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***In vitro* sonothrombolysis enhancement by transiently stable microbubbles produced by a flow-focusing microfluidic device**

Adam J. Dixon, Ph.D.^{*}, John Marschner Robert Rickel^{*}, Brian D. Shin^{*}, Alexander L. Klibanov, Ph.D.^{*†}, and John A. Hossack, Ph.D.^{*}

^{*}Department of Biomedical Engineering, University of Virginia. Charlottesville, VA 22908

[†]School of Medicine – Cardiovascular Division, University of Virginia, Charlottesville, VA 22908

Abstract

Therapeutic approaches that enhance thrombolysis by combining recombinant tissue plasminogen activator (rtPA), ultrasound, and/or microbubbles (MBs) are known as sonothrombolysis techniques. To date, sonothrombolysis approaches have primarily utilized commercially available MB formulations (or derivatives thereof) with diameters in the range 1 – 4 μm and circulation lifetimes between 5 – 15 min. The present study evaluated the *in vitro* sonothrombolysis efficacy of large diameter MBs ($d_{\text{MB}} = 10 \mu\text{m}$) with much shorter lifetimes that were produced on demand and in close proximity to the blood clot using a flow-focusing microfluidic device. MBs with a N_2 gas core and a non-crosslinked bovine serum albumin shell were produced with diameters between 10 – 20 μm at rates between $50 - 950 \times 10^3$ per second. Use of these large MBs resulted in approximately 4.0 – 8.8 fold increases in thrombolysis rates compared to a clinical rtPA dose and approximately 2.1 – 4.2 fold increases in thrombolysis rates compared to sonothrombolysis techniques using conventional MBs. The results of this study indicate that the large diameter microbubbles with transient stability are capable of significantly enhanced *in vitro* sonothrombolysis rates when delivered directly to the clot immediately following production by a flow focusing microfluidic device placed essentially *in situ* adjacent to the clot.

Key terms

thromboembolism; sonothrombolysis; microfluidics; microbubbles; ultrasound

Introduction

Ischemic stroke, venous thromboembolism, and pulmonary embolism result from blood vessel occlusion caused by either *in situ* thrombus formation or by migration of a thrombus from an upstream blood vessel to a smaller downstream vessel.¹ Together, these conditions affect over 1.3 million individuals in the United States and over 20 million worldwide each year.² Intravenous or intra-arterial administration of recombinant tissue plasminogen activator (rtPA), either peripherally or through a catheter, remains the most common therapeutic intervention for these conditions.^{3,4} Unfortunately, rtPA is ineffective in many individuals, and the majority of patients presenting with stroke or venous thromboembolism are contraindicated for the full course of rtPA therapy due to bleeding and hemorrhagic risk.⁵ Therefore, there is a significant need for improved therapeutic interventions, addressing

both efficacy and safety, across the entire spectrum of diseases that result from thromboembolisms.

Thrombolysis assisted by ultrasound and/or microbubbles (MBs), referred to as sonothrombolysis, has been studied extensively in *in vitro*, *in vivo*, and clinical settings as a means to safely accelerate thrombolysis. Clinical studies of sonothrombolysis have reported improved recanalization rates accompanied by an increased risk of off-target bleeding or intracerebral hemorrhage (ICH).^{6–8} However, given that each clinical trial used ultrasound parameters and MB formulations optimized for diagnostic imaging, and not for therapy, there remains an opportunity to increase recanalization rates and mitigate the risk of off-target bleeding by designing an ultrasound parameter set and MB platform specifically for localized therapeutic delivery for thrombolysis applications.

The presence of MBs reduces the acoustic pressure required for cavitation, and it has been demonstrated that acoustically-driven MBs mechanically disrupt the fibrin mesh, increase fluid mixing via MB microstreaming, and expose deeper regions of the fibrin mesh for rtPA enzymatic activity.^{9–12} Accordingly, significant effort has been applied to determining the influence of ultrasound parameters on sonothrombolysis efficacy. Parameters studied include: ultrasound frequency, duty factor, pressure, and waveform cycle length.^{9–15} However, apart from an *in vitro* study by Borrelli *et al.*,¹⁶ which demonstrated improved clot lysis efficacy by 3 μm MBs versus 1 μm MBs, MB design parameters (e.g. size, composition, stability) remain largely unexplored, in part due to safety concerns associated with gas embolism in capillary networks caused by large, stable MBs.

