Microbubble mediated dual-frequency high intensity focused ultrasound thrombolysis: An *In vitro* study

Dingjie Suo,[1] Zhiyang Jin,[2] Xiaoning Jiang,[1] Paul A. Dayton,[3] and Yun Jing[1,a)]
[1]*Department of Mechanical and Aerospace Engineering, North Carolina State University, Raleigh, North Carolina 27695, USA*
[2]*Department of Physics, Nanjing University, Nanjing, Jiangsu 210093, China*
[3]*Joint Department of Biomedical Engineering, University of North Carolina and North Carolina State University, Chapel Hill, North Carolina 27599, USA*

(Received 3 November 2016; accepted 28 December 2016; published online 10 January 2017)

High intensity focused ultrasound (HIFU) has recently emerged as a promising alternative approach for thrombolysis. However, the high acoustic energy required by HIFU could elicit thermal damage bioeffects, impeding the clinical translation of this technique. This paper investigates the use of dual-frequency focused ultrasound (DFFU) mediated by microbubbles (MBs) to minimize the acoustic power required for thrombolysis *in vitro*. It was found that MBs, with sufficient concentration, could significantly lower the power threshold for thrombolysis for both DFFU and single-frequency focused ultrasound (SFFU). In addition, SFFU needs about 96%–156% higher energy to achieve the same thrombolysis efficiency as that of DFFU. The thrombolysis efficiency is also found to increase with the duty cycle. The measured cavitation signals reveal that the enhanced inertial cavitation is likely responsible for the improved thrombolysis under DFFU and MBs. *Published by AIP Publishing*. [http://dx.doi.org/10.1063/1.4973857]

To date, the only Food and Drug Administration (FDA) approved treatment for acute ischemic stroke is intravenous injection of tissue plasminogen activator (tPA). However, this technique is only effective within 4.5 h of the symptom onset. Furthermore, the use of tPA can exacerbate intracerebral hemorrhage.[1] Finally, this technique is characterized with a low recanalization rate[2] as the chronic components of the thrombi have fibrous network which are stiff, retracted, and not responsive to thrombolytic drugs.[3,4]

Ultrasound has been suggested to be a potential alternative approach for recanalization. Ultrasound could be used in combination with tPA or contrast agents,[5] or even as a standalone treatment.[6–8] When combined with tPA, ultrasound can increase the recanalization rate at diagnostic intensities[9] but could also be associated with an increased symptomatic hemorrhage.[10–12] Researchers have also demonstrated *in vivo* and *in vitro* that relatively short-pulsed, high intensity focused ultrasound (HIFU) alone can mechanically disintegrate a clot within minutes.[6,7,13–15] Although this technique is drug free (therefore avoiding the associated side effects), non-invasive, and yields rapid lysis, there are still issues that need to be addressed before HIFU can be applied in a clinical setup for treating acute stroke patients.

Frequencies must be in the MHz range for safe treatment of stroke using HIFU because a higher frequency yields a smaller focal size, reducing the risk of tissue erosion outside the desired region.[8,16] However, due to the strong attenuation of the skull as well as high inertial cavitation thresholds at high frequencies, a large amount of acoustic power is required for thrombolysis through an intact skull at a frequency over 1 MHz.[17] To achieve this, advanced transducer arrays are required for beam focusing through the skull,[17] which are complicated and expensive, reducing the likelihood of clinical translation. In addition, a large amount of acoustic power delivered at high frequencies not only raises concern for brain tissue damage around the focus, but also could potentially overheat the skull and damage neighboring tissue. One existing solution is to use microbubbles (MBs) since they are known to be able to considerably lower the required acoustic power for initiating cavitation.[18] Notably, recent studies have shown that MBs and ultrasound mediated thrombolysis can be effective *in vitro* and *in vivo* without using thrombolytic drugs[5,15,19–22] and without apparent side effects. To further reduce the power required by HIFU for thrombolysis, additional methods other than using MBs should be developed.

Recent studies from a few groups including ours showed that dual-frequency focused ultrasound (DFFU) holds great potential in enhancing ablation efficacy, thrombolysis efficiency, and cavitation.[23–29] Liu and Hsieh found that single element transducers with dual frequency could enhance both inertial and stable cavitation.[23] Recent papers by Guo *et al.*[26] and Yang and Jo[27] reported that the detected inertial cavitation signal was stronger for DFFU than single-frequency focused ultrasound (SFFU). Other researchers also observed a nearly doubled thrombolysis efficiency at certain power levels during thrombolysis experiments.[28,29] In this study, we investigate an approach for HIFU-based clot dissolution, where DFFU and MBs are combined to enhance the inertial cavitation and therefore the thrombolysis efficiency.

