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Preparation and in vivo evaluation of ligand-conjugated polymeric microbubbles as targeted ultrasound contrast agents

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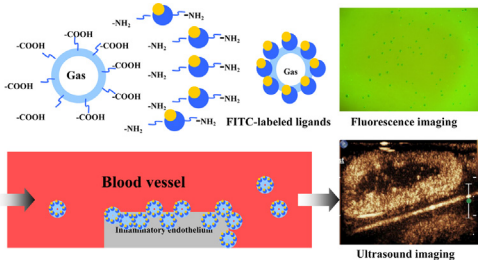
HIGHLIGHTS

- The polymeric microbubbles show narrow average size and size distribution.
- The carboxyl groups on the polymeric microbubbles surface were activated by EDC/NHS.
- The microbubbles can locate on inflamed plaque by modifying the surface with ligand.
- The high conjugation efficiency makes the microbubbles use in clinical applications.
- The targeted microbubbles have a significant ultrasound contrast-enhanced property.

GRAPHICAL ABSTRACT



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ABSTRACT

Targeted ultrasound contrast agents (UCAs) offer new opportunities to enhance the capabilities of diagnostic ultrasound imaging to detect pathological tissue. Poly (D, L-lactic-co-glycolic acid) (PLGA) UCAs based on microbubbles (MBs) were developed to target inflamed plaque, to aid in the diagnosis of atherosclerosis. A conjugation strategy was adopted using PLGA shelled MBs to enable localized targeting on atherosclerotic plaque by modifying the surface of these polymeric MBs with anti-ICAM-1. In addition, several investigations were carried out to determine the morphology and performance of the prepared MBs, including surface morphology, size distribution and conjugation efficiency analysis. Fluorescence microscopy and flow cytometry analysis showed a specific adhesion and high conjugation efficiency of targeting ligands to MBs. Furthermore, the conjugated MBs were applied in vivo ultrasound targeted imaging and the results demonstrated the availability of conjugated MBs as a highly efficient ultrasound system in targeted ultrasound imaging and in noninvasive biological research.

1. Introduction

The formulation of new devices to be used as contrast agents with enhanced diagnosis and therapeutic functionalities is a promising area of research in biomedicine [1–3]. Ultrasonography

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is a widely available, low cost and highly safe, clinical imaging technique. Nevertheless, differentiating tissues of diagnostic importance is often hampered by similar levels of echogenicity; therefore, ultrasound contrast agents (UCAs) or microbubbles (MBs) are used to improve the visualization of specific tissues [4–7]. The MBs consist of a gas surrounded by a polymeric shell, with a typical diameter of 1–8 μm. When intravenously injected into the bloodstream, the MBs have the capability to alter the acoustic properties of the tissues which result in higher echogenicity due to the high compressibility and low density of the gas [8–10].

The next-generation of contrast agents for drug delivery will focus on active targeting delivery [11,12]. Gaio Paradossi's group reported that modifying the surface of polymeric UCAs with oxidized hyaluronic acid could be used in targeted localization [13]. The same group developed targeted UCAs via induced platelet adhesion with nitric oxide release to control arterial thrombosis [14]. As a result, the strategy usually requires an integrated approach for targeting MBs with surfaces that have been covered with cellular epitopes or ligand [15–17]. Furthermore, UCAs, targeted with ligands, recognize disease antigens and bind to specific biomarkers on receptor cells, thus enhancing the ability to distinguish diseased tissue from healthy tissue [18–20].

Atherosclerosis is a chronic and highly variable inflammatory endothelium disease involving the evolution of vulnerable inflamed plaque, often progressing silently for decades before becoming clinically evident [21,22]. The atherosclerotic plaque is characterized by cell surface proteins such as leukocyte adhesion molecules [23]. Sasha has discussed the ability of liposome UCAs to couple with ICAM-1 atherosclerotic plaques in vivo for ultrasound image enhancement [24]. Wagner reported that MBs targeted to ICAM-1 demonstrated greater adhesion strength than MBs targeted to other inflamed marker [9]. It is confirmed that the ultrasound detection of UCAs with specific ligands provides a means to noninvasively diagnose and treat the atherosclerotic plaque. Therefore, the conjugation of the surface of UCAs with ICAM-1 is envisaged for the targeting of atherosclerotic plaque in our study.

