Influences of Microbubble Diameter and Ultrasonic Parameters on In Vitro Sonothrombolysis Efficacy

Michael J. Borrelli, PhD, William D. O'Brien Jr, PhD, Eric Hamilton, MS, Michael L. Oelze, PhD, Jonah Wu, BS, Laura J. Bernock, MS, Stephen Tung, PhD, Husein Rokadia, PhD, and William C. Culp, MD

ABSTRACT

Purpose: To quantify the effects of microbubble (MB) size, elasticity, and pulsed ultrasonic parameters on in vitro sonothrombolysis (ultrasound [US]-mediated thrombolysis) efficacy.

Materials and Methods: Monodispersive MBs with diameters of 1 μm or 3 μm were exposed to pulsed US (1 MHz or 3 MHz) to lyse rabbit blood clots. Sonothrombolysis efficacy (clot mass loss) was measured as functions of MB size and concentration, ultrasonic frequency and intensity, pulse duration (PD), pulse repeat frequency (PRF), and duty factor.

Results: Sonothrombolysis at 1 MHz was more effective using 3-μm MBs and at 3 MHz using 1-μm MBs. Sonothrombolysis was more effective at 1 MHz when $\geq$ 75% of MBs remained intact, especially for 3-μm MBs; improving sonothrombolysis by increasing PRF from 100 Hz to 400 Hz at 3 MHz was associated with increasing 3-μm MB survival. However, 60% of 1-μm MBs were destroyed during maximal sonothrombolysis at 3 MHz, indicating that considerable MB collapse may be required for sonothrombolysis under these conditions.

Conclusions: The ability to control MB size and elasticity permits using a wide range of US parameters (eg, frequency, intensity) to produce desired levels of sonothrombolysis. Comparable, maximal sonothrombolysis efficacy was achieved at 20-fold lower intensity with 3-μm MBs (0.1 W/cm^2) than with 1-μm MBs (2.0 W/cm^2), a potential safety issue for in vivo sonothrombolysis. US parameters that maximized MB survival yielded maximal sonothrombolysis efficacy except with 1-μm MBs at 3 MHz where most MBs were destroyed.

ABBREVIATIONS

AFM = atomic force microscopy, MB = microbubble, PBS = phosphate-buffered saline, PD = pulse duration, PRF = pulse repeat frequency, tPA = tissue plasminogen activator

Sonothrombolysis involves using ultrasonic energy to lyse clots and thrombi. Initial preclinical and clinical sonothrombolysis studies investigated using ultrasound (US) alone to disrupt thrombi within occluded vessels (1,2), including thrombi in the heart (3,4), brain (5–7), and other locations (8–10). Combining US with thrombolytic agents, such as heparin, urokinase, or tissue plasminogen activator (tPA), yielded enhanced sonothrombolysis (11–13). Evidence suggests that ultrasonic shear forces, microstreaming, and radiation force fracture thrombi to facilitate deeper penetration of thrombolytics (14–16).

Ultrasonic cavitation was observed during sonothrombolysis (17,18) and may be one mechanism that promotes sonothrombolysis. Consequently, encapsulated microbubbles (MBs) were combined with US in vitro to provide more cavitation nuclei and produced the expected increase in sonothrombolysis efficacy (19–23). Combining MBs with US also improved sonothrombolysis efficacy in clinical trials (24–26).

US-MB interactions during sonothrombolysis have been investigated using frequencies from the kilohertz (27–29)

From the Department of Radiology (M.J.B., E.H., J.W., L.J.B., W.C.C.), University of Arkansas for Medical Sciences, 4301 West Markham Street Slot #556, Little Rock, AR 72205; Bioacoustics Research Laboratory (W.D.O., M.L.O.), Department of Electrical and Computer Engineering, University of Illinois at Urbana-Champaign, Urbana, Illinois; and Department of Mechanical Engineering (S.T., H.R.), University of Arkansas, Fayetteville, Arkansas. Received September 28, 2011; final revision received August 7, 2012; accepted August 13, 2012. **Address correspondence to** M.J.B.; E-mail: mjborrelli@uams.edu

This work was supported in part by NIH Grants R01 CA99178 (M.J.B.), R01 HL082481 (W.C.C.), and R37EB002641 (W.D.O., M.L.O.), and by a grant from the Arkansas Biosciences Institute (M.J.B. and S.T.), the major research component of the Arkansas Tobacco Settlement Proceeds Act of 2000.

None of the other authors have identified a conflict interest.

J Vasc Interv Radiol 2012; 23:1677–1684

http://dx.doi.org/10.1016/j.jvir.2012.08.019

into the lower megahertz range (20,22,30,31). Intracranial hemorrhage that occurred during clinical trials using frequencies of 100–600 kHz (32) influenced some investigators to concentrate on using low megahertz frequencies for sonothrombolysis, although clinical complications were also reported when using 2-MHz US (7,24,26).

MBs have been tagged with antibodies and other adducts that bind to fibrin, platelets, and other components of a thrombus (23,30,33,34) to increase local MB concentration near clots and thrombi to maximize sonothrombolysis. MBs have been combined with tPA to improve sonothrombolysis efficacy further (21,23,29,31,35) and possibly to minimize the amount of tPA required for sonothrombolysis to reduce bleeding and other tPA-associated clinical complications (23,35). tPA has also been encapsulated so that localized US induces MB cavitational activity to target tPA release at the thrombus (29) to minimize systemic complications.

Because there is strong evidence that ultrasonic cavitation is an important mechanism for sonothrombolysis (1,2,17,18,20,36,37), it is logical to investigate the effects that MB size, shell elasticity, and other properties relevant to cavitation (38–43) have on sonothrombolysis efficacy. However, most studies involved adjusting ultrasonic output parameters (center frequency, pressure or intensity, pulse repetition frequency, duty factor, and exposure duration) because most investigators used commercial MBs and could not adjust MB parameters. This study investigates the effects that MB size and shell elasticity have on sonothrombolysis efficacy in vitro. MB concentration and ultrasonic output parameters were tested and optimized for maximal in vitro sonothrombolysis of clots produced from fresh rabbit blood using pulsed US at a center frequency of either 1 MHz or 3 MHz.