With this in mind, it was the intent of this study to evaluate the sonothrombolysis performance of MBs with design parameters lying in a previously unexplored region of the parameter space (MB diameter > 5 μm , lifetime < 90 s, N₂ gas core). Rapidly dissolving, large diameter MBs were hypothesized to confer enhanced bioeffects in the form of accelerated thrombolysis rates, while also mitigating the risk of gas embolism due to the propensity of this MB formulation to dissolve off-target shortly after administration.^{17–19} The MBs in this study were fabricated in real time by a flow-focusing microfluidic device (FFMD) and were administered to an *in vitro* sonothrombolysis model as though they were fabricated by a catheter-mounted FFMD placed in close proximity to the blood clot.²⁰ In this way, the locally administered, short-lived MBs had time to interact with the blood clot before dissolving farther downstream. In addition, a new *in vitro* sonothrombolysis model is presented that permits the assessment of volumetric clot lysis rates by measuring the concentration of hemoglobin released from the clot during the thrombolysis process.

Materials and Methods

Flow-focusing microfluidic device fabrication

A chrome-metal photolithography mask (Applied Image, Inc, Rochester, NY) was used to fabricate an SU-8 mold of the microfluidic channel geometry on a silicon wafer.²¹ Flow-focusing microfluidic devices (FFMD) were cast from the SU-8 mold in polydimethylsiloxane (PDMS) (Sylgard 184, Dow Corning Corp., Midland, MI) and bound to a clean PDMS substrate using oxygen plasma to activate the surface. The total footprint of

the device measured $9 \times 8 \times 4$ mm (L×W×H). The nominal device channel dimensions were: height – $20 \mu\text{m}$, nozzle aperture (narrowest point) width – $8 \mu\text{m}$, liquid channel width – $50 \mu\text{m}$, and gas channel width – $30 \mu\text{m}$ (Figure 1A). Note that these nominal dimensions varied $\pm 5\%$ across devices.

Microfluidic microbubble production and characterization

Microbubbles (MBs) were produced by precise mixing of separate gas and liquid phases at the FFMD nozzle. The gas phase was comprised of 99.998% nitrogen gas (GTS Welco, Richmond, VA) and its pressure was regulated by a gas regulator and digital monometer (Model 06-664-21, Fisher Scientific, Waltham, MA). The liquid phase was a solution of 4% (w/v) bovine serum albumin (BSA) and 10% (w/v) dextrose in isotonic saline (0.9%).¹⁷ All chemicals were purchased from Sigma Aldrich (St. Louis, MO). The liquid phase was advanced into the microfluidic device channels through PTFE microbore tubing connected to a digital syringe pump (PHD2000 Harvard Apparatus, Holliston, MA). A high-speed camera (SIMD24, Specialised Imaging, Tring, United Kingdom) connected to an inverted microscope was used to measure MB production rates and diameters.²¹ Given that multiple microfluidic devices were used across multiple days of experimentation, it was not feasible to produce identical microbubbles for each experimental run. Accordingly, experiments were performed with microbubble diameters within $\pm 1 \mu\text{m}$ of the stated value and all production rates were within $\pm 5\%$ of the stated value.

MB lifetime after exiting the microfluidic device was measured using a Z2 Coulter Counter (Beckman Coulter, Brea, CA).¹⁹ An FFMD producing MBs of approximately $20 \mu\text{m}$ diameter was placed directly within 20 mL of air saturated saline at 37°C . After 10 s, the FFMD was removed and the saline was sampled by the Coulter Counter every 15 s over a period of 120 s. The saline was continuously stirred during measurement to limit MB aggregation and to ensure adequate mixing. MB size distribution and number were analyzed to determine the MB half-life in a well-mixed, air-saturated, saline environment.

Stable and inertial MB cavitation induced by acoustic excitation was characterized using a method described by Datta *et al.*²² Long-pulse acoustic excitation (1000 cycle, 1 MHz) at varying peak-negative-pressure (PNP, 50 kPa, 250 kPa, 500 kPa) was applied from a single element ultrasound transducer (V303 Olympus Panametrics, Waltham, MA) onto a 2 mm diameter PTFE tube containing slowly-flowing MBs produced by an FFMD. The FFMD was placed at the orifice of the PTFE tube and a syringe pump was used to establish a weak suction force to draw MBs into the tube for analysis. Microbubble concentrations ranged between 1×10^6 and 1×10^7 MB/mL. The acoustic backscatter from the MBs was measured by a calibrated polyvinylidene difluoride (PVDF) hydrophone (GL-0200, Onda Corp., Sunnyvale, CA) that was placed 1 cm from the tube and oriented orthogonal to the direction of ultrasound emission from the excitation transducer. The frequency content of the acoustic backscatter was analyzed to monitor for signs of stable and inertial cavitation.²²