Bovine blood clots were prepared using the same method as described in Ref. 28. Before experiments, the clots were immersed in 37 °C water for 30 min and then drained into a 1 ml latex free syringe (4.65-mm inner diameter (ID), 6.55-mm outer diameter (OD)) with saline solution for thrombolysis. The experimental system is shown in Fig. 1. All experiments were conducted in a water tank filled with degassed water at 37 ± 0.5 °C. A three-dimensional (3-D)

a)Author to whom correspondence should be addressed. Electronic mail: yjing2@ncsu.edu

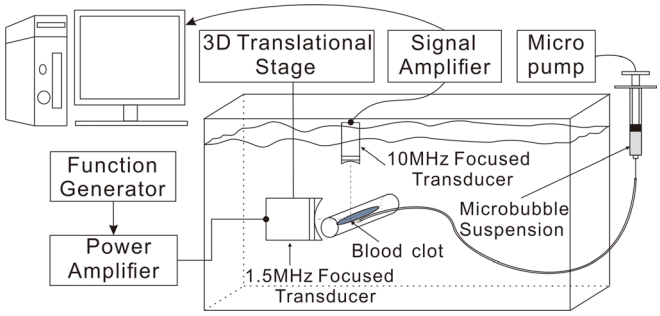

FIG. 1. Schematic of the experimental system.

translational stage (Velmex, Inc., Bloomfield, NY) was used to move a 1.5 MHz HIFU transducer (Blatek, Inc., State College, PA) to target the clot. The aperture diameter and focal length are 29.5 mm and 30 mm, respectively. A 10 MHz (Panametrics V309, Olympus, Center Valley, PA) broad-band focused transducer was placed orthogonal to the axial direction of the HIFU transducer to collect the inertial cavitation signal. The two transducer focal points coincided within the clot. An RF power amplifier (3100L, Electronics and Innovation, Rochester, NY) was used to amplify signals generated from a function generator (AFG3101, Tektronix, Inc., Beaverton, OR). A high-intensity hydrophone (HNA-0400, ONDA, Sunnyvale, CA) was used to calibrate the peak negative pressure (PNP) at the focus of the HIFU transducer. Burst waves were used with a pulse length 100 μs and 10% duty cycle except for the ones where the duty cycle was varied.

MBs were produced from lipid solutions as described in Ref. 30. Before experiments, the MBs were activated using a shaker for 45 s (Bristol-Myers Squibb Medical Imaging, Inc., N. Billerica, MA). The radius of the MB was 0.9 ± 0.45 μm at a concentration of 1×10^{10} MBs/ml after activation. 1 ml MBs was dissolved into 10 ml, 100 ml, and 1000 ml saline solution in order to vary the concentration from 1×10^9 MBs/ml to 1×10^7 MBs/ml. The MBs were subsequently drawn into a 30 ml syringe (Henke Sass Wolf, Tuttlingen, Germany) which was installed on a micropump (DUAL-NE-1010_US, New Era Pump System, Inc., Farmingdale, NY). The micropump was configured to have a flow rate of 100 μl/min during the whole process.[31] For each experiment, the pump was turned on for 30 s before the ultrasound was activated and was kept on until the end of the experiment. The syringe containing MBs was shaken manually after each experiment to stop MBs from concentrating on the surface of the suspension.

Each clot was blotted and weighted (170 ± 20 mg) before experiments. The total number of clots used in the experiments was $n = 105$. The clots were pushed into the syringes filled with saline solution. The syringes containing the clots were then connected with the pump system using a narrow tube through which the MBs were delivered to the clot site. The exit of the narrow tube was located beside the center of the clot along its axial direction (the tube was not inserted into the clot). Each clot was cut into roughly 17-mm long and 4-mm in diameter and was flushed with saline solution. During the treatment, the HIFU transducer was moved by the 3-D translational stage at 3 mm step size along the length of the clot to treat the entire clot. The system paused at each location for a period of time t/4 for each step, where t is the total treatment time. After the treatment, the debris from the syringe was injected onto a 100 μm filter and blotted gently before weighing using tissue wipers. The control group experiments were conducted the same way except that HIFU was not activated. All thrombolysis experiments were repeated 5 times, and the standard deviation and *p*-value were calculated using a T-test with one-tailed distribution. The single frequency excitation had a center frequency at 1.5 MHz while the dual-frequency one had two center frequencies at 1.5 MHz and 1.45 MHz.[28] The two frequencies were chosen because of the relatively narrow bandwidth of the HIFU transducer and also the fact that no significant differences were found between dual-frequency excitations with different frequency differences (e.g., 1.5 MHz + 1.475 MHz, 1.5 MHz + 1.45 MHz, and 1.5 MHz + 1.4 MHz).[28] The output powers at different input levels were calibrated by a power meter (UPM-DT-1000PA, Ohmic Instruments, St. Charles, MO) prior to the experiment to ensure that the output powers were the same for SFFU and DFFU.