MATERIALS AND METHODS

Production of Clot from Rabbit Blood

A 270-μL aliquot of fresh rabbit blood was placed into an 18-mm well on a Boerner glass slide and covered with an 18-mm circular glass coverslip to exclude air-blood contact. The Boerner slide was placed inside a humidified 37°C incubator for 3 hours. The clotted blood was removed and placed in a 35-mm plastic culture dish containing 3–4 mL of rabbit serum (room temperature) and was cut into square pieces weighing 7.5–10 mg (35).

MBs

MBs were prepared by sonicating decafluorobutane gas–saturated solutions of bovine serum albumin (Sigma-Aldrich Co, St. Louis, Missouri) and dextrose (Sigma-Aldrich Co) using a 20-kHz Fisher Scientific 500 Sonic Dismembrator (Thermo Fisher Scientific, Waltham, Massachusetts). The concentrations of bovine serum albumin and dextrose and the sonication parameters were varied to produce an abundance of MBs with the desired diameter (35,44). Multiple, differential buoyancy fractionation steps were used for size separation to produce uniformly sized MB preparations (44). The 1-μm diameter MBs were produced using the 1-μm MB protocol A, and the 3-μm diameter MBs were produced using the 3-μm MB protocol B, as described by Borrelli et al (44).

MB Concentration

MBs were warmed to room temperature and diluted with phosphate-buffered saline (PBS), and MB concentration was measured as a function of optical density at 530 nm using standard curves established previously (44). MB concentrations were spot checked regularly with hemocytometer counts.

In Vitro Sonothrombolysis

The experimental system and protocol for in vitro sonothrombolysis has been described and illustrated previously (35). Briefly, a clot was weighed and suspended in a vertical, acoustically transparent polyester resin material (Mylar, a polyester resin) tube that was prefilled with PBS. MBs suspended in PBS flowed into the Mylar tube, from below, at a constant rate of 0.5 mL/min (2.4 cm/min linear velocity). This Mylar chamber was suspended in a tank filled with degassed water at 25 °C. Pulsed or continuous wave US was delivered horizontally using a circular, flat, piezoelectric transducer located 6.5 cm from the center of the Mylar tube. Most US exposures were performed using a 100-Hz pulse repeat frequency (PRF) and a 2-ms pulse duration (PD).

Clots were exposed to US for 15 minutes, wicked dry with absorbent paper, and reweighed. The fractional change in clot weight was used as the endpoint for reporting sonothrombolysis efficacy. Experiments were performed without MBs to determine sonothrombolysis with US only, and sham experiments were performed identically but without transducer activation. Experiments were performed at 25°C to minimize the effects of tPA or plasmin trapped within prepared clots and to accentuate the physical sonothrombolysis of MBs and US.

MB Collapse and Destruction

The concentration of MBs entering the sonothrombolysis chamber was measured, and the MB suspension that flowed pass the clot and ultrasonic field was collected to quantify changes in MB concentration that resulted from MB destruction during sonothrombolysis (44). MB survival is reported as the fraction of original MBs remaining intact.

Atomic Force Microscopy of MBs to Determine Young's Modulus

Sample Preparation for AFM Analysis. Mica discs were treated with a 0.6% aqueous solution of 300,000 MW poly-L-lysine, rinsed with 18 MΩ water, and suspended atop a drop of MB suspension. The MBs rose and attached onto the mica surface via charge attraction; after a PBS rinse, the discs were placed MB side up into a PBS-filled holder to perform AFM measurements.

AFM Imaging and Nanoindentation. Topography scans and nanoindentations were performed using an Agilent

5500 AFM (Agilent Technologies, Inc., Santa Clara, California) in acoustic AC mode. A sharp silicon tip of radius R < 8 nm and cantilever stiffness coefficient k_c of 0.2% ± 10 N/m (Arrow-CONT; NanoWorld AG, Neuchâtel, Switzerland) was used for both imaging and nanoindentations, and all measurements were made with the MBs submerged in PBS. Topography scans were performed at a scan rate of 1 Hz, and nanoindentations were performed using the Indent module in the AFM control software. Deflection of the cantilever was calibrated by pressing the tip into the hard substrate material (mica) near the object to be indented. At each nanoindentation location, eight scanner displacement-cantilever deflection curves were obtained. In each curve, indentation information was recorded until a maximum cantilever deflection of 60 nm on the MBs was reached. A topographic scan was performed after each indentation to confirm that the indented bubble did not move or burst during the indentation process.

Data Analysis. Indentation force F on the MB was determined by multiplying the cantilever deflections d by the cantilever stiffness coefficient:

$$F = k_c \times d \quad (1)$$

The depth of the nanoindentation δ is determined by:

$$\delta = Z - d \quad (2)$$

where Z is the piezo displacement obtained from the displacement-deflection curves. To obtain Young's modulus of the MBs, the F versus δ curves are plotted and fitted by the Hertz model for conical tips (45):

$$F = \frac{4ER^{1/2}}{3(1-\sigma^2)}\delta_H{}^{3/2} \quad (3)$$

E is the Young's modulus, δ_H is the theoretical indentation predicted by the Hertz model, and σ is the Poisson's ratio. The Hertz model assumes no adhesion between the AFM tip and indented sample.

RESULTS

MB Concentration and Sonothrombolysis Efficacy

Sonothrombolysis efficacy was tested as a function of MB concentration to determine the concentration of 1-μm or 3-μm MBs that yielded maximal sonothrombolysis. These data are available online (**Figures E1a, b** and **E2a–c**; available online at *www.jvir.org*). The optimal concentration for sonothrombolysis with 1-μm MBs was 5.4 × 10^8 MB/mL and only fivefold greater than the optimal concentration of 1.1 × 10^8 MB/mL for 3 μm MBs.

