Investigation of the acoustic vaporization threshold of lipid-coated perfluorobutane nanodroplets using both high-speed optical imaging and acoustic methods

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Abstract

A combination of ultra high-speed optical imaging (5 x 10^6 frames/s), B-mode ultrasound and passive cavitation detection was used to study the vaporization process and determine both the acoustic droplet vaporization (ADV) and inertial cavitation (IC) thresholds of phospholipid-coated perfluorobutane nanodroplets (PFB-NDs; diameter 237 nm ± 16 nm). PFB-NDs have not previously been studied with ultra high-speed imaging and were observed to form individual microbubbles (1-10 µm) within 2-3 cycles and subsequently larger bubble clusters (10-50 µm). The ADV and IC thresholds were not statistically significantly different and decreased with increasing pulse length (20-20000 cycles), pulse repetition frequency (1-100 Hz), concentration (10^8-10^{10} ND/ml), temperature (20-45^{o}C) and decreasing frequency (1.5-0.5 MHz). Overall, the results indicate that at frequencies of 0.5, 1.0 and 1.5 MHz, PFB-NDs can be vaporized at moderate peak negative pressures (< 2.0 MPa), pulse lengths and pulse repetition frequencies. This finding is encouraging for the use of PFB-NDs as cavitation agents, as these conditions are comparable to those required to achieve therapeutic effects with microbubbles, unlike those reported for higher boiling point NDs. The differences between the optically and acoustically determined ADV thresholds, however, suggest that application-specific thresholds should be defined according to the biological/therapeutic effect of interest.

Keywords

Nanodroplets, Perfluorobutane, High-intensity focused ultrasound, Acoustic droplet vaporization, Cavitation, Threshold, High-speed imaging.

Introduction

Gas-filled microbubbles, stabilized by a coating material such as phospholipids, denatured human serum albumin or synthetic polymers, have been the subject of extensive investigation both as ultrasound contrast agents and therapeutic carriers e.g. for drug delivery and gene therapy (Hernot and Klibanov 2008; Liu et al. 2006). Their size (1-10 μm), however, limits both their circulation time and their ability to extravasate and accumulate in a target tissue (Kaya et al. 2010). Lipid-coated perfluorocarbon (PFC) "nano"droplets [1] (NDs) with diameters of a few hundred nanometres have been explored as a means of addressing these limitations (Zhou et al. 2013). The lipid shell coating the PFC core can help to stabilize the NDs, facilitates biocompatibility and also functionalization of the ND surface to enable targeting and/or attachment of therapeutic species (Hatziantonioy and Demetzos 2008; Peetla et al. 2013; Unger et al. 2004). PFC NDs are not easily detected by ultrasound imaging because of their liquid core and size. Upon exposure to ultrasound of sufficient intensity, however, they can be converted into echogenic gas-filled microbubbles, through a process termed acoustic droplet vaporization (ADV) (Kripfgans et al. 2000; Matsuura et al. 2009; Sheeran et al. 2011c). Due to the high Laplace pressure and corresponding increase in the energy required to vaporize the encapsulated liquid, much higher acoustic pressures are typically required for ADV than those required for stimulating microbubbles (Mannaris et al. 2019). This can increase the probability of unwanted bio-effects (Dalecki 2004; Leighton 2012) and consequently, a range of different methods have been explored for reducing the pressure threshold for ADV.

Perfluoropentane (PFP) and perfluorohexane (PFH) have been most commonly used to form the core of NDs, but these both require substantial acoustic pressures to achieve vaporization

[1]The NDs described here do not meet the strict definition of "nano," i.e. smaller than 100 nm, but the term has become widely used in the literature.

(Fabiilli et al. 2009; Kripfgans et al. 2000; Matsuura et al. 2009; Peng Zhang and Porter 2009; Vlaisavljevich et al. 2015b; Vlaisavljevich et al. 2015a). Even for therapeutic applications, in which higher ultrasound intensities are normally used, vaporization efficiency may be poor and recondensation of droplets can occur after vaporization (Reznik et al. 2013; Shpak et al. 2014a). One approach to solve this has been to use a mixture of droplets and microbubbles. The inertial collapse of the microbubbles at moderate ultrasound intensities is thought to trigger ADV through the localized generation of shockwaves (Healey et al. 2016a; Lo et al. 2007). "Acoustic cluster therapy" (ACT) is an example of this approach, although currently the size of the clusters used limits its application to targets where vascular embolization is desirable (Healey et al. 2016b; Sontum et al. 2015; Wamel et al. 2016). Incorporation of solid nanoparticles to act as nuclei within the droplets has also been used to successfully lower the ADV threshold of NDs (Lee et al. 2015), but it is not always desirable to include additional materials in the formulation and there remain safety concerns over the biomedical use of nanoparticles. Using alternative PFCs with lower boiling-points is another way to reduce the ADV threshold (Rojas et al. 2019; Sheeran et al. 2011c; Sheeran et al. 2011a; Sheeran et al. 2011b). Sheeran et al. proposed a method whereby perfluorobutane (PFB) and octafluoropropane (OFP), which are gaseous at room temperature, can be used to produce both nano and microdroplets (MDs , i.e. > 1 μm diameter) by a microbubble condensation technique (Sheeran et al. 2011a; Sheeran et al. 2012). They found that ND/MDs produced in this way required significantly lower pressures for ADV compared with similar droplets of PFP or PFH.

In addition to the droplet composition, it has been shown that many other parameters influence the ADV threshold of PFC ND/MDs. These include environmental parameters (such as temperature, viscosity of the surrounding fluid, and boundary conditions); droplet characteristics (size and concentration as well as core and shell composition); and the acoustic exposure

parameters (frequency, pulse repetition frequency, pulse length and exposure duration). Perhaps as a consequence of this sensitivity to multiple parameters, there is considerable variation in the published values for ADV thresholds in the literature as shown in Table 1, which summarises the results from 29 studies of PFC ND/MD vaporization. There are some qualitatively consistent trends. For example, the ADV threshold typically decreases with increasing environmental temperature, tube diameter, droplet size and concentration, pulse repetition frequency and pulse length (Aliabouzar et al. 2018; Fabiilli et al. 2009; Kripfgans et al. 2000; Lo et al. 2007; Porter and Zhang 2008; Rojas et al. 2019). There are however differences across studies concerning the effect of ultrasound frequency. In some studies, the ADV threshold increases with increasing the ultrasound frequency (Aliabouzar et al. 2018; Kripfgans et al. 2004; Sheeran et al. 2013b; Vlaisavljevich et al. 2015a), which is consistent with classical nucleation theory (Vlaisavljevich et al. 2016). However, an opposite effect has also been reported (Kripfgans et al. 2000; Kripfgans et al. 2002; Schad and Hynynen 2010a; Williams et al. 2013). These contradictory results have been attributed variously to limitations of the experimental apparatus, droplet deformation (Kripfgans et al. 2004) and, in the case of microdroplets (MD), to nonlinear propagation and super-harmonic focusing (Miles et al. 2016; Shpak et al. 2014b).

