Characterization of Microbubble Activity Under Ultra-Short Focused Pulses Delivered by an Imaging Phased Array

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***Abstract*—Theranostic Ultrasound (ThUS) has been recently developed to combine focused pulses for blood-brain barrier (BBB) opening with Power Cavitation Imaging (PCI) for treatment monitoring in a single repurposed imaging transducer. ThUS employs ultra-short focused pulses for microbubble cavitation, which enables high PCI resolution; however there have not been extensive studies into the nature of microbubble cavitation under these ultra-short pulses. In this study, we investigate the acoustic signal of microbubbles under 1.5 MHz ThUS pulses in an *in vitro* flow channel, analyzing the types of cavitation present at various pulse lengths and pressures, and assessing the *in vivo* safety of these pulses in mice. We observe significant spectral broadening in the frequency domain. However, analysis of the time domain signal for multiple pulses reveals a stable cavitation activity at the shortest pulse length of 1.5 cycles. A higher rate of microbubble collapse is observed at longer pulse lengths and higher pressures, as evidenced by a large decrease in acoustic signal amplitude over time. The *in vivo* study in mice showed no visible damage in mice treated with 1.5- and 5-cycle pulses, and only small erythrocyte extravasation for the mouse treated with 10-cycle pulses at 1.00 MPa. Therefore, this study shows that, despite the dominant broadband activity at ultra-short pulse lengths, there is limited microbubble collapse, which leads to safe BBB opening. This was an initial study to generate the parametric space for safe and efficient ThUS-mediated BBB opening.**

***Keywords*—Theranostic ultrasound, Microbubbles, Cavitation, Blood-brain barrier opening**

I. Introduction

The safe and transient opening of the blood-brain barrier (BBB) using Focused Ultrasound (FUS) and systemically administered microbubbles (MB) has been extensively studied as a drug delivery [1], gene editing [2] and immunotherapeutic technique [3]. Over the past years, numerous studies have explored the parameters that govern the efficacy and safety of FUS-mediated BBB opening, paving the way towards clinical applications and commercialization. Currently, most systems rely on the coordination of two transducers, one to induce FUS either by geometric or electronic focusing, and one to monitor microbubble cavitation activity during treatment. While this has become an established paradigm, there have been efforts to combine the therapeutic and diagnostic functionalities into a single "theranostic" transducer to enhance treatment monitoring and simplify clinical translation. Theranostic Ultrasound (ThUS) utilizes a repurposed phased array to induce microbubble cavitation and obtain simultaneous Power Cavitation Imaging (PCI) maps, thus allowing for better localization of microbubble activity within the brain. Recently published studies show successful Theranostic Ultrasound (ThUS)-mediated BBB opening and Adeno-associated Virus (AAV) delivery in mice [4].

The activity of microbubbles under ultrasonic pulses has a direct effect on the safety of FUS- and ThUS-mediated BBB opening. Microbubble cavitation activity has traditionally been classified as stable, where microbubble oscillation is sustained over time, and inertial (or transient), where microbubbles expand to more than twice their original radius and collapse, causing shock waves and microjetting [5]. Previous studies by our group have shown that safe FUS-mediated BBB opening can occur with predominantly stable cavitation, and that increased levels of inertial cavitation can lead to tissue damage and erythrocyte extravasation [6]. The nature of microbubble activity can be analyzed in the frequency domain, as stable cavitation often manifests by increases in amplitude at harmonic and ultraharmonic frequencies, whereas inertial cavitation produces a broadband non-specific acoustic signal. In the realm of ultra-short pulses, such as those employed by ThUS, the small duration of the signal in the time domain causes spectral broadening in the frequency domain, leading to uncertainty in the determination of harmonic and ultraharmonic signal, and increased detection of broadband signal, an indication of inertial cavitation. Since inertial cavitation can be detrimental to the safety of ThUS-mediated BBB opening, it is important to characterize the microbubble activity under ultra-short ThUS pulses to establish a safety and efficacy framework for the new theranostic technology. In this study, we investigate the effect of pulse length and pressure on the spectral signature of microbubble activity *in vitro*, the induced microbubble collapse, and the associated effects in mice *in vivo*.