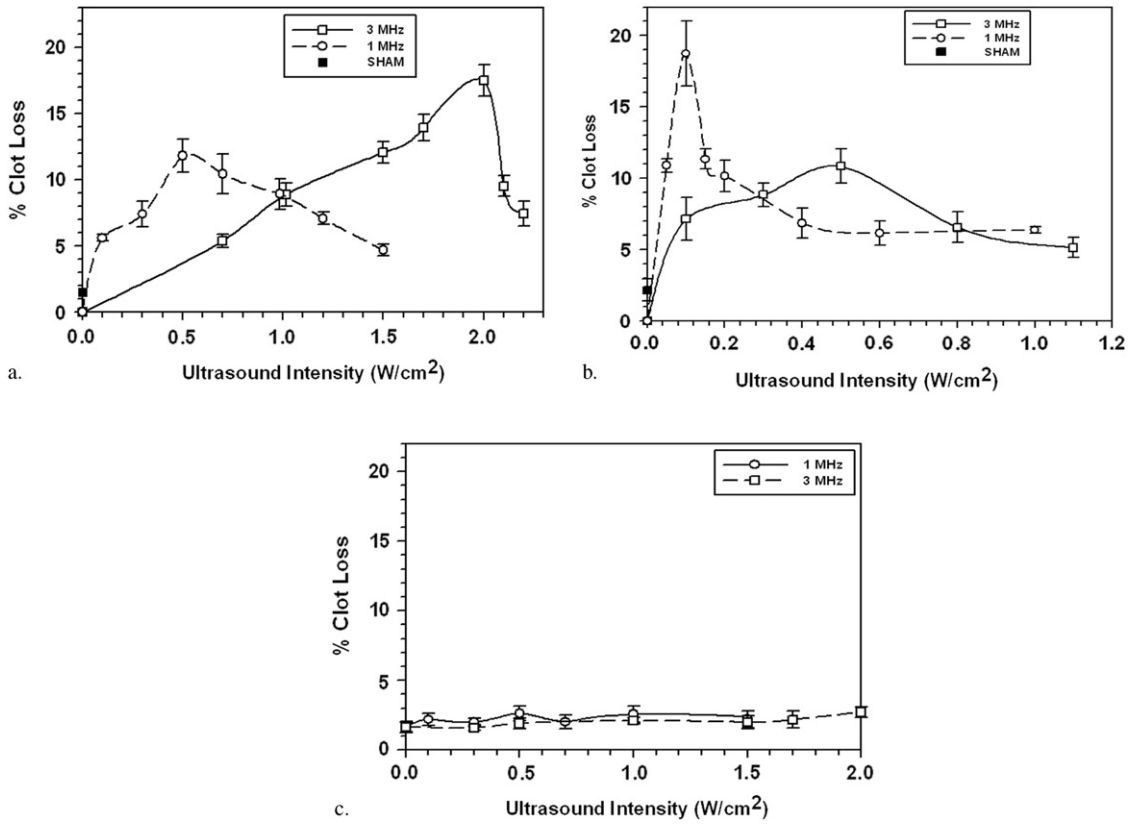

Figure 1. Sonothrombolysis efficacy as a function of ultrasonic intensity at 1 MHz and 3 MHz (pulsed US with 100 Hz PRF, 2 ms PD, and 20% duty factor). Each symbol represents the mean of a minimum of four measurements, and error bars indicate standard deviation. Data points near minima and maxima are the results of 6–10 measurements to ensure accuracy. **(a)** With 1-μm diameter MBs (5.4 × 10^8 MB/mL). **(b)** With 3-μm diameter MBs (1.1 × 10^8 MB/mL). **(c)** Without MBs.

Ultrasonic Intensity and Sonothrombolysis Efficacy

The relationships between sonothrombolysis efficacy and pulsed ultrasonic intensity (100 Hz PRF and 2 ms PD) for the 1-μm and 3-μm diameter MBs are presented in **Figure 1a** and **b**, obtained using the optimized concentration of 5.4×10^8 MB/mL for 1-μm MBs and 1.1×10^8 MB/mL for 3-μm MBs. As expected, sonothrombolysis efficacy was greater for each different diameter MB at the frequency closest to its natural resonant frequency.

Sonothrombolysis efficacy as a function of US intensity, without MBs, is presented in **Figure 1c**. These data demonstrate clearly that sonothrombolysis without MBs was minimal at 25°C and that the sonothrombolysis illustrated in **Figure 1a** and **b** was due predominantly to MB-US interactions.

During selected experiments, the tip of a 0.25-mm diameter thermocouple was inserted into the center of the clot to record temperature changes during sonothrombolysis, without or with MBs. The temperature increase during sonothrombolysis never exceeded 0.5°C during 15 minutes of sonothrombolysis (**Fig 2**) demonstrating that a temperature increase did not cause or influence clot loss during sonothrombolysis.

The general trend was that as ultrasonic intensity increased there was little MB destruction as sonothrombolysis efficacy approached and reached maximum (**Fig 3a–d**). Continuing to increase intensity reduced sonothrombolysis efficacy with a rapid reduction in MB survival. Maximal sonothrombolysis occurred at the highest ultrasonic intensity at which most MBs remained intact.

The 1-μm diameter MBs exhibited markedly different relationships between MB survival, ultrasonic intensity, and sonothrombolysis at 3 MHz. Sonothrombolysis did not increase significantly until an ultrasonic intensity that destroyed ≥25% of 1-μm MBs (**Fig 3b**). Sonothrombolysis efficacy continued to increase as MB survival decreased, and sonothrombolysis efficacy reached a maximum at 2 W/cm^2, which destroyed 60% of the MBs. MB survival remained at 40% even though sonothrombolysis efficacy decreased sharply to 30% of the maximum level as intensity was increased further.

MB Young's Modulus and Sonothrombolysis

The protocol used to produce the 3-μm MBs used for the experiments illustrated in **Figures 1b** and **3c** and **d** (3 μm MB-B(44)) was altered to change MB shell strength and elasticity (Young's modulus). One new 3-μm MB (3-μm MB-A(44)) was produced by reducing the serum albumin concentration threefold (from 5% to 1.67%) but keeping the sonication steps unchanged, whereas another 3-μm MB (3 μm MB-C(44)) was produced by keeping the bovine serum albumin and dextrose concentrations unchanged but increasing the duration of the first sonication step from 30 seconds to 40 seconds and the second sonication step from 20 seconds to 30 seconds.