A further confounding factor is the fact that the definition of the threshold itself may vary between studies and according to the measurement technique(s) used. Both direct and indirect methods have been applied. Direct measurements include high-magnification microscopy and high-speed imaging, enabling direct observation of the vaporization process (Kripfgans et al. 2004; Sheeran et al. 2013b; Vlaisavljevich et al. 2015a). However, optical observation is not suitable for measuring the initial size of droplets below 800 nm due to the resolution limits of brightfield imaging, nor can it be applied in tissue. To address this, indirect methods, such as ultrasound imaging (Fabiilli et al. 2009; Kripfgans et al. 2000; Lo et al. 2007; Porter and Zhang 2008) and/or

115 monitoring of acoustic emissions (Aliabouzar et al. 2018; Vlaisavljevich et al. 2015a) have been
116 used to identify ADV. In all cases the sensitivity and/or spatial resolution of the system will affect
117 the pressure at which a bubble (or bubbles) or its emissions are first detected and hence the
118 recorded threshold. A further important distinction with acoustic methods is whether it is the first
119 appearance of a gas bubble(s) that is being detected, i.e. true ADV, or its subsequent oscillation
120 and collapse. Under the acoustic exposure conditions typically required for ADV the resulting
121 bubble will be likely to undergo inertial cavitation (IC), i.e. when a bubble grows to a diameter
122 that is at least twice its original diameter during a single cycle of acoustic pressure and then
123 collapses violently under the inertia of the surrounding fluid, potentially fragmenting into many
124 smaller bubbles (Fabiilli et al. 2009; Neppiras 1980). The measured threshold, however, will
125 depend upon the signal amplitude selected by the experimenter as representing ADV or IC. This
126 is discussed further later.

127 The thresholds determined by different methods may also vary on account of the stochastic
128 nature of both ADV and IC. If a droplet of a given size has a fixed probability of vaporising at a
129 given ultrasound frequency and pressure, then the larger the number present, the more likely it
130 is that an ADV event will occur. The same applies to bubbles and IC. The field of view in an optical
131 experiment will typically be considerably smaller than that of an ultrasound transducer and so
132 contain fewer ND/bubbles. This can potentially lead to a higher threshold being measured by
133 optical compared with acoustic methods. In addition, there will also likely be a range of
134 ND/bubble sizes present, the probability of ADV/IC may vary with other parameters e.g.,
135 differences in coating integrity; and, once some bubbles have formed, then their collapse may
136 promote ADV as mentioned above.

137 Despite the desirability of using PFB or OFP to minimize the ADV threshold, there have been
138 relatively few studies that systematically investigate their vaporization dynamics. Sheeran et al.

investigated the effect of Laplace pressure on the vaporization threshold of different PFC MDs (1-13 μm), and found the vaporization thresholds of PFB MDs were lower than thresholds of the higher-boiling point PFC MDs and decreased as the MD diameter increased (Sheeran et al. 2011c). More recent studies by Sheeran et al. showed that the vaporization threshold for PFB NDs increased with ultrasound frequency (Sheeran et al. 2013b). These findings are further supported by Rojas et al. who investigated the effect of environmental parameters (including hydrostatic pressure, boundary constraints and viscosity) on the vaporization threshold of PFB NDs (Rojas et al. 2019). There remains, however, considerable uncertainty regarding the activation and subsequent dynamics of low boiling point PFC NDs. The aim of this study is therefore to undertake a comprehensive investigation of both the ADV and IC thresholds of lipid-coated PFB NDs using a combination of high-speed video microscopy, B-mode ultrasound imaging and passive cavitation detection methods. The effects of acoustic parameters (pulse repetition frequency, pulse length and frequency), in addition to droplet parameters (droplet composition, size and concentration) and temperature on the vaporization threshold of PFB NDs are all investigated.

Materials and Methods

Materials

1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and 1,2-distearoyl-sn-glycero-3-phosphoethanol-amine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG2000) were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Cholesterol, glycerol, propylene glycol and phosphate buffered saline (PBS) were obtained from Sigma-Aldrich (Gillingham, Dorset, UK). PFB and PFP were obtained from FluoroMed, L.P. (Round Rock, TX, USA). PFB was chosen in preference to OFP for this study on the basis of preliminary experiments in which it was found to be difficult to form a stable population of exclusively submicrometre droplets using OFP either directly or by microbubble condensation. This is consistent with the report of Sheeran et al. (Sheeran et al. 2011b).

Formulation and characterization of NDs

A lipid mixture was prepared by mixing 13.7 mg (17.4 µmol) of DSPC, 1.9 mg (4.8 µmol) of cholesterol, and 5.4 mg (1.9 µmol) of DSPE-PEG2000 from stock solutions in chloroform (25 mg/mL). The solvent was evaporated under reduced pressure and the resulting lipid films were hydrated in 4 mL of PBS/propylene glycol/glycerol (16:3:1 volume ratio). The resulting lipid suspension was dispersed by brief sonication at room temperature, at which point it can be stored at 4 ℃ for later use. To 4 mL of the lipid suspension, 100 µL of liquid PFB (obtained by condensation of PFB gas at -10 ℃) were added and the biphasic mixture was cooled in an ethanol ice bath maintained between -7 ℃ and -12 ℃. The mixture was then sonicated using a probe sonicator (Q125, QSonica, LLC. USA) at 50% power for 3 minutes (125 W, 20 kHz, 2 s on and 4 s off) to form the NDs. The low freezing point of the solvent mixture prevented sample freezing

during this process. To remove excess free lipids and any gas bubbles, the NDs were centrifuged at 10000 rpm (11292 *g*) for 6 min and resuspended in PBS. The centrifugation and washing process were repeated three times. The NDs were then centrifuged at different speeds to obtain NDs with different size ranges. Finally, the prepared NDs were stored at 4 °C for later use. As a comparison, higher-boiling point droplets made with PFP were prepared in a similar manner.

The size distribution of the NDs was determined by dynamic light scattering (DLS) (Zetasizer Nano, Malvern Instruments, Malvern Worcestershire, UK). The concentration of the NDs was measured using nanoparticle tracking analysis (NTA) (NanoSight, Malvern Instruments, Malvern Worcestershire, UK) by capturing 60-s videos (3 videos per sample). The analysis was carried out using the instrument manufacturer's NTA software (Version 3.0, Build 0066, Malvern Instruments). To investigate the stability of PFB NDs, the produced PFB NDs were stored at both 20 ^{o}C (room temperature) and 37 ^{o}C (physiological temperature). The changes in size and concentration at each time point were quantified by DLS and NTA respectively.