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II. Materials and Methods

A. Theranostic Ultrasound System

A repurposed phased array (P4-1, ATL Philips, Center Frequency: 2.5 MHz, Bandwidth: 1.5-3.5 MHz, 96 elements) was driven by a research ultrasound system (Vantage 256, Verasonics Inc., Kirkland, WA) at a frequency of 1.5 MHz. The transducer was electronically focused at an axial distance of 35 mm, where hydrophone calibration measurements show a -6dB focal area of 1.3 mm, 8 mm and 12.40 mm in the lateral, elevational and axial directions respectively. The system was programmed to transmit bursts of 100 focused pulses at a pulse repetition frequency (PRF) of 1000 Hz and a burst repetition frequency (BRF) of 0.5 Hz. Power Cavitation Images were calculated after each burst using a previously published GPU-accelerated delay and sum beamforming algorithm [7].

B. In vitro Flow Phantom Experiments

A schematic of the setup for the *in vitro* study is shown in Figure 1. A 3 cm piece of regenerated cellulose micro-dialysis hollow fiber (Spectra/Por, Spectrum Laboratories, Rancho Dominguez, CA) with inner diameter 200 μm and outer diameter 216 μm was fixed with glue between two pieces of silicone tubing with inner diameter 500 μm and outer diameter 940 μm (Item 51845K66, McMaster-Carr, Elmhurst, IL) and was in turn attached to two poles and immersed in a tank of degassed and deionized water in such way that there was excess silicone tubing on both sides of the water tank. A rubber absorber was placed at an approximately 30^o angle underneath the flow channel. The P4-1 array was fixed on a 3-axis positioning system (VXM, Velmex Inc., Bloomfield, NY) and using B-mode imaging the transducer was immersed in the water tank and placed at a distance 35 mm above the cellulose tube segment. A 3.5 MHz center frequency single element transducer (C384-SU, Olympus, Waltham, MA) was used as a passive cavitation detector (PCD) and was placed in the water tank perpendicularly to the P4-1 transducer and at the same height as the flow channel. The PCD was connected to a pulser/receiver (5072PR, Olympus, Waltham, MA), which was in turn connected to an oscilloscope (Picoscope 5000 Series, Pico Technology, St. Neots, UK). The transducer and PCD were aligned by moving the transducer laterally and elevationally while transmitting focused ThUS pulses, until the signal registered by the oscilloscope did not increase further. An infusion pump (Genie Plus, Kent Scientific) was used to control the flow of microbubble solution through the flow channel at a constant rate of 100 μL/min.

In this study, in-house synthesized, lipid-shelled, polydisperse microbubbles were synthesized and activated according to a previously published protocol [8]. The average concentration of microbubbles with diameter between 1 and 10 μm in the vial was 1.14×10^{10} MB/mL. Baseline measurements, where degassed and deionized water was flown through the channel were taken first, followed by a solution of lipid microbubbles diluted to an approximate concentration 8×10^8 MB/mL. The acoustic response of the flow channel was recorded by the PCD for baseline and microbubble solution conditions for pressures 0.75, 1.00, 1.25, and 1.50 MPa and pulse lengths of 1.5, 5, 10, and 20 cycles. The oscilloscope was set to record one entire burst per buffer at a sampling rate of 62.5 MHz.

The acquired results were analyzed in the frequency domain for cavitation dose calculation. A Fast-Fourier Transform of each pulse was performed on MATLAB using 2^{19} points. Frequency bands with 50 kHz bandwidth were centered around the harmonic and ultraharmonic frequencies and the maximum amplitude within these bands was used to calculate stable cavitation dose. The inertial cavitation dose was determined by the amplitude of the frequency spectrum outside the harmonic and ultraharmonic bands.