In vitro sonothrombolysis model and optical assay

Blood clots were comprised of 35% (v/v) citrated human red blood cells (RBC) and 65% (v/v) citrated platelet-rich plasma (PRP, $> 5 \times 10^6$ platelets/ μL) and were intended to simulate

the average composition of clots retrieved from 50 ischemic stroke patients (61 ± 21 % fibrin/platelets, 34 ± 21 % RBCs, and 4 ± 2 % white blood cells).²³ This blood mixture was placed within glass tubes of 2.5 mm inner diameter and vinyl sutures were threaded through the tubes. Introducing CaCl_2 at a concentration of 15 mM initiated the clotting process, which was allowed to proceed for 4 hours at 37 °C. After 4 hours, the clots were stored at 4°C for three days to promote clot retraction.²⁴ Prior to experimentation, the clots were removed from the glass tubes but remained attached to the vinyl suture. Excess clot was removed from the suture so that all clots were 2 cm long and 2.3 ± 0.1 mm in diameter. All human blood products were procured from Virginia Blood Services (Richmond, VA).

The blood clot was placed within an *in vitro* sonothrombolysis experimental apparatus that simulated a catheter-based deployment of the FFMD for intravascular production of MBs (Figure 3A). The apparatus contained a thermostated water bath (37 ± 2 °C) that was located on the stage of an inverted microscope. A flow-loop comprised of thin-walled, transparent PTFE tubing of two different diameters was submerged within the water bath (Zeus Inc, Orangeburg, SC). The FFMD, along with its gas and liquid phase input tubes, was advanced into the proximal section of the flow loop (ID = 8.13 mm) and continuously dispensed MBs while under continuous microscopic observation to monitor microbubble production rates and diameters. The blood clot was placed 2 cm downstream of the FFMD, in a section of the flow loop with ID of 3.05 mm. Human plasma (30 ml) was circulated within the flow loop by a peristaltic pump so that the average flow velocity in the 3.05 mm section of the flow loop was 10 cm/s (NE-9000B, New Era Pump Systems, Inc.). The plasma was maintained at 37 ± 2 °C by a hot plate and was continuously stirred by a magnetic stir bar. Recombinant tissue plasminogen activator (rtPA, Activase, Genentech, South San Francisco, CA) was added as a bolus to the circulating plasma in either 0, 0.1, or 1 µg/ml concentrations. An 18 µm nylon mesh filter (Tisch Scientific, Cleaves, OH) was placed in the flow loop to trap large clot fragments for additional analysis.

The blood clot was aligned within the acoustic field of a single element 1 MHz ultrasound transducer with a 2.54 cm diameter (V302-SU Olympus Panametrics, Waltham, MA). A single set of ultrasound parameters was used for all sonothrombolysis experiments in this study: 1 MHz center frequency, 100 cycle pulses, 1 kHz pulse repetition frequency, with a 2 s on/2 s off pulsing pattern to allow for microbubble replenishment on the clot face. The peak negative pressure (PNP) at the surface of the clot closest to the ultrasound transducer was 500 kPa ($I_{\text{SPTA},0} = 420 \text{ mW/cm}^2$). The sinusoidal excitation was supplied by an arbitrary waveform generator (AFG 3022B, Tektronix, Beaverton, OR) and was amplified by a 60 dB RF amplifier (A-500, ENI Ltd., Rochester, NY).

In some experiments, MBs produced via tip sonication of a decafluorobutane (C_4F_{10}) saturated solution of 4% BSA and 10% dextrose were used instead of FFMD-produced MBs as a means to evaluate sonothrombolysis efficacy of conventionally sized MBs.^{25,26} These MBs were sized and counted by a Coulter counter and were infused continuously into the flow-loop by a syringe pump.

Sonothrombolysis with varying rtPA, ultrasound, and MB conditions was performed for 30 min ($N = 4$ for each experimental condition). The volumetric clot lysis rate was quantified

