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Microbubble size isolation by differential centrifugation

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ABSTRACT

Microbubbles used as contrast agents for ultrasound imaging, vectors for targeted drug delivery and vehicles for metabolic gas transport require better size control for improved performance. Mechanical agitation is the only method currently available to produce microbubbles in sufficient yields for biomedical applications, but the emulsions tend to be polydisperse. Herein, we describe a study to generate lipid-coated, perfluorobutane-filled microbubbles and isolate their size fractions based on migration in a centrifugal field. Polydispersity of the freshly sonicated suspension was characterized by particle sizing and counting through light obscuration/scattering and electrical impedance sensing, fluorescence and bright-field microscopy and flow cytometry. We found that the size distribution was multimodal. Smaller microbubbles were more abundant. Differential centrifugation was used to successfully isolate the 1–2 and 4–5 μm diameter fractions. Isolated microbubbles were stable over two days. After two weeks, however, more dilute suspensions (<1 vol%) were susceptible to Ostwald ripening. For example, 4–5 μm microbubbles disintegrated into 1–2 μm microbubbles. This latter observation indicated the existence of an optimally stable diameter in the 1–2 μm range for these lipid-coated microbubbles. Overall, differential centrifugation provided a rapid and robust means for size selection and reduced polydispersity of lipid-coated microbubbles.

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1. Introduction

Microbubbles are being employed for several biomedical applications, including contrast enhanced ultrasound [1,2], drug and gene delivery [3,4] and metabolic gas delivery [5,6]. Microbubbles react strongly to ultrasonic pressure waves by virtue of their compressible gas cores, which resonate at the MHz-frequencies used by current clinical scanners. Oscillation of the gas core allows re-radiation (backscatter) of ultrasound energy to the transducer at harmonic frequencies and nonlinear modes, thus providing exquisite sensitivity in detection with current contrast-enhanced pulse sequences and signal processing algorithms. Additionally, microbubbles may cavitate stably or inertially to facilitate drug release [7,8] and extravascular delivery [9,10] within the transducer focus.

Current commercially available microbubble formulations are polydisperse in size. In most cases, the size distribution is broad over a range of submicrometer to tens of micrometer in diameter. This is problematic because microbubble behavior depends very strongly on size. For example, increasing the microbubble diameter from 1 to 5 μm will change the resonance frequency of an unencapsulated microbubble from 4.7 to 0.72 MHz [11]. Microbubble size also affects the biodistribution and pharmacodynamics after intravenous injection, the bioeffects during ultrasound insonification, the gas release profile, and other related behaviors. Clearly, microbubbles of a specific size with low polydispersity are desired for advanced biomedical applications [12].

Efforts to engineer monodisperse microbubble suspensions have mainly focused on microfluidic technologies. These techniques include flow focusing [12–14], T-junctions [15] and electrohydrodynamic atomization [16,17]. While these techniques provide very low polydispersity, they are rather slow at generating microbubbles [18]. Using flow focusing, for example, requires several hours to produce microbubbles at sufficient numbers for even a single small-animal trial ($\sim$0.1 mL $\times$ 10^9 mL^{-1}). Additionally, dust particles can plug microchannels, thus requiring fabrication and calibration of a new device. While engineering breakthroughs may eventually allow efficient and robust generation of monodisperse microbubbles via microfluidic strategies, these techniques currently remain untenable for biomedical studies.

Mechanical agitation has been the main method to create encapsulated microbubbles for biomedical applications, since their inception by Feinstein et al. [19]. Mechanical agitation is a common emulsification procedure in which a hydrophobic phase (i.e., gas) is dispersed within an aqueous surfactant solution by disruption of the interface. Acoustic emulsification (sonication), for example, generates large quantities of microbubbles (100 mL $\times$ 10^{10} mL^{-1}) rapidly and reproducibly within just a few seconds. Shaking a

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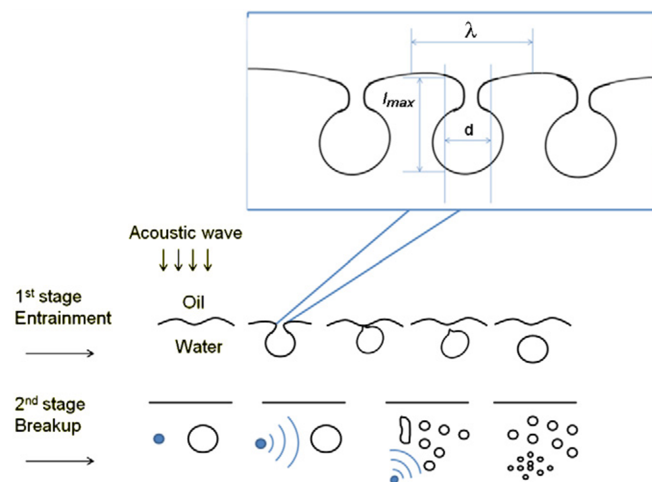

Fig. 1. Cartoon showing origins of polydispersity during acoustic emulsification. Initial bubble entrainment occurs as a capillary instability. Inset shows relevant length scales. Subsequent cavitation in the suspension (shown as filled circle with propagating waves) induces breakup of the larger bubbles to a critical diameter, where surface forces and inertial forces balance. Figure adapted from Li and Fogler [21,22].

serum vial with a device similar to a dental amalgamator produces a sufficient dose of microbubbles (2 mL $\times$ 10^{10} mL^{-1}) for a single patient study, at the bedside in under a minute [20]. While mechanical agitation is highly efficient at generating microbubbles, the size distributions tend to be highly polydisperse and thus are not optimal for biomedical applications.