The cavitation signal was processed after it was received by the 10 MHz focused transducer and amplified by a signal amplifier (AH-2020, ONDA, Sunnyvale, CA). For each cavitation detection experiment, cavitation signals were captured every 10 s, and a total of 10 signal samples were taken. The length of each capture was 4 ms which consisted of 4 bursts at a sampling rate of 50 MHz. Each signal was transformed into the frequency domain, and all 10 signal samples of that one experiment were averaged. Finally, every 100 points (a 25 kHz range in the frequency domain) of the averaged data were substituted with their root-mean-square value to generate a clear frequency-domain figure. The thrombolysis efficiency was calculated using the clot weight loss percentage.[28] An efficiency of 100% indicates a complete thrombolysis.

Various PNPs were examined by fixing the other acoustic parameters. The PNPs shown in figures below only represent those of SFFU. The DFFU results are always compared with those of SFFU under the same power, but with different PNPs. To achieve the same output power, the PNPs for DFFU was about 30%–40% higher as demonstrated in our previous study.[28] The I_{sppa} for all achieved PNPs were 159 W/cm^2, 257 W/cm^2, 364 W/cm^2, 551 W/cm^2, and 733 W/cm^2. The I_{spta} could approximately be calculated using $I_{spta} = I_{sppa} \times$ duty cycle. For 10% duty cycle, the corresponding I_{spta} were 15.9 W/cm^2, 25.7 W/cm^2, 36.4 W/cm^2, 55.1 W/cm^2, and 73.3 W/cm^2, respectively.

Figure 2 shows the thrombolysis efficiencies with 10^9 MBs/ml, 3 min treatment time, and 10% duty cycle. Figures 2(a) and 2(b) indicate that DFFU excitation could achieve an efficiency above 25% at a pressure level as low as 2.5 MPa and the efficiency was 11% higher than the control group. On the other hand, SFFU could only achieve about the same efficiency at a pressure level between 3.5 and 4.0 MPa. In terms of energy, SFFU needs about 96%–156% higher acoustic energy since the energy is roughly proportional to pressure square. It should be noted from Fig. 2(b) that once the pressure threshold for thrombolysis is achieved, e.g., around 2.5 MPa for DFFU and around 3.0 MPa for SFFU, there is a dramatic increase in thrombolysis efficiency which indicates that inertial cavitation possibly occurred and this

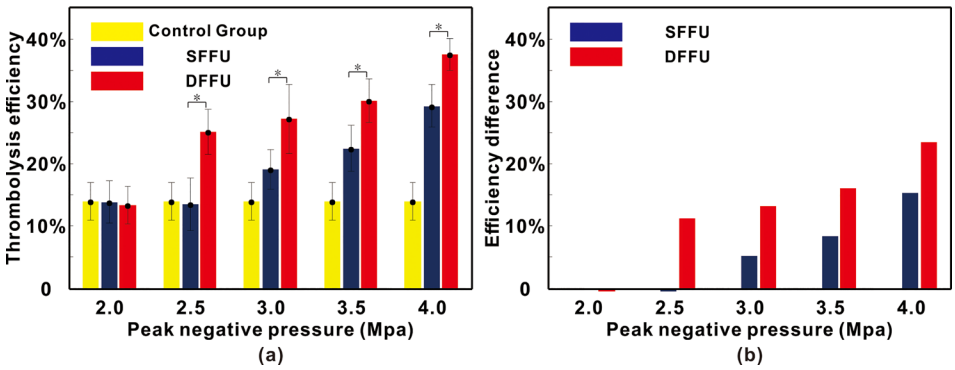

FIG. 2. Thrombolysis efficiency vs. PNP. Acoustic parameters: 10^9 MBs/ml MB concentration, 10% duty cycle, 100 μs pulse length, and 3 min treatment time. (a) Thrombolysis efficiency; (b) thrombolysis efficiencies of SFFU and DFFU minus the thrombolysis efficiency of the control group. (*$p < 0.005$.)