Optical experimental setup

A schematic of the setup for high-speed optical imaging only, is shown in Figure 1(a). A single element spherically focused ultrasound (FUS) transducer (0.5 MHz centre frequency, H107, Sonic Concepts, USA) was used to excite the NDs. The aperture and the geometric focus of the transducer were 64 mm and 63.2 mm, respectively. The transducer was driven by a programmable arbitrary waveform generator (33220A, Agilent, USA) and the US field was focused on a polyethylene tube of 1.2 mm inner diameter and 0.2 mm wall thickness (Advanced Polymers, Salem NH, USA). The signal was amplified by a 300 W RF power amplifier (A-300, ENI, USA) and sent to the FUS transducer via a 50 Ω matching network. The transducer and tube were placed

within a tank of degassed and deionized water. A low-pulsatility peristaltic pump (Minipulse Evolution, Gilson, Middleton, WI, USA) was used to create a flow of NDs in degassed PBS through the polyethylene tube at a constant rate of 0.3 mL/min (4.42 mm/s mean velocity). The flow rate was chosen to be in agreement with previously published data of tumour perfusion (Kallinowski et al. 1989). The NDs were excited by a single 100-cycle pulse with different peak negative pressures. An objective lens with a numerical aperture of 0.45 and working distance 8.2-6.9 mm (S Plan Fluor, Nikon Instruments Europe BV, Amsterdam, The Netherlands) was focused on the mid-plane of the tube and coupled to a high-speed camera (HPV-X2, Shimadzu, Tokyo, Japan). The high-speed camera was triggered using the output from the waveform generator. After a delay of 40 μs to allow for propagation of the ultrasound pulse to the focal region, the camera recorded 256 frames at 5 million frames per second (Mfps), with a 200 ns exposure time per frame providing a temporal resolution of 0.2 μs. Digital images of 400 × 250 pixels were recorded; the image resolution was 0.34 μm/pixel, determined using a hemocytometer as a reference standard (Bright-Line, Hausser Scientific, Horsham, PA, USA). Illumination was provided by a cold cathode fiber optic illuminator (Model 41500-55, Cole-Parmer Instrument Company) inserted through a circular cut out in the centre of the FUS transducer.

In order to capture acoustic emissions simultaneously with the high-speed imaging, a second experimental set up was used (Figure 1(b)). Another single element ultrasound transducer of centre frequency 7.5 MHz, element diameter 12.5 mm and focal length 75 mm (V320 Panametrics, Olympus, Waltham, USA) was used as a passive cavitation detector (PCD). This was inserted through the cut out in the FUS transducer to enable co-alignment of both transducers' foci (Figure 1(b)). The lateral and axial full width half amplitude dimensions of the focal volume for this transducer were 1.2 mm and 37.6 mm, respectively. The nominal bandwidth was 50%. The same objective lens and high-speed camera were used as above but the objective was

mounted with its central axis perpendicular to that of the ultrasound transducers. Illumination in this set up was provided by a high intensity light source (SOLIS-1C, Solis® High-Power LEDs, Thorlabs LTD. Ely, United Kingdom). The peak negative pressure from the FUS transducer was increased in increments of 330 kPa from 0 to 2.64 MPa. The acquired PCD signal was filtered using a 5 MHz high pass filter (F5081-5P00-B, Allen Avionics, Inc., Il, US; 20 dB bandwidth of 3.125 MHz) to reject strong reflections from the tube at the fundamental FUS frequency and lower harmonics caused by non-linear propagation. It was then amplified five times with a low noise amplifier (Stanford Research Systems, SR445A). The amplified PCD signal was recorded with a 14-bit PCI Oscilloscope device (PCI-5122, National Instruments, USA) at a rate of 100 MHz.

Acoustic experimental setup

A similar experimental setup, containing a FUS transducer, PCD, polyethylene tube (1.2 mm inner diameter and 0.2 mm wall thickness) and a diagnostic ultrasound imaging probe (L12-5 linear array, operated at 7 MHz using an iU22 imaging system, Philips, Bothell, WA, USA), was used to further investigate the acoustic response of the PFB NDs, as shown in Figure 2. A second single-element spherically focused FUS transducer with a centre frequency of 1.0 MHz (H102 Sonic Concepts, Bothell, WA, USA) was also used to excite the NDs in this set up; and the third harmonic of the H107 transducer was used for excitation at 1.5 MHz. The aperture and the geometric focus of both FUS transducers were 64 mm and 63.2 mm, respectively. In each experiment, both the FUS transducer and PCD were focused on the polyethylene tube through which NDs were pumped at 0.3 mL/min. The peak negative pressure was increased in increments of ~0.24 MPa. The acquired PCD signal was processed and recorded as above. The ultrasound imaging probe was used to simultaneously record B-mode images with the aim of detecting ND

vaporization. The water in tank was passively heated to the desired temperature by heating water in an auxiliary tank.

The beam profiles and focal pressures for the FUS transducers were measured in water using a needle hydrophone (400 μm, HNA-0400, Onda Corporation, USA). In water, the H107 transducer focal volume had lateral and axial full width half amplitude dimensions of 4.1 mm and 25.2 mm respectively when driven at 0.5 MHz; and 1.4 mm and 8.4 mm when driven at 1.5 MHz. The lateral and axial full width half amplitude dimensions of the focal volume for the H102 transducer driven at 1.0 MHz were respectively 1.4 mm and 10.2 mm. The same set up was also used to determine the attenuation of the field produced by the polyethylene tube. The pressure in the tube was measured using the hydrophone in a 1 x 2 mm hole drilled through one side of tube. The pressure in the tube was 96 ± 2% of the pressure in the free field for the H102 transducer driven at 1.0 MHz.

Detection of ADV and IC

In the high-speed camera images, ADV was detected by the appearance of an optically resolvable bubble or bubble cluster, manifest initially by a change in grayscale contrast in the optical focal region that was above that due to noise. The number of pixels with a grayscale value of less than 100 (i.e. darker than the mean background level of 174) was counted as an indicator of the volume of bubbles formed. Counts were made from the last 5 frames of the videos for each set of exposure conditions and compared with the count for the first frame i.e., before ultrasound exposure. Since the pressure was increased in relatively large increments (330 kPa from 0 to 2.64 MPa) a threshold was not defined from these measurements. Rather the pressure at which a statistically significant change in optical density (i.e. the number of pixels with a grayscale value

<100) was compared with that at which a detectable change in B-mode intensity or the energy of acoustic emissions was seen.

To determine an ADV threshold from the B-mode images, the mean echo amplitude (MEA) in a fixed region of interest (ROI) was used to quantify the scattering from the gas bubbles produced by ADV. The ROIs (1.2mm x 4mm) were positioned downstream of the FUS transducer focus in the tube to allow for the movement of the bubbles in the flow (Figure 3a). The MEA was calculated as the sum of the amplitude (A) at pixel (i,j) for the images having dimensions M by N pixels in a given ROI.

$$MEA = \frac{1}{MN}\sum_{i=1}^{M}\sum_{j=1}^{N}A(i,j) \quad (1)$$

The MEA of the ROI before ultrasound exposure was subtracted from the MEA of ROI after ultrasound exposure to compute the relative echo amplitude (REA), which should be zero in the absence of any bubbles.

$$REA = MEA_{after} - MEA_{before} \quad (2)$$

The REAs from five separate images (corresponding to the period over which the MEA reached a stable level) were used to obtain an average REA for each set of exposure conditions. This was then normalized by the maximum value of each average REA to enable comparison between the groups. The normalized REAs were plotted as a function of peak negative pressure (Figure 2(b)). The point at which the normalized REA was >80% was defined as the ADV threshold. This selection was made to be consistent with the IC threshold definition described in the next paragraph.

