Acta Biomaterialia 6 (2010) 3542–3549

Contents lists available at ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

Novel ultrasound contrast agent based on microbubbles generated from surfactant mixtures of Span 60 and polyoxyethylene 40 stearate

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ARTICLE INFO

Article history:
Received 21 August 2009
Received in revised form 15 January 2010
Accepted 3 March 2010
Available online 10 March 2010

Keywords:
Microbubbles
Monolayer
π–A isotherms
Ultrasound contrast agent

ABSTRACT

In this study, novel perfluorocarbon-filled microbubbles as ultrasound contrast agent were fabricated using ultrasonication of a surfactant mixture of sorbitan monostearate (Span 60) and polyoxyethylene 40 stearate (PEG40S) in aqueous media. The microbubbles generated from a 1:9 mixture of PEG40S/Span 60 exhibited an average diameter of 2.08 ± 1.27 μm. More than 99% of the microbubbles had a mean particle diameter less than 8 μm, indicating that they were appropriately sized for intravenous administration as ultrasound contrast agent. The stabilization mechanism of the microbubbles was investigated by the Langmuir–Blodgett technique including the measurements of surface pressure–area (π–A) isotherms and compression–decompression cycles with a two-dimensional monolayer of Span 60 and PEG40S. The dependence on molar fraction of PEG40S in π–A isotherms of mixed monolayers provided a strong evidence of interactions between the two microbubble-forming materials. It is suggested that the monolayer shell imparts good stability to the microbubbles by three means: (1) a low surface tension monolayer hinders dissolution through the reduction of surface tension, which introduces a mechanical surface pressure that counters the Laplace pressure; (2) the presence of a monolayer shell imparts a significant barrier to gas escaping from the core into the aqueous medium; and (3) encapsulation elasticity stabilizes microbubbles against diffusion-driven dissolution and explains the long shelf-life of microbubble contrast agent. The preliminary in vivo ultrasound imaging study showed that such stabilized microbubbles demonstrated excellent enhancement under grey-scale pulse inversion harmonic imaging and power Doppler imaging.

1. Introduction

Ultrasound contrast agents (UCAs) have great potential to improve the diagnostic capabilities of ultrasound imaging [1–6]. Enhancement of Doppler signals from small and/or deep-lying vessels, as well as increases in the difference in echo texture between normal and adjacent abnormal tissue in organs such as the liver and spleen, are feasible with UCAs [7,8]. Apart from their diagnostic utility, contrast microbubbles have also gained notable attention from the medical community due to their excellent potential as ultrasound-facilitated drug and gene delivery vehicles [9–14] and metabolic gas transport [15–17]. Therefore, the development of new UCAs has become one of the most promising fields in ultrasound medicine.

The ideal UCAs should be nontoxic, injectable intravenously, capable of traversing the pulmonary, cardiac and capillary circulations as well as stable for recirculation [18]. A typical microbubble is composed of an inner gaseous core that is coated with a thin shell. Most microbubbles are stabilized against dissolution and coalescence by the presence of additional materials at the gas–liquid interface. Proposed shell materials include protein [19–21], surfactant [22,23], polymer [24,25], and lipid [26,27] and they are used to form a protective shell layer to prevent gas from escaping from the core as well as to avoid the microbubbles' coalescence. Lipids [11,26] and surfactants [22,23] are currently used as the main materials to make bubbles because they have the property of lowering the surface tension, and are able to stabilize microbubbles by forming a coating layer. It has been demonstrated that the emulsification and sonication of mixtures of two non-ionic surfactants or lipids can produce stable microbubbles for ultrasound contrast agent. Amphiphilic molecules can self-assemble into a monolayer shell at the interface between the gas core and surrounding medium, allowing better control of the shell surface architecture and greater flexibility [28].

Both Span 60 and PEG40S are known to be biocompatible, degradable, and nontoxic non-ionic surfactants that are widely used in food products and pharmaceuticals [29,30]. Their toxicity

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doi:10.1016/j.actbio.2010.03.007

and safety have been extensively studied. In short-term and long-term studies with mouse, rat, hamster, dog, monkey, and human, both of them exhibited extremely low acute and chronic toxicities, no reproductive and developmental toxicity, genotoxicity, and carcinogenicity [31–37]. Herein, the commercially available surfactant of Span 60 was used as the main material to fabricate novel microbubble-based ultrasound contrast agent while PEG40S was introduced to enhance the stability of microbubbles [38,39]. An additional advantage using Span 60 and PEG40S in humans could be their non-mammalian source, which eliminates the risk of contamination with pathogenic agents.

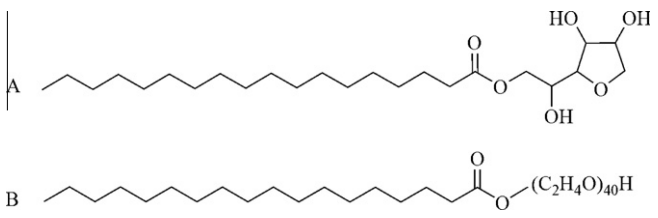
Thus far, only a few studies have been performed to develop the relationships among composition, structure and property for microbubbles in comparison to the vast majority of the clinical research with gas microbubbles [39,40]. From the viewpoint of microbubble structure, the stability of microbubbles depends on their physiochemical properties, especially the molecular interactions between the constituents in the monolayer structures. Since a monolayer at the gas–liquid interface is similar in some respects to the gas–liquid interface in the microbubbles, the study of mixed monolayer behaviors of microbubble components by the Langmuir–Blodgett (LB) technique may provide important information on intermolecular interactions in an oriented system that result in stable contrast agent [39,40]. Wheatley et al. developed a series of surfactant-stabilized microbubbles for use in diagnostic ultrasound by employing Span-type and Tween-type surfactants. The stability of the microbubbles was investigated by using a Langmuir trough to measure the π–A isotherms of the surfactant monolayer [41,42].