via an optical absorption assay that measured the concentration of hemoglobin present in the circulating plasma over time. Every 3 min, a 1 ml sample of the circulating plasma was removed, placed in a 2 ml Eppendorf tube, and kept on ice. At the completion of the 30 min sonothrombolysis experiment, the entirety of the clot that remained on the suture was lysed by the addition of 1 $\mu\text{g/ml}$ rtPA in a total volume of 2 mL plasma within an Eppendorf tube. This served to liberate all RBCs remaining within the clot into solution for the following analysis: each Eppendorf tube containing plasma plus free RBCs was lightly centrifuged to pellet the RBCs and all plasma was removed. The RBCs were lysed in distilled water to release hemoglobin into solution (total volume 100 μl). The samples were centrifuged at high centrifugal force one final time to remove RBC membranes from solution. The concentration of hemoglobin in each sample was measured via optical absorption ($A_{540\text{nm}}$) in a 96 well plate reader (FLUOStar Optima, BMG Labtech) and compared against the optical absorption of a reference concentration of human hemoglobin (Sigma Aldrich, St. Louis, MO). Thus, the circulating hemoglobin concentration was quantified every three minutes during the sonothrombolysis experiment, and the total amount of hemoglobin within the unlysed clot that remained on the suture was also measured. Together, these measurements permitted the calculation of the rate at which RBCs were removed from the clot through time, which was considered to be a volumetric measurement of the clot lysis rate. Least-squares linear regression was performed on the time-series data to derive a volumetric clot lysis rate for each experimental condition. Statistical significance between slopes was determined using two-sided t-tests ($\alpha=0.05$). All statistical analysis was performed in MATLAB (Mathworks, Natick, MA).

Results

FFMD microbubble production and characterization

MBs of diameters between 9.8 ± 0.3 and 31.1 ± 1.4 μm were produced at rates between $87.7 \pm 4 \times 10^3$ and $1043 \pm 94 \times 10^3$ MB/s. Gas pressures ranged from approximately 40 to 110 kPa and liquid flow rates were varied between 20 and 110 $\mu\text{l/min}$ (Figure 1).

MB stability was assessed by monitoring the change in MB size and number following production by the FFMD. The data shown in Fig 2A were acquired from an FFMD producing 20 μm diameter MBs at a rate of approximately 225×10^3 MB/s. As shown, the initial distribution at 0 s has a primary peak at approximately 20 μm that broadens through time as MBs dissolve, expand, coalesce, and aggregate. Through time, the average MB diameter, d_{MB} , remained approximately constant near 20 μm , however the standard deviation of d_{MB} measurements increased from 2.2 μm at 0 s to 5.9 μm at 120 s. The half-life of these 20 μm diameter MBs in a well-mixed saline environment was measured to be 65 ± 27 s across three separate measurements.

The onset of stable and inertial MB cavitation in response to acoustic excitation was characterized by analyzing the frequency content of acoustic backscatter from MBs excited by long-pulse acoustic excitation at 1 MHz. A representative power-spectra of a suspension of FFMD-produced MBs with 15 μm diameter is shown in Figure 2B. Ultraharmonics were not observed at a PNP of 50 kPa, but were observed at PNP of 250 kPa and 500 kPa. A significant increase (approx. 10 – 15 dB) in broadband acoustic noise was observed when

increasing the PNP from 250 kPa to 500 kPa, which corresponded to the onset of inertial cavitation. Thus, based on this analysis, both stable and inertial cavitation was observed at 500 kPa for FFMD produced MBs of 15 μm diameter.²² A similar analysis for sonication-produced MBs ($d_{\text{MB}} = 2.8 \pm 1.7 \mu\text{m}$) confirmed that both stable and inertial cavitation also occurred at 500 kPa (data not shown). In addition, long-pulse 500 kPa PNP excitation was confirmed to result in significant primary radiation force that pushed MBs out of the stream of flow and onto the clot face during the sonothrombolysis experiments (see Supplementary Video 1).

In vitro sonothrombolysis assay

A schematic depiction of the colorimetric assay utilized to measure volumetric clot lysis rates is shown in Figure 3A. To determine the sensitivity of this assay, 10 blood clots of uniform size and composition were completely lysed by 1 $\mu\text{g/ml}$ rtPA in a 30 ml volume of human plasma for 8 hours at 37 C. Following complete lysis, the free RBCs were centrifuged, plasma was removed, and the pelleted RBCs were lysed to release free hemoglobin in distilled water. The optical absorption ($A_{540\text{nm}}$) of a dilution series of the released hemoglobin, from 50% to 0.097%, was measured to determine the smallest fraction of eroded clot that could be measured accurately by the assay. As shown in Figure 3B, hemoglobin concentrations corresponding to less than 1.5% of the total clot could not be distinguished from one another as the absorption measurement was too close to the sensitivity limit of the plate reader. Fractions above 1.5% of the total clot could be accurately and significantly detected ($p < 0.05$).

Images of the clot at 4X magnification were acquired by a microscope-mounted camera, as shown in Figure 3C and Supplementary Video 2, although these images were not used to quantify clot lysis rates. As shown, clot erosion occurred approximately uniformly along the clot face, and no large clot fragments were observed to be released from the clot. This observation is further strengthened by an analysis of clot fragments observed on the 18 μm nylon mesh filter. Figure 3D is a histogram of all clot fragments contained on the mesh filter across all clots and across all experimental conditions that used FFMD-produced MBs (Groups E – K, as described below). The largest clot fragment was 78 μm measured along its longest dimension.