For the IC measurements, 5000 μs of acoustic emissions were recorded simultaneously with the start of every 5th pulse from the FUS transducer. The IC threshold was determined from the

processed PCD traces as follows. The frequency spectra of the emissions recorded by the PCD were calculated by Fast Fourier Transform (FFT) and the harmonic components and broadband noise were separated using a comb filter (width 300 kHz) in MATLAB (R2017b, The Mathworks, Natick, MA, USA). IC was deemed to occur when the mean-squared value of the broadband signal was at least 20 times (i.e. e^3) larger than the background noise. The probability of inertial cavitation (PIC) was calculated as the fraction of total pulses for which IC was detected. The PIC was plotted as a function of peak negative pressure (Figure 3). The IC threshold was defined as the peak negative pressure corresponding to a PIC > 80% (denoted in Figure 3 by an arrow). This selection was based on previous work as corresponding to the level at which a consistent bioeffect could be achieved (please see the Discussion section for additional information).

Parameter ranges investigated

NDs in the size range 200-600 nm were investigated as this is the range for which enhanced circulation times and tissue extravasation would be expected, as above. The range of concentrations used was 10^8-10^{10} ND/ml, corresponding to a blood volume fraction of PFC of 10^{-6}-10^{-4} and hence comparable to that of microbubble contrast agents. Ultrasound driving frequencies of 0.5, 1.0 and 1.5 MHz, pulse lengths of 20-20,000 cycles and PRFs of 1-100 Hz were used, corresponding to the capabilities of clinically available therapeutic ultrasound systems. All experiments were performed at 20 ℃ unless otherwise indicated. Each experiment was repeated 3 times, and the mean average and standard deviation calculated. A summary of the exposure conditions used for each experiment is shown in Table 2.

Results

ND size and concentration

For the PFB NDs used in the majority of the experiments, the mean diameter measured over five different batches by DLS was 237 ± 16 nm (mean ± standard deviation) as shown in Figure 5(b). The corresponding concentration, as measured by NTA, was 6.6 ± 0.4 $\times 10^{11}$ droplets per ml. For all experiments except those in which concentration was a variable, the suspension was diluted by a factor of 100. For the experiment in which size and composition were varied, both PFB and PFP NDs were prepared and separated by centrifugation into 2 size ranges. The PFP NDs had mean diameters of either 235 ± 21 nm or 518 ± 37 nm; whilst the PFB NDs had mean diameters of 237 ± 16 nm or 514± 28 nm. The concentration used for these experiments was 10^9 droplets per ml.

To investigate the stability of PFB NDs, we monitored the stability of NDs (initial diameter 237 ± 16 nm) in phosphate buffered saline (PBS) at 20 °C and 37 °C for one day. The effective boiling point of PFB-NDs of this size has been estimated to be ~50 °C (Sheeran et al. 2011c; Sheeran et al. 2011a). The size of PFB NDs, as measured by DLS, remained stable for the period of investigation, at both 20 ℃ and 37 ℃ (Figure 5 (c)). There was no significant change to the diameter of NDs with time ($p > 0.05$). Changes in the concentration of nanodroplets were measured using NTA. The concentration of PFB NDs decreased slowly at 20 °C. Within 6 h, only 9% of NDs were lost and 87% remained after one day. At 37 ℃, the concentration of PFB NDs reduced by 18% in the first 6 h, and 71% of NDs were still detectable after one day. The effect of a higher temperature (45°C) upon the ADV threshold was also tested as described below. Stability measurements were not performed at this temperature, however, as this would not be a practical temperature for storage, nor would it be encountered *in vivo* prior to ultrasound exposure.

Ultrafast dynamics of ADV of PFB NDs

The vaporization dynamics of PFB NDs were observed using the high-speed camera. An example of a series of high-speed images of droplet vaporization and subsequent bubble dynamics is shown in Figure 6 and Supplementary Video 1. In the first cycle, an initially undetectable ND, or group of NDs, begins to vaporize near the trough of the first rarefactional half-cycle, resulting in a bubble being produced and reaching its maximal size at ~1.0 μs. Over the compressional half-cycle, the bubble begins to visibly compress and disappears from view completely by the peak of the compression, most likely due to the optical resolution limit (~400 nm). The bubble then oscillates volumetrically, remaining approximately spherical over the next two cycles, but the size of the bubble increases. In the rarefactional phase of the fourth cycle, several bubbles appear in a cluster, either due to fragmentation of the original bubble or nucleation of additional droplets, and expand and contract. In the fifth cycle, bubbles appear that are highly non-spherical. They grow and then coalesce, appearing to form a single bubble, although this cannot be conclusively stated, again due to the optical resolution limit. Following ultrasound exposure (i.e. after 100 cycles) a small number of large bubbles (5~15 μm) persisted, possibly formed by the fusion of smaller bubbles. At peak negative pressures of 1.98 MPa and above, ADV of PFB NDs occurred within a single cycle at a driving frequency of 0.5 MHz. It was not possible to adequately capture ADV at higher frequencies due to the maximum frame rate of the camera.

Simultaneous high-speed imaging and measurement of acoustic emissions

Acoustic emissions were captured simultaneously with the high-speed footage to determine whether the appearance of visible bubbles coincided with a change in the acoustic radiation. The frequency, pulse length and pulse repetition frequency (PRF) were set to 0.5 MHz, 1000 cycles and 10 Hz respectively. Representative time traces (first column), their corresponding frequency content (second column) and optical images (third column) at different peak negative driving pressures are shown in Figure 7. PFB NDs remained unresponsive until the peak negative pressure exceeded 1.32 MPa. Above this, the number of bubbles formed by vaporization of PFB NDs increased with increasing the peak negative pressure and there was a corresponding increase in the amplitude of the acoustic emissions, all of which contained broadband noise. This indicated that the pressures required for ADV were also sufficient to induce inertial cavitation. In order to make an approximate quantitative comparison between the optical and acoustic results, Figure 8 shows how the PIC and the optical density (i.e. number of pixels whose grayscale values were less than 100) varied with peak negative pressure.

Effect of acoustic parameters on droplet activation threshold

Pulse repetition frequency (PRF)

To study the effect of the PRF on the ADV and IC thresholds, the frequency and pulse length were set to 1 MHz and 5000 cycles respectively. The PRF was varied from 1 Hz to 100 Hz. The mean diameter and concentration of PFB NDs were 238 ± 16 nm and 10^9 droplets per mL respectively. The results are shown in Figure 9(a) and indicate that both thresholds decrease substantially with increasing PRF. At a PRF of 100 Hz, the ADV and IC threshold were found to be 1.80 and 2.05 MPa, respectively, increasing to 2.79 and 3.03 MPa at a PRF of 2 Hz. Also as expected,

the ADV threshold is lower than the IC threshold in all cases, although the difference is not statistically significant (p-value of >0.05 in all cases).