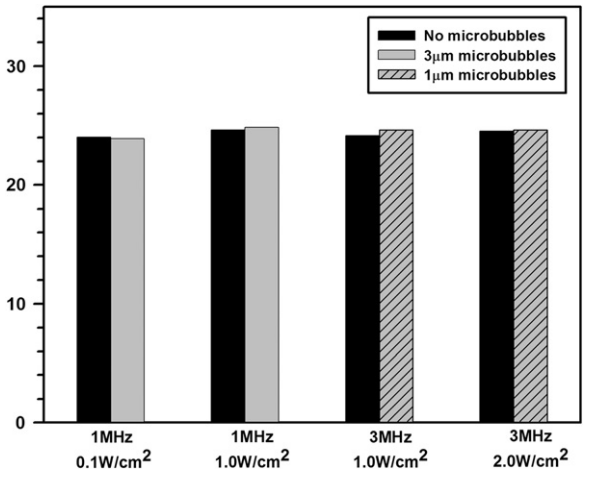

Figure 2. Temperature measurements during sonothrombolysis with MBs and pulsed US (100 Hz PRF, 2 ms PD, 20% duty factor). The tip of a 0.25-mm diameter thermocouple was inserted into the middle of the clot, and temperature recordings were made before and during sonothrombolysis with pulsed US (100 Hz PRF, 2 ms PD, 20% duty factor). These measurements were done using acoustic intensities corresponding to the maximal sonothrombolysis rates achieved with 1-μm and 3-μm MBs at 1 MHz and 3 MHz. The data show the average, steady-state temperature increase (mean of three measurements) that was observed during sonothrombolysis and that this was identical with and without MBs. The temperature increase never exceeded 0.4°C and was the same with or without MBs, although sonothrombolysis was about 10-fold higher in the latter case. The minimal US-induced temperature increase was not a contributing factor to the process of sonothrombolysis.

Figure 4 presents data for sonothrombolysis efficacy versus ultrasonic intensity at 1 MHz for these two other 3-μm MBs superimposed on the data for the original 3-μm MB (3-μm MB-B) from **Figure 1b**, with the AFM-measured Young's modulus for each 3-μm MB indicated. The 3-μm MB-A with the reduced Young's modulus was more susceptible to ultrasonic lysis at 1 MHz and exhibited a markedly lower sonothrombolysis efficiency. The 3-μm MB-C was more resistant to ultrasonic lysis and exhibited a more consistent maximal sonothrombolysis level over a wider intensity range, albeit with a significantly lower sonothrombolysis maximum than that obtained with 3-μm MB-B. Increasing the Young's modulus might have increased the resonant frequency of 3-μm MB-C and reduced sonothrombolysis at 1 MHz. However, this possibility was not investigated.

Effect of Pulse Duty Factor on Sonothrombolysis

Ultrasonic pulse parameters were modified to determine how duty factor (PD×PRF) affected sonothrombolysis efficacy. For the 1-μm MBs at 1 MHz and 3 MHz and for the 3-μm MBs at 1 MHz, maximal sonothrombolysis was achieved with a duty factor of 20%, and all attempts to increase duty factor produced reduced levels of sonothrombolysis (**Fig E2a–c**; available online at *www.jvir.org*).

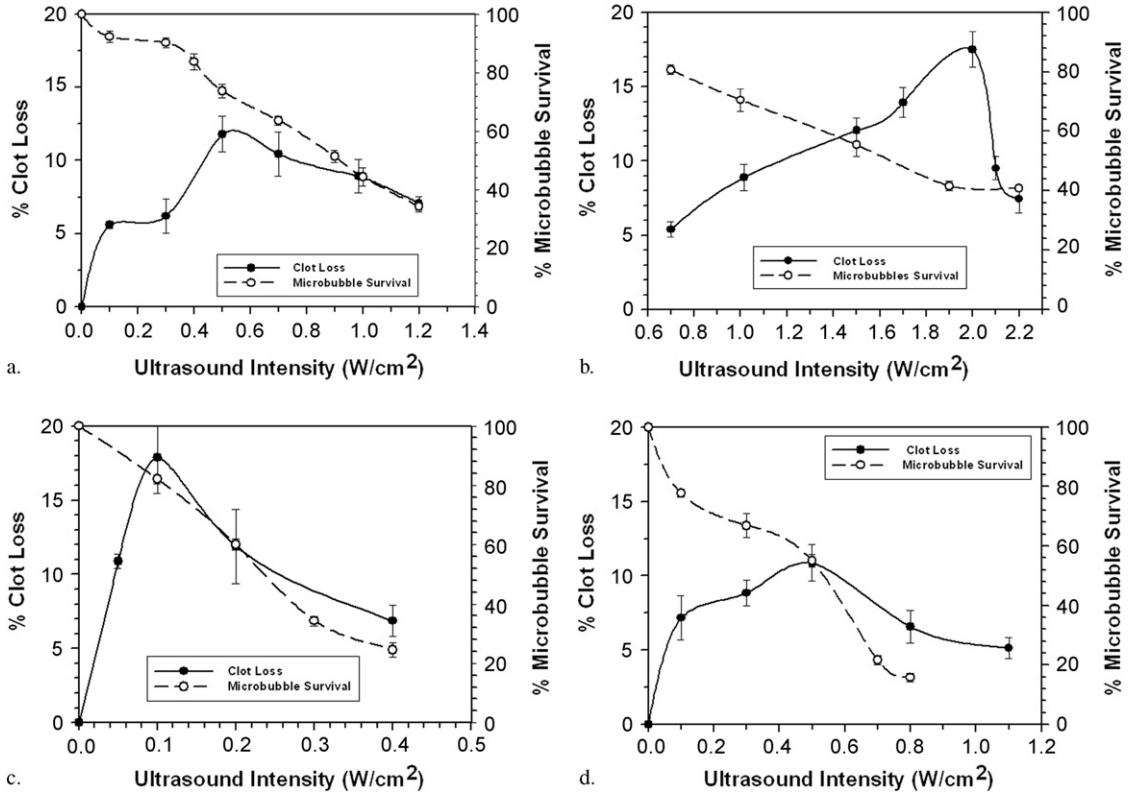

Figure 3. MB survival as a function of ultrasonic intensity. Each symbol represents the mean of a minimum of four measurements, and error bars indicate standard deviation. **(a)** With 1-μm diameter MBs at 1 MHz. **(b)** With 1-μm diameter MBs at 3 MHz. **(c)** With 3-μm diameter MBs at 1 MHz. **(d)** With 3-μm diameter MBs at 3 MHz.