In vitro sonothrombolysis with MBs produced by FFMD

The sets of experimental sonothrombolysis conditions that were evaluated in this study are described in Table 1. Two conditions without MBs and two conditions with MBs produced via sonication were evaluated in order to establish a baseline control for comparison to the MBs produced by the FFMD. The approximate volume of gas and total number of MBs introduced into the flow loop over the entire 30 min experiment are also listed in the table (C_4F_{10} or N_2).

Volumetric clot lysis curves for four experimental conditions are shown in Figure 4A. In all cases, the volumetric erosion rates were observed to be approximately linear over the first 30 minutes, although erosion rates in Group F began to plateau once approximately 30% of the clot had eroded.¹⁵ The slopes derived from a linear regression of these four datasets

correspond to the volumetric clot lysis rates (% of clot per minute) and are presented as a bar graph in Figure 4B. Clot lysis in the absence of rtPA, ultrasound, or MBs (Group A) was below the sensitivity of the assay (less than 1.5% of clot eroded over 30 minutes). To approximate a clinical-dose of rtPA,³ 1 µg/ml rtPA was circulated in the flow loop (Group B), and the clot lysis rate was 0.13 ± 0.03 %/min. Sonication-produced MBs (1×10^6 MB/ml, $d_{MB} = 2.8 \pm 1.7$ µm) and 1 MHz ultrasound were added to 1 µg/ml rtPA to match approximate clinical sonothrombolysis conditions (Group C^{6,8}) and the clot lysis rate approximately doubled to 0.29 ± 0.04 %/min. When FFMD-produced MBs with $d_{MB} = 15$ µm and a production rate of 250×10^3 MB/s were used with 1 µg/ml rtPA and 1 MHz ultrasound (Group F), the clot lysis rate was 0.97 ± 0.06 %/min.

Additional studies were performed to evaluate the effects of MB concentration, rtPA concentration, and MB diameter on rate on the sonothrombolysis process. Figure 5A shows the sonothrombolysis rates observed as the MB concentration (Groups C, D) or the FFMD production rates were increased (Groups E, F, G). As shown, sonothrombolysis rates increased as FFMD production rate increased, and rates observed for Groups F and G were significantly greater than Group D, which represented an approximate 100-fold greater MB dose than clinical MB dose. The effect of increasing rtPA concentration on sonothrombolysis rates is shown in Figure 5B. As expected, sonothrombolysis rates increased with increasing rtPA dose across Groups J, K, F. Finally, the effect of MB diameter was investigated and results are shown in Figure 5C. The approximate volume of gas administered in groups H, F, I was held constant while the MB diameter was varied across the range 10, 15, and 20 µm. The sonothrombolysis rates decreased with increasing MB diameter, but this effect may be due to the dramatic decrease in MB number as the MB diameter increased. When the production rates of 10 and 15 µm diameter MBs were approximately the same (Groups H and G), the observed sonothrombolysis rates were not significantly different ($p = 0.377$).

p-values were computed to test for similarity among the experimental conditions and are listed in Table 2 (two-tailed t-test). The ratio of clot lysis rates for all experimental conditions relative to an approximate clinical dose of rtPA (Group B, 1 µg/ml) are listed in Table 3.

Discussion

Microbubble formulation and delivery strategy

This study investigated the sonothrombolysis efficacy of large diameter, low-stability MBs produced in real-time by a flow-focusing microfluidic device. Unlike sonothrombolysis performed using a systemic injection of small-diameter, long-circulating MBs,^{6–8} the MBs evaluated in this work are intended to be administered from a catheter placed in close proximity to or embedded within the blood clot.^{20,27} While this approach is more invasive than intravenous therapies, it is consistent with existing catheter-directed interventions for venous thromboembolism⁴ and investigational therapies for ischemic stroke²⁸. Further, focal delivery also ensures that the majority of MBs reach the therapeutic target site and permits the use of MBs that otherwise could not be administered systemically.¹⁷ While this study primarily focused on the efficacy of the microbubble formulation for enhancing

thrombolysis, it must be noted that significant effort is still required to miniaturize the FFMD to human-compatible dimensions for future intravascular deployment on a catheter.²⁷