Pulse length

The effect of pulse length is shown in Figure 9(b). In this case the frequency and PRF were set to 1 MHz and 10 Hz respectively. Both the ADV and IC thresholds were found to decrease in a similar fashion with increasing pulse length. When the number of cycles was increased from 20 to 20000, the ADV and IC thresholds decreased from 3.06 MPa to 2.08 MPa and 3.36 MPa to 2.30 MPa, respectively. Additionally, the ADV and IC thresholds are relatively constant for short excitation pulses (< 1000 cycles), which is consistent with the measurements of PFP MDs reported by Lo et al. (Lo et al. 2007). As in Figure 9(a), the ADV threshold was found to be lower than the IC threshold but not by a statistically significant amount.

Ultrasound Frequency

To study the effect of ultrasound frequency on the threshold of PFB NDs, transducers operating at center frequencies of 0.5 MHz, 1 MHz and 1.5 MHz were used. The PRF was set to 10 Hz and different pulse lengths were investigated. Figure 10(a) shows the PIC as a function of peak negative acoustic pressure in PFB ND suspensions with a 5 ms pulse length. Only PIC results are shown since the previous experiments indicated the ADV and IC thresholds were statistically indistinguishable. At the lowest ultrasound frequency used, IC occurred at peak negative pressures as low as 1.62 MPa, while at 1.5 MHz, it was not observed consistently until the peak negative pressure reached 3.14 MPa (the locations of the IC thresholds for PFB NDs are denoted in Figure 10(a) by arrows). Figure 10(b) shows the IC threshold at all three frequencies with varying pulse length. The threshold was found to increase substantially with increasing frequency and, as above, with decreasing pulse length.

Effect of ND parameters on the ADV threshold

ND core and size

As above, different sizes of both PFB and PFP NDs were prepared and separated into four groups: small PFB (mean size: 237 ± 16 nm); large PFB (mean size: 514 ± 28 nm); small PFP (mean size: 235 ± 21 nm) and large PFP (mean size: 518 ± 37 nm) all with the same concentration of 10^9 ND/ml. Figure 11 shows how the ADV threshold varied with pulse length for each of these groups at a fixed driving frequency of 1 MHz and PRF of 10 Hz, respectively. As above, the ADV thresholds were found to decrease with increasing pulse length for all groups. At each pulse length, the ADV thresholds for larger NDs were higher than those of the smaller NDs, consistent with the results of PFP NDs by Aliabouzar et al. (Aliabouzar et al. 2019), but the differences were not statistically significant. The ADV thresholds for PFP NDs were higher than for PFB NDs, e.g. for a 5 ms pulse length the ADV thresholds were: 2.29 ± 0.16 MPa for small PFB NDs; 2.06 ± 0.21 MPa for large PFB NDs; 3.88 ± 0.19 MPa for small PFP NDs and 3.43 ± 0.20 MPa for large PFP NDs.

Nanodroplet Concentration

To study the effect of ND concentration on the ADV threshold of PFB NDs, different concentration suspensions (10^8, 10^9, 10^{10} NDs/ml) were exposed to ultrasound at 1 MHz driving frequency, PRF 10 Hz and pulse lengths of 1 ms, 5 ms or 10 ms (1000, 5000 or 10,000 cycles). Figure 12 shows that the ADV threshold decreased with increasing ND concentration. For example, for a pulse length of 5 ms, the ADV thresholds were 2.65 ± 0.22 MPa, 2.30 ± 0.16 MPa, and 2.13 ± 0.17 MPa for concentrations of 10^8, 10^9 and 10^{10} NDs/ml respectively.

Effect of Temperature on the ADV threshold

To study the effect of temperature on the ADV threshold, PFB NDs were exposed to ultrasound at different temperatures (20 ^{o}C, 37 ^{o}C, and 45 ^{o}C). The ultrasound parameters were set to 1 MHz driving frequency, PRF 10 Hz and pulse lengths of 200, 1000 or 5,000 cycles. The concentration was 10^{9} NDs/ml. Figure 13 shows that the ADV threshold decreased with increasing environmental temperature. For example, for a pulse length of 5,000 cycles, the ADV threshold was 2.29 ± 0.16 MPa, 1.66 ± 0.16 MPa, and 0.77 ± 0.13 MPa at 20 ^{o}C, 37 ^{o}C, and 45 ^{o}C respectively.

Discussion

Effect of PRF and pulse length

Both the ADV and IC thresholds decreased in a similar fashion with increasing PRF and increasing pulse length (Figures 9 and 10). This is consistent with studies of PFP NDs (Fabiilli et al. 2009; Lo et al. 2007) and is likely associated with increasing probability of ADV or IC. If the probability of ADV or IC for a single ND or bubble has a fixed value, then increasing either the PRF or pulse length would increase the expected number of events over the course of the experiment.

Effect of driving frequency

As discussed in the introduction, different effects have been reported for varying the driving frequency in previous studies. Vlaisavljevich et al. (Vlaisavljevich et al. 2015a) found that the ADV threshold of PFP NDs increased from 7.4 MPa to 13.2 MPa upon increasing the frequency from 0.345 MHz to 3 MHz. A similar trend has been observed by other groups[11,26,33,39], but the opposite trend has also been reported. Williams et al. (Williams et al. 2013) found that vaporization threshold for PFP NDs decreased with increasing ultrasound frequency. The same relationship has

453 also been observed by Kripfgans et al. (Kripfgans et al. 2000) and Schad et al. (Schad and Hynynen
454 2010b) for MDs. The IC threshold has always been found to increase with increasing ultrasound
455 frequency as would be expected, due to the longer exposure of bubbles to negative pressure at
456 lower frequencies (Apfel and Holland 1991). In this study, both the ADV and IC thresholds were
457 found to increase with driving frequency. The most likely explanation is again the increased
458 probability of vaporization and collapse, due to the longer times that NDs are exposed to negative
459 pressures at lower frequencies. This is also consistent with the findings of Sheeran et al.[39]

460

461 ***ADV vs. IC threshold***

462 Similarly consistent with previous studies, it was found that ADV occurred at lower peak
463 negative driving pressures than IC (Figure 9). This indicates that, whilst microbubble collapse may
464 promote ADV, (Lo et al. 2007) IC is not required to initiate it. Contrary to the results of Fabiilli et
465 al. (Fabiilli et al. 2009) with PFP MDs, however, the difference between the ADV and IC thresholds
466 was not statistically significant. This discrepancy may be due to differences in the definition of the
467 thresholds. As described above, the ADV and IC thresholds were defined respectively as the peak
468 negative driving pressures producing a normalized REA of >80% and a PIC >80%. This level was
469 chosen as providing an acceptable degree of repeatability between experiments, but some
470 previous studies (Fabiilli et al. 2009; Maxwell et al. 2013; Schad and Hynynen 2010b; Vlaisavljevich
471 et al. 2015a) including that of Fabiilli et al., have used smaller changes in B-mode signal amplitude
472 or PIC to define the thresholds. How this impacts the difference between IC and ADV thresholds
473 is illustrated in Figure 14, which shows the normalized REA and PIC of PFB NDs as a function of
474 peak negative acoustic pressure in degassed water at 1.0 MHz. At the peak negative pressure
475 corresponding to >80% normalized REA, a reasonable number of bubbles would already have

been formed. Hence the PIC would be relatively high and the difference between the ADV and IC thresholds small. In addition, the frequencies investigated in this study were lower than those investigated by Fabiilli et al. (Fabiilli et al. 2009) (0.5, 1 and 1.5 MHz vs. 3.5 MHz) and Schad et al. (Schad and Hynynen 2010b) found the difference between the ADV and IC threshold for PFP MD narrows as the frequency decreases. Furthermore, there were differences in the droplet size and composition which may have affected the results as discussed in the next section.