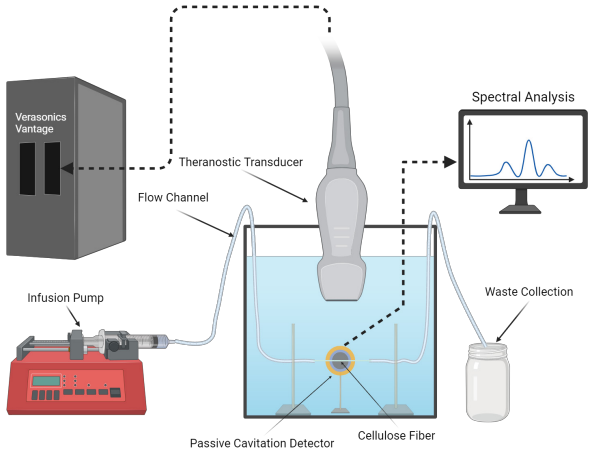

Fig. 1. Experimental Setup for *in vitro* flow phantom experiment. Created using Biorender.com.

C. In vivo Safety Assessment

Three wild-type black male mice (C57BL/6) were sonicated bilaterally at both hippocampi, using a different pulse length for each mouse, either 1.5, 5, or 10 cycles. The mice were anesthetized with isoflurane and placed in a stereotaxic apparatus. Their heads were shaven and depilated, degassed ultrasound gel was applied on the head, and a degassed water bath was placed on top of the head. The P4-1 transducer was immersed in the water bath and targeted over the lambdoid suture using B-mode imaging. The hippocampi were targeted by moving the transducer 2.2 mm anterior of lambda and 2.3 mm right or left of the center of the brain. The right hippocampus was sonicated first at a pressure of 1.00 MPa for 2 minutes and then the transducer was moved over the left hippocampus, which was sonicated at a pressure of 0.75 MPa for 2 minutes. A 50 μL solution of microbubbles (approximate concentration: 8×10^8 MB/mL) was systemically injected through the tail vein before each sonication using a winged catheter and a Hamilton syringe.

BBB opening was confirmed through contrast-enhanced T1-weighted MRI. After the sonication, each mouse was intraperitoneally injected with 0.2 mL of gadodiamide contrast

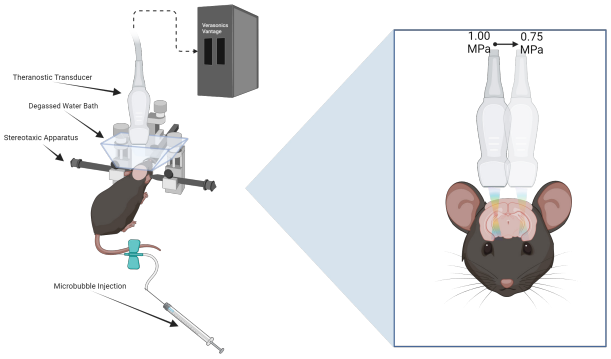

Fig. 2. Experimental Setup for *in vivo* mouse safety assessment. Created using Biorender.com.

agent (Omniscan™, GE HealthCare, Princeton, NJ) and placed in a 9.4 T Bruker vertical bore magnet. T1-weighted 2D FLASH sequences were used to acquire coronally- and axially-oriented images of the brain, and BBB opening was confirmed as regions of hyperintensity due to increased diffusion of the contrast agent. The hyperintense areas were subsequently quantified using a custom MATLAB script.

Twenty-four hours after sonication, the mice were exsanguinated and transcardially perfused first with phosphate-buffered saline (PBS) for 5 minutes, followed by 4% paraformaldehyde (PFA) solution for 5 minutes. The brains were extracted and placed in 4% PFA for 48 hours, and then in PBS for 24 hours. The samples were then paraffin embedded, coronally sectioned, mounted on slides, and stained with hematoxylin and eosin (H&E). The stained sections were imaged using an optical microscope (Leica DM6 B, Leica Microsystems Inc., Buffalo Grove, IL, USA) at 10x and 20x magnifications.