Increased MB concentrations are associated with accelerated sonothrombolysis rates, but may also increase the incidence of undesirable off-target effects (e.g. hemorrhage, hemolysis, embolism).^{12,15,16} Thus, we sought to develop a MB delivery technique and formulation that would permit high MB concentrations in the vicinity of the thrombus but low concentrations elsewhere. The N₂ gas MBs used in this work exhibited a half-life of 65 ± 27 s in well-mixed saline and were also observed to dissolve within minutes in the sonothrombolysis experiments.^{17,19} Notably, a similar FFMD MB formulation was evaluated in a mouse model, and circulation half-lives of less than 30 s were observed.¹⁸ MB production by a FFMD at the site of therapeutic delivery is hypothesized to permit real-time adjustment of MB size, concentration, composition, and stability in order to modulate sonothrombolysis efficacy and safety.^{18,20} The stability of FFMD-produced MBs can be tuned to a specific application by changing the shell material (e.g. using a lipid²⁹) or by using a gas with different solubility in water (e.g. C₄F₁₀¹⁹, CO₂, or Xe).

MB gas volumes introduced into the sonothrombolysis assay over 30 minutes ranged from 1.4 µl to 3 ml. The condition in which 3 ml N₂ gas was administered (Group G) represented the upper limit of MB production rates attainable by the FFMD, and the safety of administration of these large gas volumes must be considered when translating this technique to *in vivo* models. Rapid intra-arterial administration of even 1 ml of gas is considered unsafe due to the risk of gas embolism, but smaller volumes (e.g. 100 µl) have been administered safely in rabbit models,³⁰ suggesting that several hundred microliters of high solubility gas could be administered intra-arterially over a long time period. In contrast, venous administration of large gas volumes poses much less of a risk, so this technique may be more translatable for the treatment of venous thromboembolisms than for stroke.⁴ In any case, additional study of the use of large volumes of high solubility gases in *in vivo* models is required to determine if this approach is feasible *in vivo*.

In vitro sonothrombolysis

The sonothrombolysis model used in this work produced results that are in broad agreement with those presented in the literature.^{14–16} As shown in Figure 4, adding ultrasound and sonicated MBs at a concentration of 1 × 10⁶ MB/ml increased the *in vitro* thrombolysis rate by 2.1-fold (95% C.I. 1.9 – 2.4) relative to thrombolysis rates observed when using rtPA alone at an approximate clinical dose (1 µg/ml). Previously reported results for similar experimental conditions demonstrated increased thrombolysis rates between approximately 1.5 and 3-fold, depending on ultrasound conditions and the composition of the blood clot.^{14–16} In addition, studies that have tracked clot erosion at multiple timepoints during sonothrombolysis have also reported approximately linear erosion rates until approximately 30 – 40 % of the clot has eroded, at which point thrombolysis rates begin to decrease, presumably due to less clot remaining for lysis.¹⁵ A common limitation of many *in vitro* models, including the model used in this work, is the use of circulating plasma devoid of RBCs. The presence of RBCs at physiological hematocrit may alter the interactions of MBs with the clot, as has been shown in the case of molecular targeting using MBs,³¹ although

further study is required to characterize RBC-MB interactions in the context of therapeutic applications.

Overall, sonothrombolysis rates when using MBs produced by the FFMD were observed to increase with increasing MB gas volume, MB concentration, and MB production rates (Figure 5A). The peak thrombolysis rate occurred in Group G, which represented the maximum MB production rate and administered gas volume attainable by this FFMD architecture, and was 8.8-fold and 4.2-fold greater than the lysis rates observed for cases simulating intravenous rtPA administration (Group B) and systemic sonothrombolysis with conventionally sized MBs (Group C). This result underscores the potential of this MB formulation and delivery platform to significantly outperform conventional thrombolysis and sonothrombolysis approaches. In fact, Groups D and E were evaluated to compare sonothrombolysis rates in approximately gas volume-matched conditions for sonication-produced MBs of diameter $2.8 \pm 1.7 \mu\text{m}$ and FFMD-produced MB of $15 \mu\text{m}$. The sonothrombolysis rates were not statistically different ($p = 0.124$), but Group D is representative of a 100-fold increase in the maximum clinically permitted concentration of high-stability MBs,²⁸ which is prohibitive *in vivo*. These *in vitro* results suggest that, on a per-MB basis, the larger FFMD-produced MBs may be more effective at accelerating sonothrombolysis rates, as found by Borelli *et al*¹⁶ and Bader *et al*,⁹ but there is a need for additional study to establish optimal MB size, injected gas volume, MB number, and MB concentration for accelerating thrombolysis.