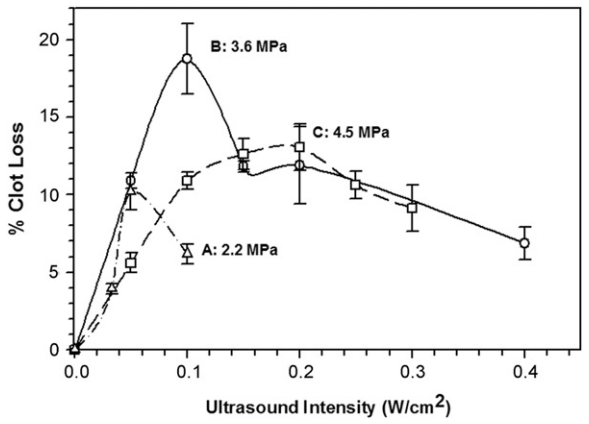

Figure 4. Effect of adjusting MB elasticity on sonothrombolysis efficacy. Three different 3-μm diameter MB preparations (preparations A, B, and C) were produced to have distinctly different values of Young's modulus (as described in the text). In vitro sonothrombolysis was performed with each MB preparation at 1 MHz and at different ultrasonic intensities. The effect that changing Young's modulus had on sonothrombolysis efficacy is evident.

The one exception was increasing duty factor with a 400-Hz PRF with 3-μm MBs at 3 MHz, which produced more efficient sonothrombolysis for duty factors > 20% (**Fig 5**). The fraction of surviving MBs as a function of duty factor is also indicated in **Figure 5** and shows that more MBs were preserved with a 400-Hz PRF.

DISCUSSION

This study provides quantitative data concerning how combining MBs with US improves sonothrombolysis and how MB size and shell elasticity affect sonothrombolysis as a function of ultrasonic frequency and intensity. As expected, the MB concentration required for maximal sonothrombolysis with 1-μm and 3-μm MBs was different, with MB concentration for 1-μm MBs being five times greater than that for 3-μm MBs. This was significantly less than the 9-fold difference in cross-sectional area or 27-fold difference in gas volume between these MBs. The fact that these experiments involved using a cloud of MBs, with complex MB-MB interactions, affected the relationship between MB diameter and the MB concentration required for maximal sonothrombolysis.

Using MBs increased sonothrombolysis efficacy markedly compared with that achieved with US only, as anticipated based on results of earlier studies (19–26,35). Experiments showed that at least 70%–80% of either 1-μm or 3-μm MBs remained intact when exposed to 1-MHz US that produced maximal sonothrombolysis (**Figure 1a, b**). As more MBs collapsed, sonothrombolysis efficacy decreased proportionately, indicating that intact MBs are required for

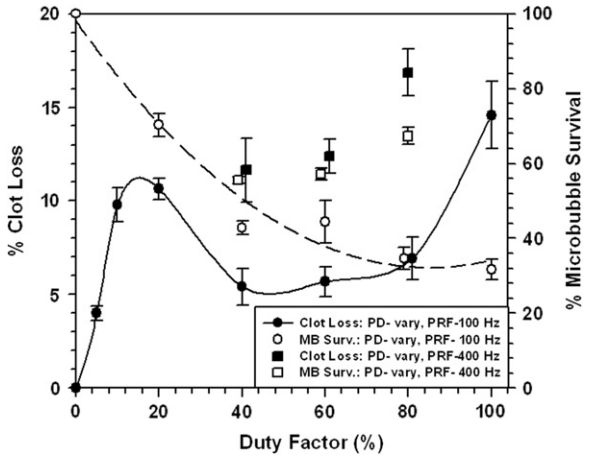

Figure 5. Effect of changing duty cycle on sonothrombolysis efficacy for 3-μm MBs at 3 MHz. Sonothrombolysis was performed at 3 MHz, as a function of duty factor, using pulsed US with a PRF of 100 Hz or 400 Hz and an acoustic intensity of 0.5 W/cm^2. Data for MB survival are also presented. Each symbol represents the mean of a minimum of four measurements, and error bars indicate standard deviation. Data points near minima and maxima are the results of 6–10 measurements to ensure accuracy at these critical transition regions.

maximal sonothrombolysis at 1 MHz; this supports the contention by Datta et al (36) that stable cavitation is an important mechanism for sonothrombolysis. However, these results do not eliminate the possibility that collapsing MBs also contributed to maximal sonothrombolysis directly via shock waves, releasing encapsulated perfluorocarbon gas, which cavitated to produce sonothrombolysis (rebound cavitation) (41), or stimulating greater nonlinear responses among intact MBs.

The relationship between sonothrombolysis efficacy and intact MBs is less clear at 3 MHz where maximal sonothrombolysis was achieved when 55%–60% of 3-μm MBs were destroyed during insonation that yielded maximal sonothrombolysis (**Fig 3d**). One possibility is that an acoustic intensity high enough to collapse approximately 40% of the MBs was required to stimulate the remaining intact MBs sufficiently to achieve maximal sonothrombolysis—that is, approximately 60% of the MBs driven to the limits of collapse produced more sonothrombolysis than a greater number of MBs driven at a lower intensity. Alternatively, the hypothesis that a combination of MBs collapsing during inertial cavitation plus intact, stably cavitating 3-μm MBs is required for maximal sonothrombolysis at 3 MHz remains valid.

The relationship between intact MBs and sonothrombolysis efficacy is especially complicated for 1-μm MBs at 3 MHz. **Figure 3b** illustrates that 1-μm MB survival decreased steadily as sonothrombolysis efficacy improved with increasing ultrasonic intensity, and the sharp decrease in sonothrombolysis efficacy observed as intensity was increased slightly $>$ 2 W/cm^2 is not accompanied by a further decrease in MB survival. One plausible explanation is that at maximal sonothrombolysis efficacy MB collapse occurred sometime shortly after the MBs flowed past the clot; they contributed to clot destruction but collapsed before being collected for assay. The small increase in intensity that caused the marked decrease in sonothrombolysis efficacy (**Fig 1b**) caused MBs to collapse before they could contribute significantly to sonothrombolysis. Visual observation showed that the MBs were not destroyed before reaching the clot. The experimental system was incapable of determining exactly what occurred.