The origins of polydispersity in acoustically generated emulsions were elucidated three decades ago by Li and Fogler [21,22]. Emulsification was reported to occur in two stages. Instability at the water surface results in entrainment of drops (or bubbles) into the aqueous medium, and subsequent cavitation in the medium results in droplet breakup to a critical size (Fig. 1). The first stage, entrainment, occurs as the unstable growth and eventual eruption of interfacial capillary waves produced by sonication. The second stage of acoustic emulsification involves the continual cavitation-induced breakdown of larger particles as a function of sonication time until a stable size is reached. The breakdown mechanism depends on the type of deformation and flow pattern around the droplet. The stable size results when surface tension forces balance the inertial forces on the droplet.

Initial and final droplet size is difficult to predict *a priori*. The analysis provided by Li and Fogler for liquid droplets points to the origin of polydispersity as a consequence of multiple mechanisms acting simultaneously on the multi-body system. Given that emulsion polydispersity is inherent in mechanical agitation processes, it is desirable to find a means of separating subpopulations of the particles based on size. This will allow improved microbubble formulations for advanced biomedical applications.

Previous reports have described the use of flotation to isolate subpopulations from polydisperse microbubble suspensions. In principle, larger microbubbles are more buoyant and rise faster, thus allowing separation based on different migration rates in a gravitational field. Kvale et al. described a model for the size fractionation of air-filled microbubbles by simple flotation [23]. Microbubbles were injected at the bottom of a stagnant water column and allowed to rise under normal gravity. The model predicted the size distribution of microbubbles at certain distances from the bottom of the column as a function of time. The form of the model was a second-order PDE that accounted for the convective motion of the bulk dispersed phase (liquid moved down the column as microbubbles moved up) as well as the Brownian (thermal) diffusive motion of the particles. The crowding effect of the microspheres was accounted for by using a modified version of Einstein's derivation for the effective viscosity in a dilute suspension [24].

Wheatley et al. reported the isolation of submicrometer bubbles using differential centrifugation [25]. Isolation was accomplished by flotation at normal gravity, or centrifugation at a relative centrifugal force (RCF) of 16 or 45 for pre-determined time intervals. This method allowed isolation of the submicrometer bubble fractions. The use of centrifugation reduced the flotation time, but led to destabilization of the surfactant-stabilized microbubbles during subsequent insonification. Microbubbles centrifuged at 45 RCF for 1 min were not stable, whereas those spun at 16 RCF for the same time were relatively stable. Destabilization was attributed to the extra hydrostatic pressure exerted on the microbubbles, which increased toward the bottom of the column and in proportion to centrifugation speed. Flotation at normal gravity was more time consuming, but less detrimental to microbubble stability.

In contrast to surfactant-coated microbubbles, lipid-coated microbubbles have been shown to be stable after centrifugation up to several hundred RCF [26,27]. The lipid shell is highly viscous [28] and relatively impermeable to gases [29]. We therefore sought to further develop the differential centrifugation method of Wheatley et al. [25], but as a rapid and facile means to isolate subpopulations of lipid-coated microbubbles. Below, we report on the experimental characterization of the initial polydisperse suspension, the development of a method to isolate size fractions of interest for biomedical applications, and characterization of the long-term stability of the isolated fractions.

2. Methods and materials

2.1. Materials

All solutions were prepared using filtered, 18 MΩ deionized water (Direct-Q, Millipore, Billerica, MA). All glassware was cleaned with 70 vol% ethyl alcohol solution (Sigma-Aldrich; St. Louis, MO) and rinsed with deionized water. The gas used to form microbubbles was perfluorobutane (PFB) at 99 wt% purity obtained from FluoroMed (Round Rock, TX). 1,2-Distearoyl-*sn*-glycero-3-phosphocholine (DSPC) was purchased from Avanti Polar Lipids (Alabaster, AL) and dissolved in chloroform (Sigma-Aldrich) for storage. Polyoxyethylene-40 stearate (PEG40S) was obtained from Sigma-Aldrich and dissolved in deionized water. The fluorophore probe 3,3′-dioctadecyloxacarbocyanine perchlorate (DiO) solution (Invitrogen; Eugene, OR) was used to label the microbubbles for part of the experiments.

2.2. Microbubble generation

Microbubbles were coated with DSPC and PEG40S at molar ratio of 9:1. The indicated amount of DSPC was transferred to a separate vial, and the chloroform was evaporated with a steady nitrogen stream during vortexing for about ten minutes followed by several hours under house vacuum. 0.01 M phosphate buffered saline (PBS) solution (Sigma-Aldrich) was filtered using 0.2-μm pore size polycarbonate filters (VWR, West Chester, PA). The dried lipid film was then hydrated with filtered PBS and mixed with PEG40S (25 mg/mL in filtered PBS) to a final lipid/surfactant concentration of 1.0 mg/mL. The lipid mixture was first sonicated with a 20-kHz probe (Model 250A, Branson Ultrasonics; Danbury, CT) at low power (power setting dialed to 3/10; 3 W) in order to heat the pre-microbubble suspension above the main phase transition temperature of the phospholipid (∼55 °C for DSPC) and further disperse the lipid aggregates into small, unilamellar liposomes [30]. PFB gas was introduced by flowing it over the surface of the lipid suspension. Subsequently, higher power sonication (power setting dialed to 10/10; 33 W) was applied to the suspension for about

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