Sonothrombolysis rates were also evaluated when the diameter of FFMD-produced MBs was varied from 10 to 20 μm and the total amount of gas held constant at approximately 800 μl . The sonothrombolysis rates decreased with increasing MB diameter (Figure 5C), but this effect may be due to the decrease in MB number and concentration as the MB diameter increased. In support of this view, when the production rates of 10 and 15 μm diameter MBs were approximately the same (Groups H and G), the observed sonothrombolysis rates were not significantly different ($p = 0.377$). Thus, this result is consistent with the findings of others, that increasing the number of MB-clot interactions by increasing the administered MB dose is a reliable way to increase sonothrombolysis rates, but as mentioned above, there are limits to the maximum concentration of conventional, high-stability MBs that may be administered systemically. The transient stability of the MB formulation studied in this work may permit higher local concentrations of MBs *in vivo*, as supported by a recent investigation of these MBs in a murine model, in which there were no gross adverse effects following intravenous administration of large doses of FFMD-produced MBs.¹⁸

Developing thrombolysis techniques that utilize reduced rtPA doses, or even no rtPA, is of significant clinical importance given that many patients presenting with stroke or venous thromboembolisms are contraindicated for rtPA therapy.⁵ The results of this study suggest that accelerating thrombolysis with MBs produced by FFMDs may provide the opportunity for rtPA dose reduction, as the thrombolysis rates for Group B (rtPA dose of 1 $\mu\text{g/ml}$, no MBs) and Group J (rtPA dose of 0 $\mu\text{g/ml}$, with FFMD-produced MBs) were statistically similar ($p = 0.457$). This also implies a significant degree of mechanical disruption from MB interactions with the clot, given that limited enzymatic breakdown of the fibrin mesh could occur in Group J. This observation is consistent with previous findings that have described

significant clot erosion from MBs and ultrasound alone,^{9,12,32} especially when using ultrasound parameters that elicit both stable and inertial cavitation (Figure 5-2).^{12,15,22} However, this mechanical interactions between this MB formulation and the thrombus must be studied in other clot types, such as soft, RBC-rich clots representative of those observed in the acute phase of DVT⁴ and hard, fibrin enriched clots that are often the cause of ischemic stroke.³³

Overall, the results of this study suggest that the larger MBs produced by the FFMD are capable of significantly enhanced sonothrombolysis rates compared to conditions in which only clinical doses of either rtPA or rtPA with small MBs were used (Table 3). The notion that large MBs confer enhanced bioeffects is supported by experimental and theoretical studies that demonstrate higher energy inertial cavitation,³⁴ larger microstreaming fields,³⁵ more violent jetting,³⁶ and increased acoustic radiation forces³⁷ from MBs of large diameter compared to MBs of small diameter. Further enhancement of sonothrombolysis rates may be possible by matching the ultrasound frequency to the resonance frequency of the MB. It should be noted that most clinical applications of sonothrombolysis for ischemic stroke therapy have utilized ultrasound frequencies between 1 – 3 MHz^{6,8,28} and that lower frequencies have been linked to standing wave formation and increased hemorrhagic transformation.³⁸ Thus, while the safety of *in vivo* administration of this MB formulation has been evaluated in a mouse model,¹⁸ additional study is required in large animal *in vivo* models to balance thrombolysis efficacy with safety concerns related to gas embolism, hemorrhage, and potentially destructive bioeffects caused by cavitating MBs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Characteristics of microbubbles produced by flow-focusing microfluidic devices
 (A) (Left) Schematic of the flow-focusing microfluidic device (FFMD). There are two liquid inlets (L), one gas inlet (G), filters to prevent the nozzle from clogging (F), and an outlet where MBs are emitted (O). (Right) 20X magnified view of the device nozzle. Dimensions are $w_g = 30\mu m$, $w_l = 50\mu m$, $w = 8\mu m$, $\theta = 25^\circ$, $\theta_b = 50^\circ$. (B) High speed images of the FFMD producing 20 μm (left) and 10 μm (right) diameter MBs. (C) MB production rates as a function of liquid flow rate, Q_l , for four different gas pressures. (D) MB diameter as a function of liquid flow rate, Q_l , for four different gas pressures.

Figure 2. FFMD microbubble dissolution and acoustic properties

(A) (left) MB distributions over time as measured by a Coulter counter immediately after production by a flow-focusing microfluidic device (FFMD). MB diameters were confirmed via optical microscopy to be approximately 20 μm . (Right) Change in MB diameter (top) and number (bottom) over time, as measured from the Coulter distributions. (B) Frequency analysis of the acoustic backscatter from 15 μm diameter MBs produced by a FFMD when exposed to long-pulse acoustic excitation at 1 MHz and three different pressures. The generation of ultraharmonic responses (e.g. 1.5, 2.5 MHz) is associated with stable MB cavitation. Increase in broadband noise is associated with the onset of inertial cavitation.

