filled droplet structure containing Sim was formed to facilitate the efficacy of high-energy ultrasound. The DCM was evaporated from the polymer droplets with the lipophilic Sim sitting in the shell of the polymer droplet. As polymeric droplets became concentrated and solidified into polymeric MBs, carboxyl
groups on the surface of the MBs were activated by EDC/NHS. Carboxyl groups activated by EDC/NHS and amino groups on the ligands were then conjoined by covalent linkage. Ultimately, S-MBs were formed as a result of this aforementioned procedure.

**Fig. 1** Schematic process for the fabrication of S-MBs.

### 3.2 Drug content and encapsulation efficiency

Drug content and encapsulation efficiency are significant indices for drug delivery systems. Lipophilic Sim of scanty water-solubility was dispersed in the DCM solution by stirring and ultrasonication, resulting in most of the Sim that encapsulated and unencapsulated in the MBs being discharged into the water. Reasonable loading and encapsulation efficiency were achieved using PBS solution (pH = 7.4) via the UV–VIS method. Each experiment was performed in triplicate. Tab. 1 illustrates the results obtained using different Sim to polymer percentages (40, 60, 80, 100, wt %). Sim content was increased with the increase of the Sim-polymer ratio; however, the Sim content of S3 was closed to S4 when the Sim-polymer ratio was increased. With the increase in Sim concentration more Sim molecules diffused into the aqueous phase during emulsification. Therefore, fewer Sim molecules remained in the emulsified droplets to interact with polymer molecules so that only a rare increase in the Sim content occurred. This is consistent with existing reports in the literature. Furthermore, Sim encapsulation efficiency increased with the increase
in drug concentration, with the exception of S4. Thus, S3 was ultimately chosen for its high Sim content and encapsulation efficiency in biomedical application for upcoming experiments.

**Tab. 1** Drug content and encapsulation efficiency of S-MBs

<table>
<thead>
<tr>
<th>Entry</th>
<th>Sim to polymer (weight ratio)</th>
<th>Drug content (%)</th>
<th>Encapsulation efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-1</td>
<td>40</td>
<td>10.6±0.3</td>
<td>21.7±2.9</td>
</tr>
<tr>
<td>S-2</td>
<td>60</td>
<td>17.1±0.1</td>
<td>24.3±4.1</td>
</tr>
<tr>
<td>S-3</td>
<td>80</td>
<td>20.5±0.4</td>
<td>25.6±4.3</td>
</tr>
<tr>
<td>S-4</td>
<td>100</td>
<td>21.3±0.3</td>
<td>21.3±3.2</td>
</tr>
</tbody>
</table>

### 3.3 Morphology and size distribution characterization

SEM is a powerful tool to identify size and surface morphology of the polymeric MBs. The blank MBs exhibited well-defined spherical shapes and smooth surfaces, and the particle sizes were not very uniform with an average diameter range from hundreds of nanometres to several microns (Fig. 2a). The surface morphology of S-MBs (S3), as identified by SEM measurement (Fig. 2b and Fig. 2c) presented smooth-surfaced, spherical-shaped particles, with a narrow size distribution, not significantly different from blank MBs. Currently, UCAs must be smaller than 7 μm in diameter in order to get through the pulmonary capillaries and produce the systemic enhancement. The SEM images analysed with Image Pro Plus indicate that the estimated mean diameter of S-MBs was about 4.8 μm. Moreover, the average size of S-MBs is 4.3±1.2 μm, as measured by the DLS technique (Fig. 2d), is in accordance with the data obtained by Image Pro Plus. The S-MBs with this unique size range meet the requirement as a UCA for clinical application.
3.4 Ultrasound-triggered drug releases in vitro

Apart from acting as UCAs to improve the quality of ultrasound imaging, one of the notable features of the gas-filled MBs for clinical use is its application as an ultrasound-triggered drug delivery system. As a poor solubility drug, the ultrasound-triggered release of Sim can enable sufficient drug to concentrate on a specific area.\textsuperscript{27,28} The results are presented in Fig. 3. After an initial burst upon introduction into the Sim release medium (within 5 min) attended by a gradual decrease in release rate over a measured period, the cumulative Sim release was 55% of all samples. The preliminary release stage was more likely due to the rapid release of Sim molecules on the surface and near the exterior surface during S-MBs destruction.\textsuperscript{29} Sim was still released from S-MBs at a fast rate for 30 min, after which the release rate was
minimal. This may be due to the faster diffusion of Sim molecules in the interior core of S-MBs during the application of ultrasonic waves, resulting in polymer fragments as well as accelerated polymer degradation. The release of Sim, over the course of 30 min, observed in this study demonstrates that the Sim-loaded MBs are capable of providing a fast release of drug with comparable release profiles as reported in other papers.

Fig. 3 In vitro drug release profiles of S-MBs when exposed to ultrasound.

The release profiles of S-MBs exposed to ultrasound and uninsonated controls were examined in vitro (Fig. 4). Both S-MBs exposed to ultrasound and the uninsonated controls revealed an immediate burst of roughly 50% of the total encapsulated Sim. Nevertheless, 44% of the Sim were released in 2 min when exposed to ultrasound control compared to 12% released in 2 min without ultrasound. Therefore, the application of ultrasonification can effectively enhance Sim release from MBs during ultrasound imaging procedures, and the Sim continued to
release at a fast rate for 30 min. The release rate was initially faster and became slower as time passed; it ultimately reached 71.2% within 200 min without ultrasonification.