Figure 1a and **b** shows that 3-μm MBs yielded greater sonothrombolysis efficacy than 1-μm MBs at the same acoustic intensity using a 20% duty factor and 100 Hz PRF. The 3-μm MBs were more efficient at transducing ultrasonic energy into sonothrombolysis on a per-MB basis. The 1-μm MBs were more resistant to collapse with increasing intensity at 3 MHz than the 3-μm MBs; they could be used to produce greater sonothrombolysis at 3 MHz. However, when PRF and PD were adjusted at 3 MHz such that 3-μm MB integrity was maintained better while insonating with a higher duty factor (**Fig 5**), sonothrombolysis efficacy at 0.5 W/cm^2 increased markedly and approached the efficacy produced with the 1-μm MBs using 2.0 W/cm^2.

The issue of MBs transducing US energy to sonothrombolysis more efficiently is important. Preclinical and clinical sonothrombolysis studies have encountered significant bleeding and other US-related complications. These complications can arguably be reduced by using lower intensity US, which could be accomplished using MBs that are more efficient at transducing US to sonothrombolysis. Based on this study, it appears that using MBs $>$ 1 μm in diameter would be better at doing this. The 3-μm diameter MBs produced greater sonothrombolysis at 1 MHz, which can penetrate tissues further and requires less incident energy to yield the necessary intensity at the site where sonothrombolysis is required.

Increasing the Young's modulus of 3-μm MBs from 2.2 MPa to 3.6 MPa increased the maximally attainable sonothrombolysis by twofold because the latter were able to be insonated with a twofold higher intensity (0.1 W/cm^2 compared with 0.05 W/cm^2) before a significant fraction of MBs collapsed. However, when the Young's modulus was increased further to 4.5 MPa, the maximally attainable sonothrombolysis was 40% lower, although attainable over a wider intensity range. Increasing the Young's modulus from 3.6 MPa to 4.5 MPa may have stiffened the MB shell and increased its resonance frequency such that more acoustic energy was required for sonothrombolysis at 1 MHz. Insonation at a higher frequency would putatively improve sonothrombolysis.

The observation that increasing the PRF to 400 Hz permitted using higher duty factors to improve sonothrombolysis efficacy with 3-μm MBs at 3 MHz is notable. The higher PRF may have preserved MBs because the shorter PDs reduced the duration of sustained cavitation without pause, which might have reduced MB shell mechanical fatigue to preserve MB integrity. Shorter PDs also maintain a richer ultrasonic frequency spectrum in each pulse that

might enhance nonlinear MB oscillations in a manner that improves sonothrombolysis. This approach to increasing sonothrombolysis by adjusting duty factor and PRF needs to be explored further using other combinations of MB diameter, MB Young's modulus, ultrasonic intensity, and ultrasonic frequency to produce more effective sonothrombolysis, and such experimentation may also affect how US and MBs are employed for other applications (eg, sonoporation and sonophoresis).

In conclusion, this study illustrated that MB size and elasticity affect sonothrombolysis efficacy markedly and demonstrated this with specific experiments using uniformly sized 1-μm and 3-μm diameter MBs. Experiments also showed how adjusting the frequency, intensity, pulse duration, and duty cycle of the actinic US affected sonothrombolysis with these two different-sized MBs. Being able to control both MB and US parameters provides more options for optimizing MB-US interactions for sonothrombolysis, sonoporation, and other ultrasonic modalities. As is often the case, this study might have initiated more questions than it answered, but it is hoped that the study helps identify which questions need to be addressed to understand further how the physical and acoustic properties of MBs affect MB-US–induced sonothrombolysis.