Figure 3. Schematic of *in vitro* sonothrombolysis assay and assay characterization

(A) Schematic of *in vitro* sonothrombolysis assay. Briefly, a blood clot was placed in a circulating flow loop and was exposed to ultrasound, microbubbles, and rtPA. The circulating plasma was sampled every minute and hemoglobin concentration was quantified using a plate reader. Changes in hemoglobin concentrations over time were used to derive a volumetric clot lysis rate for each experimental condition. (B) Sensitivity of the assay to percentage of the initial clot volume based on the concentration of released hemoglobin. The assay is able to detect hemoglobin concentrations corresponding to greater than 1.5% of the initial clot volume. (C) Representative image of a clot before (top) and after (bottom) 30 min of sonothrombolysis under experimental conditions of Group F, as described in Table 1. (D) Distribution of clot fragments observed on the 18 micrometer nylon mesh filter across all clots studied in experimental Groups E – K.

Figure 4. *in vitro* clot lysis curves for four experimental conditions
 (A) Clot lysis curves for experimental groups A, B, C, F. Data points are mean $\pm$ S.E. (B) Volumetric clot lysis rates for the four experimental groups shown in (A). * indicates $p < 0.05$.

Figure 5. *in vitro* volumetric clot lysis rates for multiple experimental conditions

(A) Volumetric clot lysis rates for experimental groups that investigate the effect of changes in MB concentration and production rate. (B) Volumetric clot lysis rates that investigate the effect of changes in rtPA concentration. (C) Volumetric clot lysis rates that investigate the effect of changing MB diameter. * indicates $p < 0.05$

Table 1Parameters for different experimental *in vitro* sonothrombolysis conditions

Group	[rtPA] ($\mu\text{g/ml}$)	US	MB production method	MB conc. (ml^{-1})	MB production rate ($1 \times 10^3 \text{ s}^{-1}$)	MB diameter (μm)	MB gas volume administered (μl)*	Total MB administered (N)*
A	0	–	–	–	–	–	0	0
B	1	–	–	–	–	–	0	0
C	1	Yes	Sonication	1×10^6		2.8 ± 1.7	1.4	3×10^7
D	1	Yes	Sonication	1×10^8		2.8 ± 1.7	140	3×10^9
E	1	Yes	FFMD	–	50	15	160	9×10^7
F	1	Yes	FFMD	–	250	15	800	4.5×10^8
G	1	Yes	FFMD	–	950	15	3020	1.7×10^9
H	1	Yes	FFMD	–	850	10	800	1.5×10^9
I	1	Yes	FFMD	–	100	20	800	1.8×10^8
J	0	Yes	FFMD	–	250	15	800	4.5×10^8
K	0.1	Yes	FFMD	–	250	15	800	4.5×10^8

Table 2

p-value for comparison of clot lysis rates under differing

	A	B	C	D	E	F	G	H	I	J	K
A	–	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
B	–	–	0.0241	< 0.001	0.113	< 0.001	< 0.001	< 0.001	< 0.001	0.457	< 0.001
C	–	–	–	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.0238	< 0.001
D	–	–	–	–	0.124	0.084	0.0481	< 0.001	0.003	< 0.001	0.183
E	–	–	–	–	–	0.017	< 0.001	< 0.001	0.312	< 0.001	0.0387
F	–	–	–	–	–	–	0.042	0.214	0.009	< 0.001	< 0.001
G	–	–	–	–	–	–	–	0.377	< 0.001	< 0.001	< 0.001
H	–	–	–	–	–	–	–	–	0.289	< 0.001	< 0.001
I	–	–	–	–	–	–	–	–	–	< 0.001	< 0.441
J	–	–	–	–	–	–	–	–	–	–	0.008
K	–	–	–	–	–	–	–	–	–	–	–

Significant differences, *p* < 0.05, are italicized.

Table 3

Clot lysis rates relative to experimental Group B (1 µg/ml rtPa alone).

Group	Ratio of Clot Lysis Rates
A	0.06 (0, 0.11)
B	–
C	2.1 (1.9, 2.4)
D	4.3 (3.9, 4.7)
E	5.4 (4.8, 6.0)
F	7.3 (6.6, 8.0)
G	8.8 (7.9, 9.8)
H	8.1 (7.2, 9.1)
I	6.0 (5.4, 6.7)
J	1.4 (1.2, 1.6)
K	4.7 (4.2, 5.2)

Relative rates presented as mean (95% C.I.).