Figure 8 indicates how the number of bubbles detected in the high-speed camera images varied with peak negative pressure and the corresponding change in PIC as measured from the acoustic emissions. Both the pixel count and PIC curves show a significant increase above the background level at the same peak negative pressure, indicating that the bubbles produced by ADV immediately undergo IC. The curve for the pixel count does not show as pronounced an "S" shape with increasing pressure as does that for the PIC, but it is difficult to make a fair comparison as there is such a large difference in the size of the sampled volume between the optical and acoustical data. In particular, there may have been large numbers of bubbles forming that were not visible to the high-speed camera due to the limited depth of field.

Effect of ND size and composition

The ADV threshold decreased with increasing droplet size, consistent with published results for PFB MDs (Table 1). This is likely due to the higher internal pressure of smaller droplets resulting from interfacial tension (Laplace pressure) which increases the energy required for vaporization (Sheeran et al. 2011c; Sheeran et al. 2011a). The ADV thresholds for PFP NDs were higher than for PFB NDs, e.g. for at 1 ms pulse length the ADV thresholds were: 2.66 ± 0.28 MPa for small PFB NDs; 2.24 ± 0.13 MPa for large PFB NDs; 4.24 ± 0.22 MPa for small PFP NDs and 3.74 ± 0.34 MPa

for large PFP NDs. These are consistent with the values published by Sheeran et al.(Sheeran et al. 2011c; Sheeran et al. 2011a), for the effective boiling points of 238 nm PFB, 514 nm PFB, 235 nm PFP and 518 nm PFP which were ~ 50 ℃, 70 ℃, 82 ℃ and 110 ℃, respectively. In this study the effect of size was not statistically significant whereas that of composition was significant. This is also consistent with previous studies. Kumar et al. (Kumar 2018) and Vlaisavljevich et al.(Vlaisavljevich et al. 2015b) presented the following equation for ADV threshold pressure:

$$P_{\mathbf{threshold}} = P_{sat} - \sqrt{\frac{16\pi\sigma^3}{3K_B T} \frac{1}{\ln(\pi J_0 d^3 / 12 f \ln 2)}} \quad (3)$$

where $P_{threshold}$: vaporization pressure threshold of droplets, P_{sat} : vapor pressure in a bubble, σ : surface tension of liquid-vapor interface, K_B : Boltzmann's constant, T : temperature, J_0 : rate of nucleation per unit time per unit volume, d : diameter of droplet, f : frequency.

Equation (3) shows that the ADV threshold strongly depends on σ and T, whereas it weakly depends on d and f since they are inside the logarithmic term.

Effect of ND concentration

The ADV threshold was found to decrease with increasing ND concentration (Figure 12) with the change between 10^8 and 10^{10} ND/ml being statistically significant. This was as expected since increasing the concentration increases the number of NDs exposed to ultrasound within the focal volume, leading to a higher probability of ADV. It would also increase the probability of a ND being in close proximity to a collapsing bubble. This finding is consistent with results of Reznik et al. [43,] for PFP NDs and the results of Khirallah et al.[58] for PFH NDs. Zhang et al.(Zhang and Porter 2010), found that the ADV threshold for PFP NDs was insensitive to ND concentration but their study

was concerned with much higher volume fractions (0.15-0.40% compared with 0.0001-0.001%) where other effects such as acoustic shielding may have been important.

Effect of Temperature

The ADV threshold of PFB NDs decreased with increasing environmental temperature, as shown in Figure 13. This expected inverse relationship was consistent with the equation (3) and the results of previous studies (Porter and Zhang 2008; Sheeran et al. 2012). PFB NDs were vaporized at 1.66 MPa at 37^{o}C while frequency and pulse length were set to 1 MHz and 5,000 cycles, which is nearly 30% lower than the pressures needed to vaporize at 20 ℃ (2.29 MPa). These results, combined with the stability data are encouraging for the practical use of PFB NDs as therapeutic agents.

Implications for therapeutic applications of PFB NDs

The results confirm that suspensions of PFB NDs can be generated that are stable at both 20 and 37^{o}C but can still be vaporized by short ultrasound pulses (200 cycles) at moderate peak negative pressures (< 3 MPa at 20^{o}C and < 2.5 MPa at 37^{o}C) at relevant therapeutic frequencies (0.5-1 MHz) and low PRFs (<100 Hz); or at even lower pressures (~2 MPa) with moderate pulse lengths (1000 cycles). Contrary to the findings of several previous studies (Table 1), these conditions are comparable to those required to achieve therapeutic effects with microbubbles. This indicates that the benefits of NDs (increased circulation time and extravasation) can be exploited without the increased risk of harmful bioeffects associated with the use of high ultrasound intensities and/or high injected concentration. Additionally, PFB NDs required lower

542 acoustic pressures to achieve vaporization while the temperature increase to 37 °C (physiological
543 temperature), which may be preferable to vaporize and perform ultrasound imaging at lower
544 pressures in the body.

545 The finding that the ADV threshold falls with driving frequency for PFB NDs is also potentially
546 advantageous for therapeutic applications. First, the lower the frequency, the larger the potential
547 focal zone and hence tissue volume that can be treated, thus increasing treatment efficiency.
548 Second, lower frequency ultrasound is also more resistant to acoustic aberration and/or
549 attenuation from overlying tissue, resulting in deeper penetration depth, thereby increasing the
550 range of potential applications (Vlaisavljevich et al. 2013; Vlaisavljevich et al. 2015a).

551 The lack of a statistically significant difference between the ADV and IC thresholds indicates
552 that both B-mode and passive cavitation detection can be used for treatment monitoring over
553 the range of frequencies investigated here (0.5 – 1.5 MHz). As discussed above, however, the
554 definition of the threshold should be carefully considered depending on the specific therapeutic
555 effect (or avoidance of unwanted bioeffects) desired for the application and how this relates to
556 droplet/bubble behaviour. For example, the high-speed camera footage indicates that there are
557 considerable changes in droplet/bubble response over successive cycles (Figure 6; Supplementary
558 Video 1). This may affect the choice of pulse length depending on whether phenomena such as
559 bubble coalescence and fragmentation are desirable or not, e.g. to promote or avoid vascular
560 occlusion or microcapillary disruption.

