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● *Original Contribution*

IMAGE-GUIDED ULTRASOUND CHARACTERIZATION OF VOLATILE SUB-MICRON PHASE-SHIFT DROPLETS IN THE 20–40 MHZ FREQUENCY RANGE

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Abstract—Phase-shift perfluorocarbon droplets are designed to convert from the liquid to the gas state by the external application of acoustic or optical energy. Although droplet vaporization has been investigated extensively at ultrasonic frequencies between 1 and 10 MHz, few studies have characterized performance at the higher frequencies commonly used in small animal imaging. In this study, we use standard B-mode imaging sequences on a pre-clinical ultrasound platform to both image and activate sub-micron decafluorobutane droplet populations *in vitro* and *in vivo* at center frequencies in the range of 20–40 MHz. Results show that droplets remain stable against vaporization at low imaging pressures but are vaporized at peak negative pressures near 3.5 MPa at the three frequencies tested. This study also found that a small number of size outliers present in the distribution can greatly influence droplet performance. Removal of these outliers results in a more accurate assessment of the vaporization threshold and produces free-flowing microbubbles upon vaporization in the mouse kidney. (E-mail: pssheeran@gmail.com) © 2016 World Federation for Ultrasound in Medicine & Biology.

Key Words: **Perfluorocarbon, Ultrasound contrast agents, Phase-shift droplets, Acoustic droplet vaporization.**

INTRODUCTION

Phase-shift perfluorocarbon droplets, designed to convert from the liquid to the gas state by the external application of acoustic or optical energy, provide a unique solution to some current problems in medical ultrasound and photoacoustics. The ability to trigger a change in particle size and echogenicity with high spatial and temporal specificity has been utilized for applications such as temporary embolization, drug/gene delivery, tissue ablation and contrast-enhanced/molecular imaging (Kripfgans et al. 2000; Sheeran and Dayton 2012; Sirsi and Borden 2014). Much of the research in the past 5 years has focused on developing nanoscale phase-shift droplets that are capable of exiting the vascular network at the site of solid tumors *via* the enhanced permeability and retention effect (Torchilin 2011), which is not possible with the microbubble blood-pool contrast agents typically used in contrast-enhanced ultrasonography (Rapoport et al. 2009; Reznik et al. 2011; Sheeran et al. 2012). Once deposited in the tumor interstitium, droplets could be vaporized to produce microbubbles that can be used for diagnosis or image-guided therapy.

Efforts to drive phase-shift droplets toward clinical translation have produced droplet-specific imaging sequences that combine activation and contrast monitoring on a single clinical transducer (Arnal et al. 2015b; Couture et al. 2012b; Puett et al. 2014; Sheeran et al. 2015). In these approaches, imaging is performed at incident acoustic pressures below the droplet activation threshold. Next, a sequence of high-energy pulses is used to convert the droplets to microbubbles within the imaging plane, followed by a second imaging sequence below the threshold. For activation and imaging using clinical diagnostic imaging systems, this approach requires that the activation parameters are within the maximum output limits set by the Food and Drug Administration, principally limited by the mechanical index ($\frac{Peak\ Neg\ Pressure\ (MPa)}{\sqrt{Center\ Freq\ (MHz)}}$; Apfel and Holland, 1991). Under these conditions, an observed

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inverse relationship between droplet size and vaporization threshold has limited the vaporization efficiency of typical nanoscale droplet formulations with cores composed of dodecafluoropentane (C_5F_{12}) and perfluorohexane (C_6F_{14}) (Rapoport et al. 2009; Reznik et al. 2013; Sheeran et al. 2011b; Williams et al. 2013). Alternative approaches that improve vaporization efficiency include incorporation of nanoparticles that increase the probability of droplet nucleation (Matsuura et al. 2009), formulation with cores that include volatile perfluorocarbons such as decafluorobutane (DFB, C_4F_{10}) and octafluoropropane (C_3F_8) (Li et al. 2015; Mountford et al. 2015; Phillips et al. 2013; Sheeran et al. 2012) and optimization of the conversion pulse length and frequency (Lo et al. 2007; Williams et al. 2013).