REFERENCES

1. Sobbe A, Stumpff U, Trubestein G, Figge H, Kozuschek W. Die Ultraschall-Auflossung von Thromben [Thrombolysis by ultrasound]. Klin Wochenschr 1974; 52:117–121.
2. Siegel RJ, Fishbein MC, Forrester J, et al. Ultrasonic plaque ablation: a new method for recanalization of partially or totally occluded arteries. Circulation 1988; 78:1443–1448.
3. Kotsuka Y, Furuse A, Matsunaga H, Chikada M. Ultrasonic angioplasty for ostial stenosis of the left coronary artery. Cardiovasc Surg 1993; 1: 192–194.
4. Eccleston DS, Cumpston GN, Hodge AJ, Pearne-Rowe D, Don Michael TA. Ultrasonic coronary angioplasty during coronary artery bypass grafting. Am J Cardiol 1996; 78:1172–1175.
5. Cintas P, Le Traon AP, Larrue V. High rate of recanalization of middle cerebral artery occlusion during 2-MHz transcranial color-coded Doppler continuous monitoring without thrombolytic drug. Stroke 2002; 33: 626–628.
6. Eggers J, Koch B, Meyer K, König I, Seidel G. Effect of ultrasound on thrombolysis of middle cerebral artery occlusion. Ann Neurol 2003; 53: 797–800.
7. Alexandrov AV, Demchuk AM, Burgin WS, Robinson DJ, Grotta JC. Ultrasound-enhanced thrombolysis for acute ischemic stroke: phase I. Findings of the CLOTBUST trial. J Neuroimaging 2004; 14:113–117.
8. Drobinski G, Brisset D, Philippe F, et al. Effects of ultrasound energy on total peripheral artery occlusions: initial angiographic and angioscopic results. J Interv Cardiol 1993; 6:157–163.
9. Kuo WT, Gould MK, Louie JD, Rosenberg JK, Sze DY, Hofmann LV. Catheter-directed therapy for the treatment of massive pulmonary embolism: systematic review and meta-analysis of modern techniques. J Vasc Interv Radiol 2009; 20:1431–1440.
10. Larsson J, Carlson J, Olsson SB. Ultrasound enhanced thrombolysis in experimental retinal vein occlusion in the rabbit. Br J Ophthalmol 1998; 82:1438–1440.
11. Luo H, Steffen W, Cercek B, Arunasalam S, Maurer G, Siegel RJ. Enhancement of thrombolysis by external ultrasound Am Heart J 1993; 125:1564–1569.
12. Frenkel V, Oberoi J, Stone MJ, et al. Pulsed high-intensity focused ultrasound enhances thrombolysis in an in vitro model. Radiology 2006; 239:86–93.
13. Holland CK, Vaidyaa SS, Datta S, Coussios CC, Shawa GJ. Ultrasound-enhanced tissue plasminogen activator thrombolysis in an in vitro porcine clot model. Thromb Res 2008; 121:663–673.
14. Diamond SL, Anand S. Inner clot diffusion and permeation during fibrinolysis. Biophys J 1993; 65:2622–2643.
15. Francis CW, Blinc A, Lee S, Cox C. Ultrasound accelerates transport of recombinant tissue plasminogen activator into clots. Ultrasound Med Biol 1995; 21:419–424.
16. Sakharov DV, Hekkenberg RT, Rijken DC. Acceleration of fibrinolysis by high-frequency ultrasound: the contribution of acoustic streaming and temperature rise. Thromb Res 2000; 100:333–340.
17. Rosenschein U, Yakubov SJ, Guberinich D, et al. Shock-wave thrombus ablation, a new method for noninvasive mechanical thrombolysis. Am J Cardiol 1992; 70:1358–1361.
18. Makin IR, Everbach EC. Measurement of pressure and assessment of cavitation for a 22.5-kHz intra-arterial angioplasty device. J Acoust Soc Am 1996; 100:1855–1864.
19. Tachibana K, Tachibana S. Albumin microbubble echo-contrast material as an enhancer for ultrasound accelerated thrombolysis. Circulation 1995; 92:1148–1150.
20. Porter TR, LeVeen RF, Fox R, Kricsfeld A, Xie F. Thrombolytic enhancement with perfluorocarbon-exposed sonicated dextrose albumin MBs. Am Heart J 1996; 132:964–968.
21. Mizushige K, Kondo I, Ohmori K, Hirao K, Matsuo H. Enhancement of ultrasound-accelerated thrombolysis by echo contrast agents: dependence on microbubble structure. Ultrasound Med Biol 1999; 25: 1431–1437.
22. Culp WC, Porter TR, Xie F, et al. Microbubble potentiated ultrasound as a method of declotting thrombosed dialysis grafts: experimental study in dogs. Vasc Interv Radiol 2001; 11:351–358.
23. Culp WC, Flores R, Brown AT, et al. Successful microbubble sonothrombolysis without tissue-type plasminogen activator in a rabbit model of acute ischemic stroke. Stroke 2011; 42:2280–2285.
24. Molina CA, Ribo M, Rubiera M, et al. Microbubble administration accelerates clot lysis during continuous 2-MHz ultrasound monitoring in stroke patients treated with intravenous tissue plasminogen activator. Stroke 2006; 37:425–429.
25. Slikkerveer J, Dijkmans PA, Sieswerda GT, et al. Ultrasound enhanced prehospital thrombolysis using microbubbles infusion in patients with acute ST elevation myocardial infarction: rationale and design of the Sonolysis study. Trials 2008; 9:72.
26. Molina CA, Barreto AD, Tsivgoulis G, et al. Transcranial ultrasound in clinical sonothrombolysis (TUCSON) trial. Ann Neurol 2009; 66:28–38.
27. Wu Y, Unger EC, McCreery TP, et al. Binding and lysing of blood clots using MRX-408. Invest Radiol 1998; 33:880–885.
28. Wang B, Wang L, Zhou XB, et al. Thrombolysis effect of a novel targeted microbubble with low-frequency ultrasound in vivo. Thromb Haemost 2008; 100:356–361.
29. Shaw GJ, Meunier JM, Huang SL, Lindsell CJ, McPherson DD, Holland CK. Ultrasound-enhanced thrombolysis with tPA-loaded echogenic liposomes. Thromb Res 2009; 124:306–310.
30. Alonso A, Dempfle CE, Della Martina A, et al. In vivo clot lysis of human thrombus with intravenous abciximab immunobubbles and ultrasound. Thromb Res 2009; 124:70–74.
31. Ribo M, Molina CA, Alvarez B, Rubiera M, Alvarez-Sabin J, Matas MJ. Intra-arterial administration of microbubbles and continuous 2-MHz ultrasound insonation to enhance intra-arterial thrombolysis. Neuroimaging 2010; 20:224–227.
32. Daffertshofer M, Gass A, Ringleb P, et al. Transcranial low-frequency ultrasound-mediated thrombolysis in brain ischemia: increased risk of hemorrhage with combined ultrasound and tissue plasminogen activator: results of a phase II clinical trial. Stroke 2005; 36:1441–1446.
33. Culp WC, Porter TR, Lowery J, Xie F, Roberson PK, Marky L. Intracranial clot lysis with intravenous microbubbles and transcranial ultrasound in swine. Stroke 2004; 35:2407–2411.
34. Marsh JN, Senpan A, Hu G, et al. Fibrin-targeted perfluorocarbon nanoparticles for targeted thrombolysis. Nanomedicine (Lond) 2007; 2: 533–543.
35. Brown AT, Flores R, Hamilton E, Roberson P, Borrelli MJ, Culp WC. Microbubbles improve sonothrombolysis in vitro and decrease hemorrhage in vivo in a rabbit stroke model. Invest Radiol 2010; 46: 202–207.
36. Datta S, Coussios CC, McAdory LE, et al. Correlation of cavitation with ultrasound enhancement of thrombolysis. Ultrasound Med Biol 2006; 32: 1257–1267.