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Limitations and future work

There is inevitably quite a large uncertainty in the measured threshold values due to: (i) the inherent uncertainty in the hydrophone measurements (calibration uncertainty is quoted as ±15%); (ii) reflections from other components in the experimental set up, e.g. from the objective in the configuration shown in Figure 1(a); (iii) attenuation of the incident pulse by the polymer tube; (iv) distortion of the field due to nonlinear propagation; and (v) changes in bubble dynamics due to confinement within the tube. The fact that there were no significant differences between the results obtained between the configurations shown in panels (a) and (b) of Figure 1 suggests that there was a minimal effect upon the incident field in this case. As indicated above, the effects of attenuation in the tube were smaller than the uncertainty in the hydrophone calibration; and the tube diameter was significantly larger than the microbubbles formed. Similarly, the harmonic content in the transmitted signal was also <10% over the range of frequencies and pressures tested. Nevertheless, these are all important considerations when comparing threshold values between experiments, and especially when predicting behaviour *in vivo*.

A further important consideration both for threshold definition and designing treatment monitoring is the sampled volume. As above, the differences in the field of view between the high-speed camera and PCD measurements are likely to have affected the shape of the curves shown in Figure 8. The volume sampled by the PCD was constant in all of the experiments reported here, but the focal volume of the FUS transducers decreased substantially with increasing frequency (please see Materials and Methods above). Due to the confining effect of the tube, in all cases the sampled volume was either smaller or comparable to the FUS transducer focal volume and thus there should have been no effect of driving frequency upon the probability of detection. At higher frequencies, or in a different environment, however, this might not be the case.

There are several important considerations for future work. Recently, it has been shown that the commercial contrast agent Definity™ can be used to prepare droplets by microbubble compression (Sheeran et al. 2017) and these have been used successfully in large animal models for cardiovascular imaging. These reports are extremely encouraging, but the use of lower boiling point PFCs still carry a higher risk of spontaneous vaporization resulting in rapid clearance and increased safety concerns over embolism. In the present study, the large bubbles observed following vaporization disappeared very quickly. Given the significant differences between the experimental set up and the tissue environment in terms of gas saturation, vessel size and the presence of biological surfactants, it would be inappropriate to assume that bubbles would similarly dissolve *in vivo*. Further studies investigating the stability of PFB NDs in serum and/or whole blood and under varying pressures corresponding to the injection process should therefore be conducted. Similarly, *in vivo* studies to quantify circulation time and clearance mechanisms are needed; and also to assess the degree of extravasation in target tissue with and without ultrasound exposure. The impact of the change in bubble dynamics over successive cycles upon the surrounding tissue should also be investigated and the feasibility of detecting these changes via PCD and/or B-mode imaging assessed.

Conclusions

The aim of this study was to investigate the vaporization of low boiling point (PFB) NDs using both optical and acoustic methods over a range of therapeutically relevant exposure conditions. The results complement those of previous studies, as shown in Table 1, by extending the range of parameters investigated, thus enabling a more comprehensive understanding of the behavior of these agents. To the best of the authors' knowledge this is also the first reported high-speed

camera (>1 Mfps) study of PFB ND vaporization; or of the simultaneous capture of acoustic emissions.

Consistent with previous studies, both the ADV and IC pressure thresholds, defined respectively as an 80% change in B-mode signal intensity or PIC, were found to decrease with increasing PRF (1-100 Hz), pulse length (20-20000 cycles) and temperature (20-45 ^{o}C). The thresholds decreased with increasing ND size and increasing ND concentration, but only the effect of concentration was found to be significant over the ranges tested (200-600 nm and 10^{8}-10^{10} ND/ml respectively). Contrary to some previous studies, the thresholds were found to increase with increasing driving frequency (0.5-1.5 MHz), likely because the NDs were too small to produce superharmonic focusing. ADV thresholds were found to be lower than IC thresholds, but there was no statistically significant difference between them for any of the parameter combinations tested. Overall the results indicate that PFB-ND vaporization can be achieved with exposure conditions that are not substantially higher than those used for therapeutic applications of microbubbles. This is encouraging for the use of PFB-NDs as cavitation agents. Future work should investigate further the observed changes in bubble dynamics over successive cycles following vaporization; confirm ND stability in vivo prior to ultrasound exposure and establish circulation times and clearance mechanisms.

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Figure Captions

Figure 1. Schematic diagram of the experimental setups employed for high-speed microscopy: (a) set up for high-speed optical imaging only; (b) set up for simultaneous optical and acoustic measurements.

Figure 2. Schematic diagram of the experimental setup for passive ADV and IC threshold measurement, containing the focused ultrasound transducer, signal generator, amplifier, PCD transducer and diagnostic ultrasound imaging device.

Figure 3. (a) B-mode images of the polyethylene tube before and after ultrasound exposure, the flow direction is denoted by an arrow, the sale bar is 5mm; (b) the plot shows the normalized relative echo amplitude as a function of applied peak negative pressure; the location of the IC threshold is denoted by an arrow. The frequency, PRF and cycles used are 1.0 MHz, 10 Hz and 1000 cycles, respectively.

Figure 4. Example of curve showing probability of inertial cavitation (PIC) as a function of peak negative acoustic pressure in degassed PBS with and without PFB NDs, the location of the IC threshold is denoted by the arrow. The frequency, PRF and no. cycles used in this experiment were 1.0 MHz, 10 Hz and 1000 cycles, respectively.

Figure 5. (a) Schematic representation of lipid coated PFB NDs. (b) Representative size distribution of PFB NDs measured by DLS. Averaged over 5 separate batches, the mean diameter ± standard deviation was 237 ± 16 nm; (c) The size changes over time at 20 °C and 37 °C. There was no statistical difference ($p > 0.05$) between diameters measured at different time points. (d) Concentration changes of PFB NDs over time at 20 °C and 37 °C. Data are averaged with error bars representing the standard deviation.

Figure 6. Example of a series of high-speed images of droplet vaporization captured over the first 5 cycles of a 100-cycle ultrasound pulse at 0.5 MHz and peak negative pressure of 1.98 MPa. The scale bar indicates 5 µm. Images were taken at 5 x 10^6 frames/s with an exposure of 200 ns per frame. The dotted lines indicate the approximate phase relationship between each frame and the incident ultrasound pulse assuming that the speed of sound in the liquid is 1481 ms^{-1}.

Figure 7. Representative acoustic emissions (first column), their corresponding frequency content (second column) and optical images (third column) from the high-speed videos for NDs exposed to different peak negative pressures. The frequency, pulse length and PRF were 0.5 MHz, 1000 cycles and 10 Hz respectively. Representative acoustic emissions (first column), their corresponding frequency content (second column) and optical images (third column) from the high-speed videos for NDs exposed to different peak negative pressures. The frequency, pulse length and PRF were 0.5 MHz, 1000 cycles and 10 Hz respectively. The optical images show the bubbles formed as the result of ND vaporisation towards the end of the high-speed camera footage, during the rarefaction phase of the ~20th cycle of the first ultrasound excitation pulse. The PCD traces show the acoustic emissions captured for this pulse. The scale bar is 20 µm. Please note that the bubbles present in the top right hand image (corresponding to a peak negative driving pressure of 0.66 MPa) were present prior to the ultrasound exposure and due to a small number of droplets vaporising upon injection into the tubing.