III. Results

A. In vitro flow phantom

Reference measurements with degassed and deionized water were taken before any microbubble solution was introduced to the flow channel, to avoid any contamination of the phantom. PCD readings for baseline measurements show a stable intensity of the reflected waves throughout the burst, without any significant fluctuation in amplitude. The amplitude peak ranged from 20 mV for the 0.75 MPa and 1.5 cycle burst, to 42 mV for the 1.50 MPa and 20 cycle burst.

When microbubbles were introduced in the phantom, a large increase in amplitude was observed. The weakest burst at 0.75 MPa and 1.5 cycles produced a peak intensity response of 210 mV and the highest intensity recorded was 489 mV for the 1.50 MPa and 20 cycle pulse. The average peak intensity of the acoustic emissions of the microbubbles increased with pressure from 164± 34 mV at 0.75 MPa to 237± 90 mV at 1.50 MPa, while for variable pulse lengths the peak amplitude increased only between 1.5 and 5 cycles, remaining almost constant for longer pulse lengths. Contrary to the baseline measurements, where a stable intensity was observed throughout the burst,

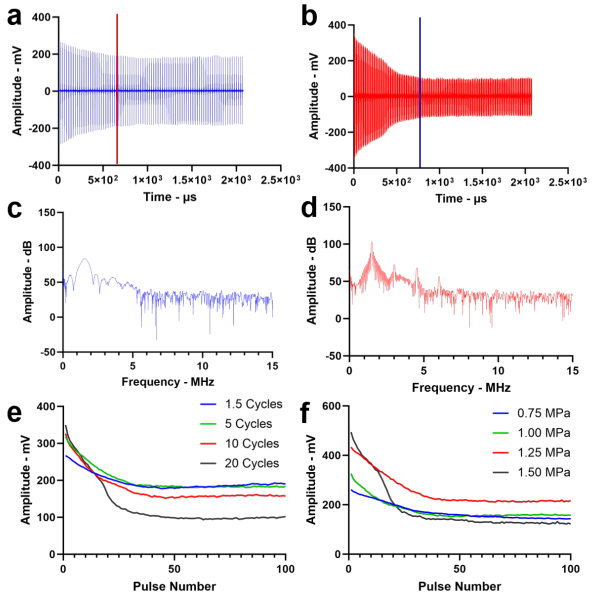

Fig. 3. Acoustic emission of flow phantom for bursts at 1.00 MPa and pulse length 1.5 cycles **(a)** and 20 cycles **(b)**, showing a larger decrease in amplitude for the longer pulse. The vertical lines signify the point where most microbubble collapse ends and stable cavitation follows. The corresponding Fourier transforms for the first pulse of the 1.5 cycle **(c)** and 20 cycle **(d)** sequence shows significant spectral broadening for the shorter pulse and distinct harmonic signals for the longer pulse. The burst amplitude decreases more rapidly and to a lower final intensity as both pulse length **(e)** and pressure **(f)** increase.

for the microbubble solution the signal amplitude decreased during the burst at a rate and to an extent dependent on pressure and pulse length (Fig. 3). It is also observed that for all pulse lengths, the burst signal exhibits an initial sharper decrease, followed by a period of stable amplitude for the remainder of the burst (Fig. 3).

Frequency analysis of the pulses revealed increased intensity of harmonic, ultraharmonic and broadband signals when microbubbles were introduced, compared to the water-only baseline. Harmonic signals were significantly broadened for the shortest, 1.5 cycle pulse, and they became more pronounced as pulse length increased (Fig. 3). Harmonic stable cavitation dose increased with pulse length, from 83.6 dB at 1.5 cycles to 93.3 dB at 20 cycles, whereas ultraharmonic and inertial cavitation doses both decreased with pulse length from 90.7 dB and 90.1 dB respectively at 1.5 cycles to 83.8 dB and 80.8 dB respectively at 20 cycles.