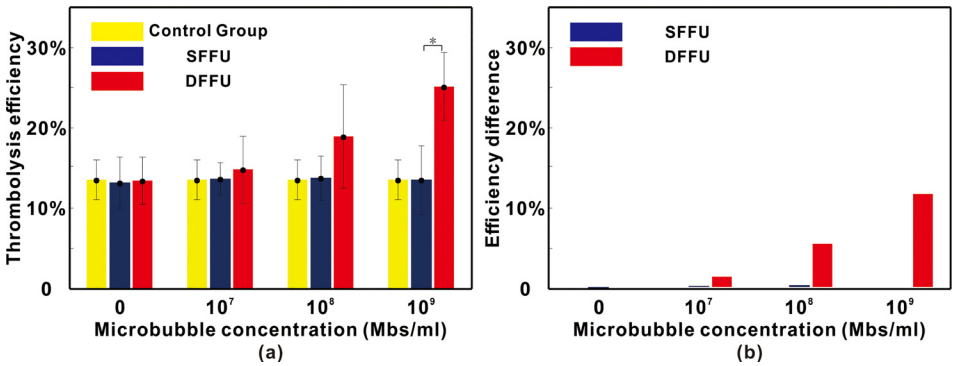

FIG. 3. Thrombolysis efficiency vs. MB concentration. Acoustic parameters: 2.5 MPa PNP, 10% duty cycle, 100 μs pulse length, and 3 min treatment time. (a) Thrombolysis efficiency; (b) thrombolysis efficiencies of SFFU and DFFU minus the thrombolysis efficiency of the control group. (*$p < 0.005$.)

will be later confirmed by our passive cavitation experiments. Beyond the threshold, the thrombolysis efficiency gradually increases with the pressure level. Except for the case where both SFFU and DFFU were ineffective at 2.0 MPa, DFFU was at least 28% more efficient than SFFU at each pressure level. The highest contrast was found at 2.5 MPa, where DFFU is 86% more efficient than SFFU (25% thrombolysis efficiency vs.14% thrombolysis efficiency). The thrombolysis power threshold for DFFU was about 30% lower than SFFU (2.5 MPa vs. 3.0 MPa). Note that this is only an approximation since the exact pressure/power thresholds were not measured in this study.

Figure 3 shows the effect of MB concentration (0 MBs/ml, 10^7 MBs/ml, 10^8 MBs/ml, and 10^9 MBs/ml) on thrombolysis efficiencies for both SFFU and DFFU at 2.5 MPa PNP. At 0 MBs/ml concentration (without MBs) and also 10^7 MBs/ml, neither SFFU nor DFFU had any effect on the clot which indicates none of them reached the threshold for thrombolysis. The SFFU thrombolysis efficiency was similar to that of the control group whereas DFFU thrombolysis efficiency increased progressively with the growing MBs concentration starting from 10^7 MBs/ml. This is possibly due to the fact that more MBs lead to more cavitation nuclei and stronger inertial cavitation activities.[32]

The effect of duty cycle was also examined for both SFFU and DFFU. The duty cycle was varied by adjusting the pulse length/pulse repetition period and three values of duty cycle were tested, i.e., 2.5%, 5%, and 10%, corresponding to 250 Hz, 500 Hz, and 1 kHz for the pulse repetition frequency. Figure 4 illustrates the relationship between thrombolysis efficiency and duty cycle at 2.5 MPa PNP and the MB concentration of 10^9 MBs/ml. At this pressure level, the thrombolysis efficiency of SFFU was similar with the control group for duty cycle less than 10%, and there was no appreciable difference between the results of the three duty cycles tested. In contrast, the thrombolysis efficiency of DFFU increased with the growing duty cycle. The increase was more significant when the duty cycle changed from 2.5% to 5% than from 5% to 10%. This suggests that 5% duty cycle could be potentially a good choice for thrombolysis as it is less energy-consuming than the 10% duty cycle and it causes less heating in the tissue.