The nature of the relationship between ultrasound center frequency and droplet vaporization threshold has been an ongoing discussion in the literature. Beginning with some of the earliest reports on phase-shift droplets, it was observed that droplets were easier to vaporize as the center frequency increased—the opposite relationship to that expected from homogeneous nucleation theory (Kripfgans et al. 2000; Williams et al. 2013). Recently, Shpak et al. (2014) suggested that this is the result of non-linear acoustic propagation focusing within the droplet core. Beyond these effects, the scaling of mechanical index by the ultrasound frequency provides a higher regulatory limit on peak negative pressures on imaging platforms that operate at higher frequencies. The majority of investigations in the literature to date have used ultrasound frequencies most relevant to human imaging and therapy through several centimeters of tissue (1–15 MHz). As a result, the performance of phase-shift droplet populations is poorly characterized at higher frequencies, such as those used for pre-clinical small animal imaging (20–40 MHz). Thus, the need exists to develop phase-shift droplets suitable for imaging and activation at these frequencies to enable more effective pre-clinical evaluation of this class of agents.

In this study, we use a pre-clinical imaging platform (Vevo2100, VisualSonics Inc., Toronto, ON, Canada) to evaluate activation of sub-micron DFB droplet populations embedded in tissue-mimicking polyacrylamide phantoms at center frequencies in the range of 20–40 MHz. Thresholds are measured for both native and size-selected populations of droplets to evaluate the impact of large outlier droplets. The measured *in vitro* thresholds are validated *in vivo* by activating droplet populations in superficial hind-limb tumors as well as in the kidneys of C3H/HeJ mice after intravenous administration. The results provide insight into proper characterization of phase-shift droplets for both pre-clinical and clinical applications.

MATERIALS AND METHODS

Droplet emulsion preparation

Nanoscale droplet emulsions were prepared by condensation of poly-disperse lipid-coated DFB microbubbles that were generated by mechanical agitation (Vialmix, Bristol-Myers-Squibb, New York, NY, USA), following the method of Sheeran et al. (2012). The encapsulating shell consisted of 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC, Avanti Polar Lipids, Alabaster, AL, USA) and 1,2-dipalmitoyl-sn-glycero-3-phosphatidylethanolamine-N-[poly(ethylene glycol)-5000] (DPPE-PEG5000, Biosolve, The Netherlands) in a 9:1 M ratio at a lipid concentration of 1 mg/mL in a solution containing phosphate-buffered saline, propylene glycol, and glycerol. All other chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA). Precursor microbubble size distributions (before condensation) were measured by Coulter counter (Multisizer III, Beckman Coulter, Brea, CA, USA) using 10 to 20 μL sample volumes. Droplet emulsions post-condensation were diluted to 0.0005% in ultra-pure water (Milli-Q, Millipore Ltd, Etobicoke, ON, Canada) and the size distributions measured using dynamic light scattering (Nano ZS, Malvern Instruments Ltd, Worcestershire, UK) with a unimodal assumption.

Two types of droplets were used for this study: native and size-selected. Native droplet emulsions were formed by the protocol described. Size-selected emulsions were formed by the addition of a decantation stage before the condensation step. In this process, the microbubbles in the vial were decanted for 15 min following the protocol of Goertz et al. (2007), resulting in visual separation between large outliers and the primary distribution of precursor microbubbles in the emulsion. The large precursor microbubbles were removed by withdrawing the small population from the inverted vial and transferring this size-selected sub-population to a second vial filled with decafluorobutane headspace. The size-selected bubbles were then condensed to the droplet state.

Polyacrylamide phantom preparation

Polyacrylamide phantoms containing droplets at 0.01% v/v were prepared according to Williams et al. (2013), with approximate dimensions of 1.3 cm (thickness) $\times$ 11 cm $\times$ 7 cm. Control phantoms were fabricated in the same way but without droplets. Ultrasound experiments were performed within 30 min

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