Figure 8. Comparison between the change in optical intensity from the high-speed video images and the PIC determined from the acoustic emissions as a function of peak negative acoustic pressure. The frequency, pulse length and PRF were 0.5 MHz, 1000 cycles and 10 Hz respectively (n=3).

Figure 9. Mean (n=3) ADV and IC peak negative pressure thresholds for PFB NDs at 1 MHz driving frequency as determined from B-mode images and PCD recordings, respectively. (a) effect of varying PRF (pulse length 5000 cycles); (b) effect of varying pulse length (PRF = 10Hz). Error bars indicate the standard deviation.

Figure 10. The effect of ultrasound frequency on the IC threshold. (a) PIC as a function of peak negative acoustic pressure in PFB NDs suspensions with a 5 ms pulse length; (b) Mean (n=3) IC thresholds of PFB NDs at frequencies of 0.5, 1 and 1.5 MHz with 1 ms, 5 ms and 10 ms pulse length respectively (* means $p < 0.05$ compared to the results of 0.5 MHz). Error bars indicate the standard deviation. Pulse length is shown in terms of ms as the number of cycles was varied with the changing driving frequency.

Figure 11. The effect of droplet core composition and size on the ADV threshold pressures of PFC NDs at a driving frequency of 1 MHz and PRF of 10 Hz with varying pulse length, n=3.

Figure 12. The effect of PFB NDs concentration on the ADV threshold pressure at different pulse lengths (1 MHz driving frequency, PRF 10 Hz, n=3).

Figure 13. The effect of temperature on the ADV threshold pressure of PFB NDs at different pulse lengths (1 MHz driving frequency, PRF 10 Hz, n=3), * means $p < 0.05$ compared to the results of 20 ^{o}C. Error bars indicate the standard deviation.

Figure 14. Normalized REA and PIC as a function of peak negative acoustic pressure. The thresholds for ADV and IC are denoted by an arrow (1 MHz driving frequency, PRF 10 Hz, pulse length 100 cycles, n=3).

PFH: Perfluorohexane; PFP: Perfluoropentane; PFB: Perfluorobutane; OFP: Octafluoropropane

Study	Core	Shell	Size (μm)	Temperature (°C)	Measurement method	Ultrasound Frequency (MHz)	Threshold (MPa)
Kripfgans et al. 2000	PFP	Albumin	90%<6	37	Acoustic/ADV	1.5~7.6	4.78~0.7
Kripfgans et al. 2002	PFP	Albumin	90%<6	37	Acoustic/ADV	2~10	3~1
Giesecke and Hynynen 2003	PFP	Albumin	1.4~2	37	Acoustic/IC	0.74~3.3	0.75~1.5
Kripfgans et al. 2004	PFP	Albumin	7~22	37	Optical/ADV	3~4	2.2~5.6
(Lo et al. 2007	PFP	Albumin	<6	37	Acoustic/ADV	1.44	3.8~5.9
Porter and Zhang 2008	PFP	Albumin	0.193	8~45	Acoustic/ADV	2	4.3~2.4
Peng Zhang and Porter 2009	PFP	Albumin	0.193	19~45	Acoustic/ADV	2	9.5~5.9
Fabiilli et al. 2009	PFP	Albumin	1~5	37	Acoustic/ADV	3.5	4.2~2.4
					Acoustic/IC		5.9~4.2
Matsuura et al. 2009	PFP	Fluorosurfactant	0.1~0.3	38	Acoustic/ADV	18	3.5
Schad and Hynynen 2010a	PFP	Lipids	1.9~7.2	37	Acoustic/ADV	1.74~2.86	1~3.9
					Acoustic/IC	0.58~2.86	2.9~4.4
Sheeran et al. 2011c	PFP	Lipids	1~13	37	Optical/ADV	5	4.47~3.13
Reznik et al. 2011	PFP	Fluorosurfactant	0.4	37	Optical/ADV	10	2.3~3.5
Williams et al. 2013	PFP	Fluorosurfactant	0.221	37	Acoustic/ADV	5~15	5.5~3.2
Reznik et al. 2014	PFP	Fluorosurfactant	0.4	37	Optical/ADV	5	3.5
Vlaisavljevich et al. 2015a	PFP	Polymer	0.178	37	Acoustic/ Optical/IC	0.345~3	7.4~13.2
Mercado et al. 2016	PFP	Albumin	2~9.75	37	Optical/ADV	2	3.7~3
Aliabouzar et al. 2018	PFP	Lipids	0.89	20	Acoustic/ADV	2.25~10	1.05~2.34
Aliabouzar et al. 2019	PFP	Lipids	0.947	20	Acoustic/ADV	2.25~15	0.4~2.57
					Acoustic/IC	2.25~15	1.6~3.5
Matsuura et al. 2009	PFH	Fluorosurfactant	0.1~0.3	38	Acoustic/ADV	18	4.6
Fabiilli et al. 2009	PFH	Albumin	1~5	44~65	Acoustic/ADV	3.5	4.6~2.8
					Acoustic/IC		6.2~4.8
Vlaisavljevich et al. 2015b; Vlaisavljevich et al. 2015a; Vlaisavljevich et al. 2016	PFH	Polymer	0.233	37	Acoustic/ Optical/IC	0.345~3	10.4~14.9
Aliabouzar et al. 2019	PFH	Lipids	0.86	20	Acoustic/ADV	2.25	2.28
			14.21			10~15	1.58~1.12
Sheeran et al. 2011c	PFB	Lipids	1~13	37	Optical/ADV	5	3.13~2.68
			0.2~0.6				3.82

Sheeran et al. 2012	PFB	Lipids	1~7	22 & 37	Optical/ADV	8	3.5~2
Sheeran et al. 2014	PFB	Lipids	0.2~0.3	37	Optical/ADV	1~8	2~3.75
Sheeran et al. 2013a	PFB	Lipids	0.2	37	Optical/ADV	1	1.4
Rojas et al. 2017	PFB	Lipids	0.2~0.3	37	Acoustic/ADV	2.25	1.83~2.5
					Optical/ADV		2.17~2.33
Rojas et al. 2019	PFB	Lipids	0.1~0.4	37	Acoustic/ADV	5	1.25~2.25
(Sheeran et al. 2012)	OFP	Lipids	1~7	22 & 37	Optical/ADV	8	2 & 0.5

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858 **Table 1：** Vaporization thresholds of PFC droplets reported in the literature and measured using

859 acoustical and optical methods.

860

		Driving Frequency		
		0.5 MHz	1 MHz	1.5 MHz
Other Parameters	PRF (Hz)	10	1~100	10
	Pulse length (cycles)	500, 2500, 5000	20~20000	1500, 7500, 15000
	ND core and size	PFB: 237 nm	PFB: 237 nm/314 nm PFP: 235 nm/518 nm	PFB: 237 nm
	Concentration (NDs/ml)	10^9	10^8, 10^9, 10^{10}	10^9
	Temperature (°C)	20	20, 37, 45	20

861 **Table 2：** Summary of experimental parameters investigated and measurements made.

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