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● *Original Contribution*

SYNTHESIS AND CHARACTERIZATION OF TRANSIENTLY STABLE ALBUMIN-COATED MICROBUBBLES *VIA* A FLOW-FOCUSING MICROFLUIDIC DEVICE

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Abstract—We describe a method for synthesizing albumin-shelled, large-diameter (>10 μm), transiently stable microbubbles using a flow-focusing microfluidic device (FFMD). The microfluidic device enables microbubbles to be produced immediately before insonation, thus relaxing the requirements for stability. Both reconstituted fractionated bovine serum albumin (BSA) and fresh bovine blood plasma were investigated as shell stabilizers. Microbubble coalescence was inhibited by the addition of either dextrose or glycerol and propylene glycol. Microbubbles were observed to have an acoustic half-life of approximately 6 s. Microbubbles generated directly within a vessel phantom containing flowing blood produced a 6.5-dB increase in acoustic signal within the lumen. Microbubbles generated in real time upstream of *in vitro* rat aortic smooth muscle cells under physiologic flow conditions successfully permeabilized 58% of the cells on insonation at a peak negative pressure of 200 kPa. These results indicate that transiently stable microbubbles produced *via* flow-focusing microfluidic devices are capable of image enhancement and drug delivery. In addition, successful microbubble production with blood plasma suggests the potential to use blood as a stabilizing shell. (E-mail: jh7fj@virginia.edu)

Key Words: **Flow-focusing microfluidic device, Monodisperse microbubbles, Albumin shell, Ultrasound-mediated drug delivery, Sonoporation.**

INTRODUCTION

Size distribution and stability are two important considerations for any microbubble formulation. Size is important as it dictates the acoustic properties of microbubbles. Microbubbles less than 1 μm in diameter produce minimal acoustic contrast (Gorce et al. 2000; Soetanto and Chan 2000). Larger microbubbles provide increased contrast (Kaya et al. 2010; Streeter et al. 2010), but as microbubbles exceed 10 μm, they quickly get filtered by the lungs and can pose an embolus risk (Butler and Hills 1979; Klibanov 2002). Microbubble size has also been reported to affect drug delivery (Burke et al. 2012; Konofagou et al. 2012). Fan et al. (2012) recently reported that after sonoporation, cell membrane pore size increases with microbubble size and different acoustic pressures can be used to selectively porate cells in contact with different-sized microbubbles. Consequently, microbubbles are often sorted by size to reduce polydispersity or select a specific microbubble size (Borrelli et al. 2012; Feshitan et al. 2009; Huh et al. 2007; Kvåle et al. 1996). Although narrow size distributions are attainable through sorting methods, microbubble yield is reduced and specific microbubble diameters are difficult to isolate from a polydisperse population.

Stability is important as it determines how well microbubbles survive the rigors of systemic circulation. Microbubbles are lost during intravenous administration (Talu et al. 2008b), filtered by the lungs, liver and spleen (Butler and Hills 1979; Iijima et al. 2006; Lim et al. 2004), and dissolve and coalesce because of the complex multi-gas environment of the vasculature (Kabalnov et al. 1998; Kwan and Borden 2010). To increase stability, newer-generation microbubbles are formulated with low-solubility gases to reduce dissolution and are stabilized with protein, polymer or lipid shells (Sirsi and Borden 2009).

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Even with control of microbubble size during production and increased stability, the *in vivo* distribution and concentration of microbubbles after microbubble administration are unknown. To overcome these limitations, we propose a new paradigm in which microbubbles are produced *in situ* directly within the vasculature, thus enabling controlled administration of microbubbles and therapeutics. Furthermore, through their production in the vasculature near the target of interest, microbubbles can be intentionally designed to be unstable. As a result, larger microbubbles (>10 μm), with their concomitant advantages, can be realized *via* transiently stable microbubbles that would mitigate the risk of embolus formation.