37. Prokop AF, Soltani A, Roy RA. Cavitational mechanisms in ultrasound-accelerated fibrinolysis. Ultrasound Med Biol 2007; 33:924–933.
38. Church CC. Prediction of rectified diffusion during nonlinear bubble pulsations at biomedical frequencies. J Acoust Soc Am 1988; 83:2210–2217.
39. Holland CK, Apfel RE. An improved theory for the prediction of microcavitation thresholds. IEEE Trans Ultrason Ferroelectr Freq Control 1989; 36:204–208.
40. Khismatullin DB. Resonance frequency of microbubbles: effect of viscosity. J Acoust Soc Am 2004; 116:1463–1473.
41. Ammi AY, Cleveland RO, Mamou J, Wang GI, Bridal SL, O'Brien WD Jr. Ultrasonic contrast agent shell rupture detected by inertial cavitation and rebound signals. IEEE Trans Ultrason Ferroelectr Freq Control 2006; 53:126–136.
42. Doinikov AA, Haac JF, Dayton PA. Modeling of nonlinear viscous stress in encapsulating shells of lipid-coated contrast agent microbubbles. Ultrasonics 2009; 49:269–275.
43. King DA, Malloy MJ, Roberts AC, Haak A, Yoder CC, O'Brien WD Jr. Determination of postexcitation thresholds for single ultrasound contrast agent microbubbles using double passive cavitation detection. J Acoust Soc Am 2010; 127:3449–3455.
44. Borrelli MJ, O'Brien WD Jr, Bernock LJ, et al. Production of uniformly sized serum albumin and dextrose microbubbles. Ultrasonics Sonochemistry 2012; 19:198–208.
45. Sen S, Shyamsundar Subramanian S, Discher DE. Indentation and adhesive probing of a cell membrane with AFM: theoretical model and experiments. Biophys J 2005; 89:3203–3213.

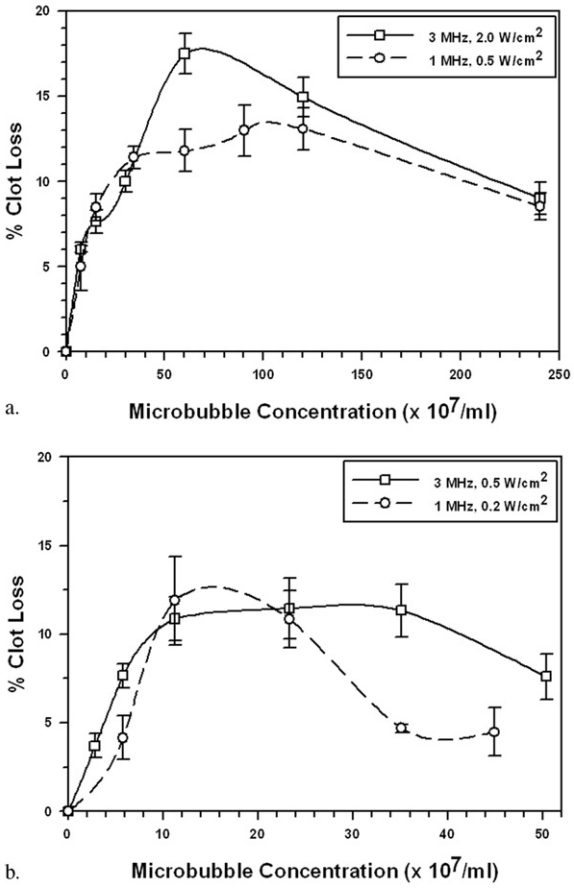

Figure E1. Sonothrombolysis as a function of MB concentration. Each symbol represents the mean of a minimum of four measurements, and error bars indicate standard deviation. Data points near minima and maxima are the results of 6–10 measurements to ensure accuracy at these critical transition regions. **(a)** With 1-μm diameter MBs. **(b)** With 3-μm diameter MBs.

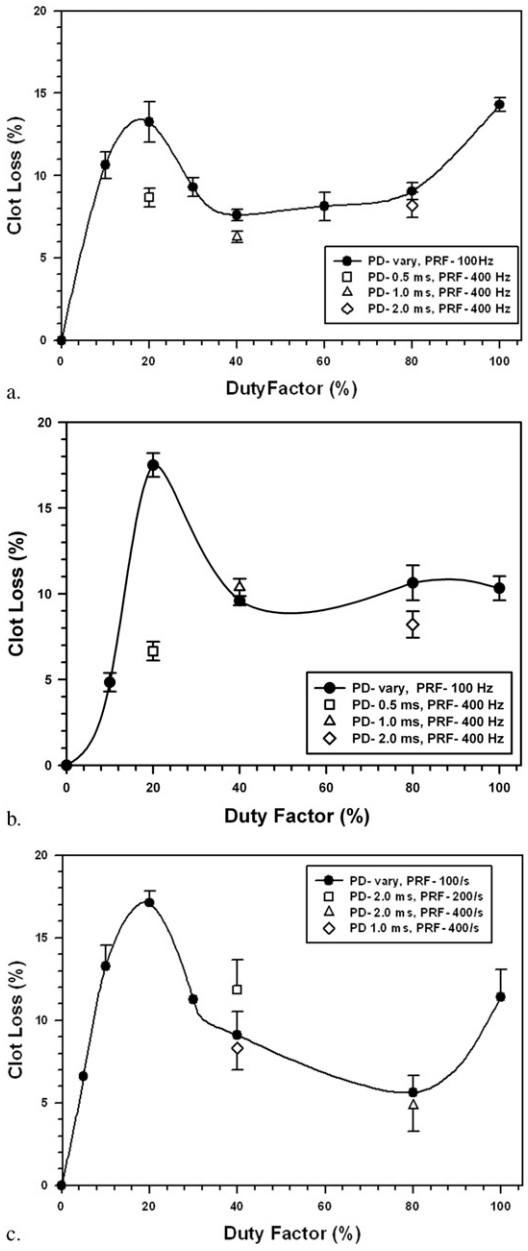

Figure E2. Effect of changing duty cycle on sonothrombolysis efficacy. Sonothrombolysis was performed using pulsed US with a PRF of 100 Hz or 400 Hz. The acoustic intensity used was that which produced maximal sonothrombolysis for each different MB at 1 MHz or 3 MHz. Each symbol represents the mean of a minimum of four measurements, and error bars indicate standard deviation. Data points near minima and maxima are the results of 6–10 measurements to ensure accuracy at these critical transition regions. **(a)** With 1-μm diameter MBs at 1 MHz and 0.5 W/cm^2. **(b)** With 1-μm diameter MBs at 3 MHz and 2 W/cm^2. **(c)** With 3-μm diameter MBs at 1 MHz and 0.1 W/cm^2 .