![Drug Release Profiles](image)

**Fig. 4** In vitro drug release profiles of S-MBs when exposed to ultrasound and uninsonated controls

Residual encapsulated Sim would eventually be released in the decomposition of the S-MBs. As a result, the release from S-MBs triggered with ultrasound was significantly faster than the uninsonated controls, having released a sufficient amount of drug during ultrasound contrast imaging procedures.

### 3.5 Antibodies conjugation efficiency measurements

Antibodies conjugation efficiency was evaluated on the S-MBs surface by fluorescence microscopy. Fig. 5a and Fig. 5b present the images of the FITC-labeled S-MBs acquired in both fluorescent mode and bright-field mode (×400). The S-MBs
clearly exhibited yellow rings and bright rings, and the intense fluorescence emitting from the outer surface can become quite clearly in almost every individual MB. This observation indicates successful deposition of targeted antibodies onto the outer surface of MBs.\textsuperscript{34}

\textbf{Fig. 5} Optical images of FITC-labeled S-MBs. (a) Fluorescence mode (×400), (b) Bright field mode (×400), (c) Fluorescence mode (×200), (d) Particle size distribution of S-MBs.

Fluorescence microscopy images of S-MBs (×200) are observed in Fig. 5c with yellow rings or filled circles, which further confirm the presence of antibodies on the surface of the S-MBs. Prepared MBs to present a wide size distribution of 4.9±1.4 μm, as shown in Fig. 5d, indicating that they meet the requirement for clinical application. Flow cytometry results demonstrated that the antibodies conjugation efficiency of S-MBs is 42.71%, compared to 0.39% for the blank MBs, as shown in
Fig. 6. The conjugation efficiency further confirmed that the targeted S-MBs can provide specific recognition of the targeted inflamed plaque.

3.6 In vivo imaging of S-MBs

In order to evaluate the acoustic behavior of the S-MBs and to identify rabbit abdominal aorta atherosclerotic plaque, in vivo imaging was performed in power Doppler mode using prepared samples. Examples of rabbit ventral aorta images acquired in power Doppler mode, before and after intravenous injection of S-MBs are described in Fig. 7. The ultrasound image with no S-MBs injected appears black due to a lack of bubbles (Fig. 7a). Additionally, two spongy areas of plaque are noted at the vascular wall of the ventral aorta of the rabbit (see white arrow). In Fig. 7b, it is observed that, when a suspension of S-MBs is injected into the rabbits. Clear power Doppler enhancement occurs immediately, revealing a dynamic blood flow in the
ventral aorta. In vivo PDI enhancement was observed with a stable acoustic enhancement of nearly 2 min, indicating that the S-MBs are durable enough for ultrasound contrast-enhanced and ultrasound-triggered drug release in clinical application.\textsuperscript{9,10,15}

![Image](image.png)

**Fig. 7** Ultrasound images of the atherosclerotic plaque in the rabbit abdominal aorta. (a) No S-MBs injection, (b) S-MBs injection.

Moreover, ultrasound contrast imaging with the S-MBs provides stronger echo intensity of atherosclerotic plaque (see white arrow) opposed to the routine ultrasound imaging, indicating that the S-MBs, which served as UCAs enhance the echoes of the intima and plaque. Therefore, areas of plaque under targeted ultrasound contrast imaging are easy to identify.\textsuperscript{34} This dynamic feature is a prerequisite for theranostic use of targeted UCAs, and can allow a more localized and, specific drug delivery.

### 4. Conclusion

In this paper, simvastatin-loaded polymeric microbubbles as targeted ultrasound contrast agents were synthesized by an emulsion solvent evaporation process to evaluate atherosclerosis. Lipophilic drug simvastatin was encapsulated in the microbubbles of well-defined average size and of narrow-size distribution.
Simvastatin content was increased with the increase in simvastatin-polymer ratio; high drug content and encapsulation efficiency were acquired when the simvastatin-polymer ratio was 80 wt%. Release profiles showed that these microbubbles were significantly faster in the release of simvastatin when triggered with continuous ultrasound waves compared to those without ultrasonification. Therefore the application of ultrasonification can effectively enhances drug release from the microbubbles during ultrasound contrast imaging procedures. Furthermore, the polymeric microbubbles, which served as targeted ultrasound contrast agents, presented a stable acoustic contrast enhancement and could assist in more effective identification of atherosclerotic plaque in vivo. We hope these results might point to a multifunctional platform for the development of more robust diagnostic and therapeutic tools to address the complications of numerous other diseases. Additional studies will focus on the release and uptake of the microbubbles which are tested in an in vivo cellular model.

Acknowledgement

This work received the support of the Scientific and Technical Research Project of Education Department of Heilongjiang Province (12521540).

References


Simvastatin-loaded polymeric microbubbles were synthesized as targeted ultrasound contrast agents and ultrasound-triggered drug carriers.