B. In vivo safety assessment

After the BBB opening and MRI procedures, the mice did not show any signs of abnormal behavior and appeared fully recovered. For all sonicated mice, two distinct regions of hyperintensity were visible upon acquisition of contrast-enhanced T1-weighted MRI, indicating successful BBB opening for all pressures and pulse lengths. The BBB opening volume was quantified with a custom MATLAB algorithm, using the axially oriented MRI slices and isolating the regions

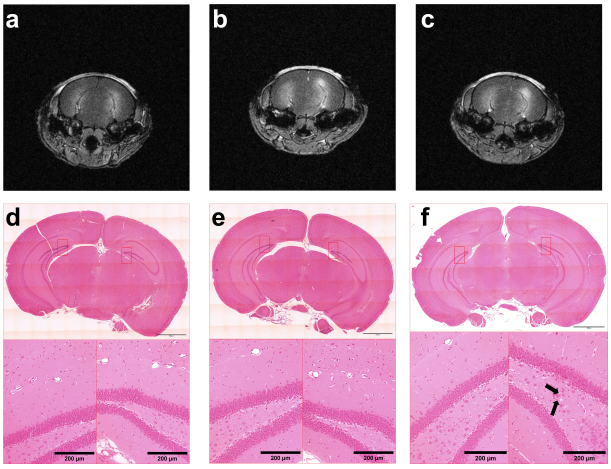

Fig. 4. Coronal MRI sections showing BBB opening for mice treated at 1.5 cycles **(a)**, 5 cycles **(b)** and 10 cycles **(c)**. Every mouse was sonicated on the right side with a derated pressure of 1.00 MPa and on the left side with a derated pressure of 0.75 MPa. H&E staining of the brains of the mice revealed no visible erythrocyte extravasation for 1.5 cycles **(d)**, and 5 cycles **(e)**, and only small extravasation for 10 cycles **(f)** at the side treated with 1.00 MPa.

of hyperintensity. The BBB opening volume increased with both pressure and pulse length; the smallest BBB opening (17.75 mm^3) was observed at 0.75 MPa and 1.5 cycles and the largest (37.31 mm^3) was observed at 1.00 MPa and 10 cycles.

Erythrocyte extravasation and cell death 24 hours after treatment were evaluated by analyzing hematoxylin and eosin (H&E) stained sections. Only in the case of the mouse treated with 1 MPa and 10 cycles was some slight erythrocyte extravasation observed, and no damage was detected for any other mouse (Figure 4).

IV. Discussion and Conclusions

This is the first study to report on the type of microbubble cavitation activity under ultra-short ThUS pulses. Due to the large spectral broadening caused by the ultra-short pulse length, increased broadband signal was observed for the 1.5 cycle pulse, leading to a calculation of higher inertial cavitation dose. However, we also observe that the microbubble activity throughout a ThUS burst of 100 pulses remains approximately stable for this pulse length, which is evidence of limited microbubble collapse. On the other hand, there is a much sharper and drastic decrease in microbubble activity for longer pulses. This shows that there is a higher rate of transient cavitation for longer pulse lengths, as the decreased signal amplitude implies that there are fewer sources of acoustic signal, thus pointing to more microbubble collapse. Therefore, our results show that the presence of large broadband signal in the frequency domain of ultra-short pulses is not directly linked with microbubble collapse.

The conclusions of the *in vitro* flow channel experiment were validated *in vivo* using a mouse model. The low rate of microbubble collapse for the 1.5 cycle pulses translated to no visible damage in the brain, while the 10 cycle pulse, which induces more transient cavitation, causes slight damage at higher pressures. Future work including more sample points can inform the parametric space of safe ThUS-mediated BBB opening.

Future studies will focus on the analysis of the microbubble behavior through transcranially-applied ThUS, using murine and non-human primate skulls. The effect of ThUS pulses on brain homeostasis, and particularly on the response of the immune system, will also be explored. In conclusion, this study provides with an initial assessment of microbubble activity under ThUS pulses, and shows stable and sustained cavitation activity that could lead to safe BBB opening with ultra-short pulses, despite the broadband activity dominance.

Acknowledgments

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