In this study, stable microbubbles were fabricated by mixing Span 60 and PEG40S with the solvent to form a mixture and then sonicating to generate microbubbles by cavitation from perfluorocarbon (PFC) in the mixture. In order to get information on the interactions between the two microbubble-forming materials in a two-dimensional monolayer, π–A isotherms for mixtures of Span 60/PEG40S were investigated using a Langmuir trough at different molar ratios. This should not only provide an explanation for the stability of Span 60/PEG40S combinations but also help in the development of optimal microbubble contrast agents for diagnostic ultrasound. To characterize the acoustic property of this microbubble agent, power Doppler imaging (PDI) and grey-scale pulse inversion harmonic imaging (PIHI) were used to demonstrate the efficacy of the enhancement in animal model.

2. Materials and methods

2.1. Materials

Sorbitan monostearate (Span 60) and emulsifier polyoxyethylene 40 stearate (PEG40S) were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and used without further purification. The chemical structures were given in Scheme 1. Perfluoropropane was purchased from Akonic S.A. Inc. and used as filling gas for microbubbles. Chloroform (HPLC grade) was purchased from Shandong Yuwang Industry Co. Ltd. and used as the spreading solvent for preparing the monolayers at the air/aqueous interface. All other chemicals used in this work were of analytical grade. The ultrapure deionized water used in all experiments was purified by a Milli-Q Plus water purification system with a resistivity of 18.2 MΩ cm. The PBS (phosphate buffer saline, pH 7.4) buffer solution was prepared by mixing 8.01 g of sodium chloride, 0.194 g of potassium chloride, 2.290 g of disodium hydrogen phosphate, and 0.191 g of potassium phosphate monobasic and then adding deionized water to 1 l and was filtered through a 0.22 μm filter prior to use.



Scheme 1. Chemical structures of two surfactants: (A) Span 60 and (B) PEG40S.

2.2. Preparation of microbubbles

PFC-filled microbubbles coated with a saturated surfactant (Span 60) and an emulsifier (PEG40S) were formed through high-power tip sonification of a vesicle/micelle aqueous solution (Fig. 1). Briefly, a suspension of Span 60 (900 mg) plus PEG40S (475 mg) and NaCl (900 mg) was prepared by dissolving the materials in powder form with 30 ml of phosphate buffer saline (PBS, pH 7.4) in a glass vial. The solution was stirred at room temperature for 10 min to ensure sufficient mixing. The mixture was then autoclaved (MLS-3780, SANYO Electric Co. Ltd., Japan) using a liquid cycle for 15 min at 120 °C. After cooling to room temperature the solution was sonicated with a probe-type (0.5″) sonicator (Sonicator 4000, Misonix Inc., USA) at the air–water interface continuously at the maximum amplitude with constant purging of PFC gas in an ice bath for 3 min. After sonication, the produced milky polydisperse suspension was transferred into a 250 ml separatory funnel, and was left to stand for 60 min. The bubbles self-segregated by flotation and stratified by size, with three distinct layers being observed: a top foam layer with large bubbles, a middle opaque layer of microbubbles, and a lower layer containing residual surfactants aggregates that failed to form microbubble shells. The microbubbles were separated off and washed three times with PBS (60 ml) buffer solution at 60 min intervals. The prepared microbubbles were dispersed in PBS buffer solution and stored under a headspace filled with the filling gas.

2.3. Physical characterization

Optical observations of microbubbles were carried out with a Leica DM 4500P microscope attached to a Leica DFC 420 digital camera.

The size distribution and concentration of the surfactant-coated microbubbles were determined using a Coulter Multisizer III (Coulter Electronics Ltd., Luton, Bedfordshire, UK), with a 30 μm aperture, a 100 μl manometer setting and aperture current of 1600 μA on diluted samples in Isoton solution. The sample was normalized to a final concentration of 1.0×10^9 bubbles ml^{-1} (using PBS) for in vivo acoustic test. Just prior to use, the glass

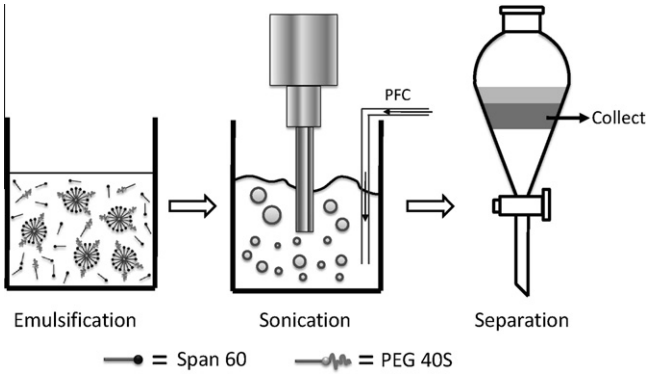


Fig. 1. Schematic illustration of the formation of microbubbles using Span 60 and PEG40S.

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