We design and develop UCAs based on polymeric MBs for targeting of inflamed plaque in the vascular system which will assist in the diagnosis and, hopefully, therapy of atherosclerosis. A gas-filled polymeric matrix, using biocompatible poly D,L-lactic-co-glycolic acid (PLGA), was prepared by using a double emulsion-solvent evaporation process. The conjugation efficiency and ultrasound imaging experiments demonstrate that conjugated MBs, used as targeted UCAs, have high conjugation efficiency and effectively identify atherosclerotic plaques in vivo. The elaboration of MBs has potential for improved targeting and stability, which can improve echogenic properties in the presence of diagnostic ultrasound, thus having widespread biomedical application.

2. Materials and methods

2.1. Materials

Poly-D,L-lactide-co-glycolide (PLGA) (lactide:glycolide ratio 50:50, MW = 10 kDa) with hydroxy end groups was provided by Daigang Bioengineering Co., Ltd, China. Rabbit anti-ICAM-1/FITC was provided by Biosynthesis Biotechnology Co., Ltd, China. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC),2-morpholinoethanesulfonic acid (MES) and N-hydroxysuccinimide (NHS) were provided by Aladdin Industrial Co., China. Dichloromethane (DCM) was provided by Tianjin Rgent, China. Polyvinyl alcohol (PVA) and other inorganic reagents were provided by Tianjin Kermel Chemical Reagents Development Center. New Zealand white rabbits were provided by Fourth Affiliated Hospital, Harbin Medical 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 used without further purification.

2.2. Preparation of PLGA MBs

PLGA MBs were fabricated using the modified solvent evaporation procedure based on the formation of water-in-oil-in-water (W/O/W) emulsion [9]. A surfactant is required to stabilize the dispersed phase, and PVA is one of the most commonly used polymeric surfactants. PLGA (0.5 g) was dissolved in 20 mL of DCM. After mutual saturation of the organic phase, the mixture was sonicated at 20 kHz using ultrasonic cell disrupter system (BILON96-II, Shanghai, China) in an ice bath with 100 W of applied power for 5 min at 3 s on and 1 s off in nitrogen atmosphere. The obtained W/O emulsion was then poured into 100 mL of (5%PVA) solution and ultrasonic 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 to analyze other characteristics.

2.3. Preparation of FITC-labeled targeted MBs

The FITC-labeled targeted MBs were fabricated via the carbodiimide covalent method [17]. Firstly, the PLGA MBs were dissolved in an MES buffer solution (0.1 mol/L, pH 5.5). Secondly, EDC (0.1 g) and NHS (0.2 g) as coupling agents were added to the MES buffer solution to activate the carboxyl group of the MBs, and the mixture was magnetically stirred at 4 °C for 30 min. Thirdly, the activated MBs were centrifugally rinsed with MES buffer solution (0.1 mol/L, pH 5.5) to remove unreacted NHS and EDC, and then dissolved in an MES buffer solution (0.1 mol/L, pH 8). Subsequently, Anti-ICAM-1/FITC was poured into the solution and the mixture was magnetically stirred at 4 °C overnight.

2.4. Microbubbles characterization

The morphology (shape and surface characteristics) of MBs was observed using a light microscope (LM, 165, Leica DM, 4500P) and a scanning electron microscopy (SEM, JEOL JSM-6480A microscope Tokyo, Japan). Samples were sputter-coated with gold using a vacuum evaporator (Edwards, Milano, Italy) and examined at 5 kV accelerating voltage. The average size and size distribution were determined by dynamic light scattering (DLS, NICOP 380ZLS).

2.5. Conjugation efficiency measurement

The conjugation efficiency of the ligands on the MBs surface was determined by fluorescence measurement of FITC-labeled ligands. Because of rapid screening of the conjugated MBs, the fluorescent images were captured with a cooling CCD (DP72, Olympus, Tokyo, Japan) equipped with an inverted microscope (IX71, Olympus). To demonstrate the targeting capabilities of our FITC-labeled MBs, the fluorescent conjugated MBs and blank MBs were added to the saline solutions, which were then subjected to flow cytometry analysis. The conjugation efficiency of the ligands during each flow cytometry test (FACScan, BD Biosciences, CA, USA) was approximately 10^6 cells/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).