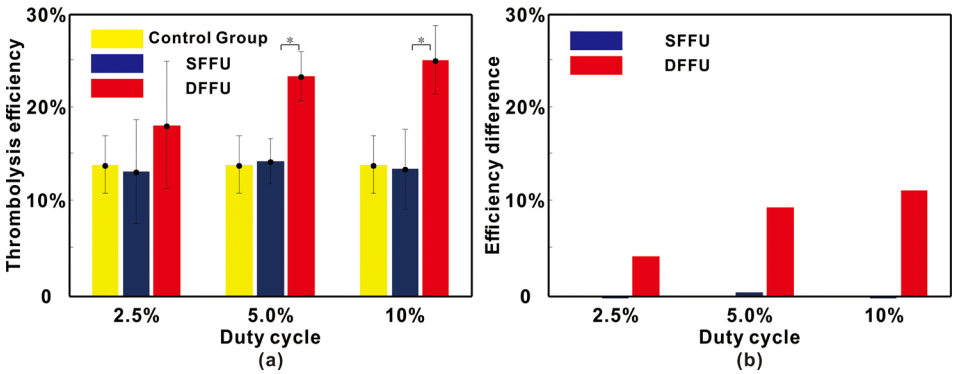

FIG. 4. Thrombolysis efficiency vs. duty cycle. Acoustic parameters: 10^9 MBs/ml MB concentration, 2.5 MPa negative peak pressure, 100 μs pulse length, and 3 min treatment time. (a) Thrombolysis efficiency; (b) thrombolysis efficiencies of SFFU and DFFU minus the thrombolysis efficiency of the control group. (*$p < 0.005$.)

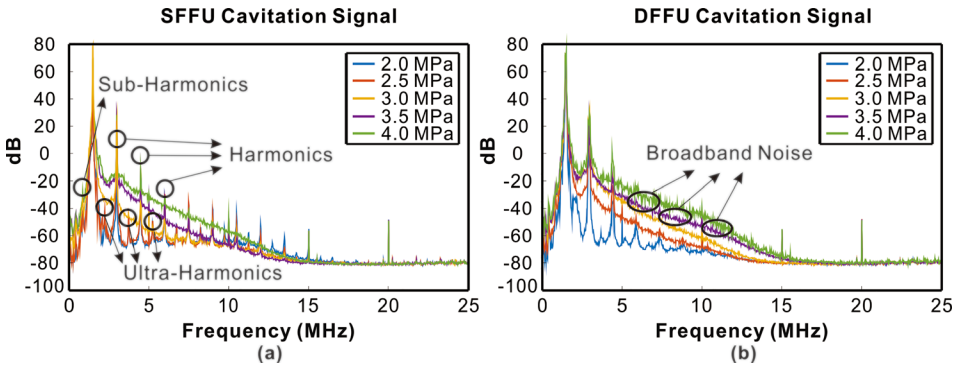

FIG. 5. Detected cavitation signals for (a) SFFU and (b) DFFU at different pressure levels.

To understand the underlying mechanism of enhanced thrombolysis efficiency of DFFU, the cavitation signal was measured at various power levels at 10% duty cycle. Figure 5 presents the wide-band cavitation spectra of SFFU and DFFU. The detected signals for low pressure levels (e.g., 2 MPa) clearly show the harmonics, sub-, and ultra-harmonics which are due to nonlinear wave propagation and stable cavitation, respectively. At higher pressure levels, sub- and ultra-harmonics disappeared and broadband noise arise due to the inertial cavitation. The results show that ultra-harmonics could be observed for SFFU at PNPs lower than 3.0 MPa and DFFU at PNPs lower than 2.5 MPa, implying most of MBs were undergoing stable cavitation. The detected broadband noise for SFFU surged as the pressure increased from 2.5 MPa to 3.0 MPa, indicating the onset of inertial cavitation. The same phenomena happened for DFFU as the pressure transitioned from 2.0 MPa to 2.5 MPa. These cavitation results are well-correlated with the previously demonstrated thrombolysis results at different pressure levels, suggesting that the enhanced thrombolysis is likely due to the enhanced inertial cavitation. The stronger inertial cavitation is possibly due to the nonlinear frequency mixing originated from the two fundamental frequencies and a higher negative peak pressure of DFFU.[28]

In conclusion, this paper demonstrated *in vitro* thrombolysis using SFFU and DFFU mediated with MBs. While it is known that DFFU and MBs can individually reduce the power threshold for thrombolysis, this study reveals that these two techniques combined can further reduce the power required for thrombolysis and enhance the thrombolysis efficiency. The detected cavitation signal indicated that the enhanced inertial cavitation is likely responsible for the enhanced thrombolysis efficiency. Our approach that integrates DFFU and MBs for enhancing inertial cavitation, not only is useful for thrombolysis, but can also find utility in tissue ablation, drug delivery, and lipid extraction.[33] Our future work will further investigate the usefulness of DFFU for thrombolysis in more realistic situations including the *in vitro* flow model and *in vivo* model, where blood flow and tissue attenuation can be taken into account.

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