Flow-focusing microfluidic devices (FFMDs) are ideal for this application as they can produce monodisperse populations of microbubbles in real time (Garstecki et al. 2004; Hettiarachchi et al. 2007; Tan et al. 2006; Wang et al. 2013) and can be miniaturized to be compatible with vasculature dimensions (Dhanaliwala et al. 2013). In addition, FFMDs are capable of producing microbubbles in sufficient quantities to provide acoustic contrast (Dhanaliwala et al. 2013) and facilitate drug delivery (Dixon et al. 2013) without the need for additional concentration or washing of the microbubbles before use. The ability to produce a monodisperse population of microbubbles at a specific microbubble diameter and concentration has implications for both imaging and drug delivery applications. Talu et al. (2007) observed that monodisperse microbubbles have reduced echo-to-echo decorrelation, which could improve signal intensity, whereas Choi et al. (2010) observed that blood-brain barrier penetration was improved when using 5-μm-diameter compared with 1-μm-diameter microbubbles.

Albumin has been extensively evaluated as a stabilizing shell material for ultrasound microbubble contrast agents (Ferrara et al. 2007; Keller et al. 1989; Sirsi and Borden 2009), and it continues to be a relevant material because of its excellent safety profile (Vincent et al. 2003). To form a stable shell, albumin is typically heated to denature and cross-link the protein (Grinstaff and Suslick 1991). Although non-cross-linked albumin can be used as a microbubble shell, these microbubbles have a higher liquid-vapor surface tension (*i.e.,* Laplace pressure) (Krishnan et al. 2004) and, thus, lower stability compared with cross-linked albumin-shelled microbubbles (Avivi and Gedanken 2002; Grinstaff and Suslick 1991). Albumin cross-linking can be difficult to achieve within an FFMD. As a result, there has been minimal investigation into albumin-stabilized microfluidic device-produced microbubbles. However, for *in situ* microbubble production, a non-cross-linked albumin shell could provide both biocompatibility and transient stability.

In this study, we describe *in vitro* production and characterization of transiently stable microbubbles by a FFMD as a step toward the goal of *in situ* microbubble production. Nitrogen-filled albumin-coated microbubbles were fabricated with a FFMD using both fractionated bovine serum albumin (BSA) and fresh bovine plasma. The FFMD was characterized for microbubble diameter, production and coalescence. Microbubble stability was characterized optically and acoustically. Finally, acoustic contrast of the microbubbles in a flow phantom and microbubble-enhanced drug delivery to an *in vitro* cell monolayer under physiologic flow conditions were investigated.

METHODS

FFMD fabrication

Flow-focusing microfluidic devices were fabricated as described previously (Dhanaliwala et al. 2013). Briefly, microfluidic devices were cast in polydimethylsiloxane (Sylgard 184, Dow Corning, Midland, MI, USA) from a custom SU-8 mold. The device was then plasma bonded to a clean polydimethylsiloxane substrate and heated at 70°C for 1 h before use to ensure hydrophobicity of the channels. With respect to final dimensions, the gas and liquid channels were 35 and 50 μm wide, respectively, the nozzle was 7 μm wide and all channels were 27 μm tall.

Microbubble fabrication

The liquid phase consisted of 3% or 5% (w/v) bovine serum albumin (BSA) dissolved in a solution of isotonic saline (0.9% NaCl) or a solution of glycerol, propylene glycol and isotonic saline (GPS). Dextrose was added to either solution as needed. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). A pharmaceutical-grade preparation of 5% human serum albumin (BSL Behring, King of Prussia, PA, USA) was also tested. Albumin concentration was determined by absorbance at 280 nm (NanoDrop 1000, Thermo Scientific, Wilmington, DE, USA) assuming a molar extinction coefficient of 6.7 M^{-1} cm^{-1}. Viscosity (η) was measured with an Ubbelohde viscometer (Cannon Instrument, State College, PA, USA) at 23°C. The gas phase consisted of 99.998% nitrogen (GTS Welco, Richmond, VA). Polytetrafluoroethylene tubing (Cole Parmer, Vernon Hills, IL, USA) 30 cm in length was used to convey the liquid and gas phases to the microfluidic device inlets. The liquid flow rate was set with a syringe pump (PHD Ultra, Harvard Apparatus, Holliston, MA, USA), and the gas pressure was set with a two-stage pressure regulator (VTS 450 D, Victor Technologies International, St. Louis, MO, USA).