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Quantitative analysis of the 5 µl thrombus dissolution process using 40 kHz – 6 MHz ultrasound

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***Abstract*—Precise quantitative analysis of the sonothrombolysis process is required to minimise the amount of thrombolytic drug dangerous for the patient, because it can cause internal hemorrhage. Verification of the effects of other drugs or other procedures for the elimination of thrombi, for example ultrasound contrast microbubbles, also requires quantitative research.**

For microscopic examination of the thrombolysis process, the Rexolite parallel plate flow chamber has been used. The internal dimensions of the chamber were 11x1x20 mm. In order to eliminate the standing wave, the incident wave was perpendicular to the reflected one. The narrowband chirp driven transducer suppressed the surface waves in Rexolite. The clot dissolution was processed at 40 kHz – 6 MHz ultrasound frequencies and 2 W/cm^2 spatial averaged, temporal averaged intensities. The thrombus was obtained from a 5 µl drop of blood placed directly inside a flow chamber. The flow chamber was filled with the cell culture medium Dulbecco's modified Eagle's medium. The flow in the chamber was forced by a peristaltic pump at a speed of 3.8 ml/min. The Actilyse tissue plasminogen activator at a concentration of 10 µg/ml was added. The similarity of the thrombolysis process obtained from 5 µl of blood with a similar volume fragment cut from a larger thrombus was experimentally verified. Thrombus volume was estimated from microscopic photographs by calculating its surface area and its optical transparency.

At 2 W/cm^2 ultrasound intensity, took the thrombus 4, 8, 9 and 12 minutes to completely dissolve for the centre ultrasound frequencies of 40.9, 149, 209 kHz and 1.02 MHz, respectively. For higher frequencies, the thrombus only reduced its volume by 82%, 69% and 27% for the frequencies 2.10, 3.34 and 6.63 MHz, respectively. Sonication for 20 - 60 minutes did not cause further dissolution of thrombi.

Keywords—ultrasound, blood, thrombus, thrombolysis, parallel plate flow chamber,

I. Introduction

Sonothrombolysis is a modern therapeutic method which involves dissolving a thrombus inside a blood vessel. The method, introduced by Alexandrov [1] is based on the interaction of the drug tissue plasminogen activator (tPA) and an acoustic wave. Its use allows the dose of the drug to be reduced and eliminates the risk of intra-systemic bleeding. The detailed selection of ultrasonic wave parameters and the amount of thrombolytic drug requires repeated experiments to dissolve thrombi in vitro. Reducing the size of the thrombus speeds up the experiment and reduces the amount of blood needed to form the thrombus.

Modern in-vitro tests are carried out on a small area (lab-on-chip), using a minimum amount of tissue. The parallel-plate flow chamber (PPFC) is popular, enabling microscopic observation of the impact of flowing liquid on the tissue being examined. The liquid contains active substances that react with the tissue and cause the studied biochemical process. PPFC vessels were used to study the process of thrombus formation [2], as well as to study the process of fibrinolysis [3].

Commercially available PPFCs do not provide effective ultrasonic wave propagation and are not suitable for sonothrombolysis studies. The authors developed their own PPFC set, made entirely of plastics with low ultrasonic losses and acoustic impedance close to water [4].

II. Methods

A. Parallel-plate Flow Chamber

To build our own PPFC, used in the experiment, the rexolite (C-Lec Plastics, Inc., Philadelphia, USA), polycarbonate and a silicone gasket (A2414, Bioptechs, Butler, USA) were used [4]. The dimensions of the gasket created the size of the channel in which the thrombi were placed. The channel cross-section had a dimension of 1 x 11 mm and the channel length was 20mm.

The ultrasonic transducer located below the PPFC generated an acoustic wave at an angle of 45 degrees relative to the PPFC surface. The wave bounced off the polycarbonate cover - air boundary and returned at a 90-degree angle to the incident wave. The perpendicularity of the incident and reflected waves eliminated the standing wave, which could unpredictably increase the intensity of ultrasound inside the PPFC vessel [5]. In order to eliminate the surface wave in the rexolite plate, the ultrasonic transducer was excited with chirp - a burst of variable frequency sinusoid.

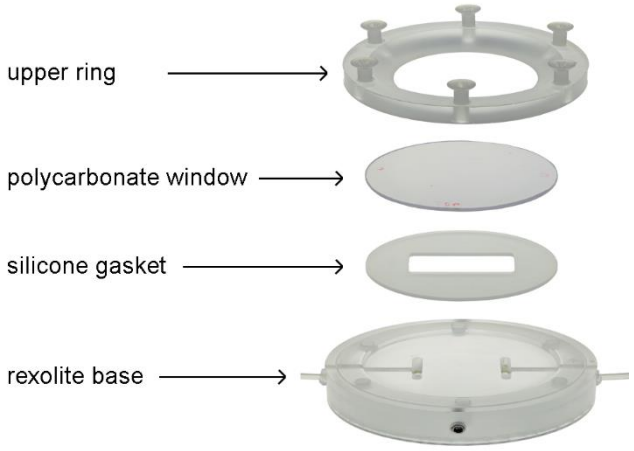

Fig. 1. Designed parallel-plate flow chamber (PPFC) [4].

PPFC was placed on the top of the polygonal polycarbonate vessel used in previous experiments [4]. The vessel was filled with deionised water at 37°C. An ultrasonic transducer and a sonic absorbing silicone rubber layer were attached beneath the PPFC. The whole vessel was put in place of the dismantled Nikon Eclipse E400 microscope stage with a Nikon E plan 4x/0.10 lens (Nikon, Tokyo, Japan). The tested thrombus sample was illuminated by an LED lamp placed under the vessel. Microscopic images were taken with a Sony α6500 camera. The field of view of the microscope was 5 mm in diameter.

A set of ultrasonic transducers for the frequency range 40 kHz - 6 MHz was prepared for the measurements (Fig.2). The Langevin transducer from an ultrasonic cleaner was used at 40 kHz. Other transducers are made of Pz26 or Pz28 piezoceramics (Meggitt, Denmark). The two highest ultrasonic frequencies 3 MHz and 6 MHz were obtained by the third harmonic drive of 1 MHz and 2 MHz transducers. The parameters of the prepared transducers are shown in Table 1.

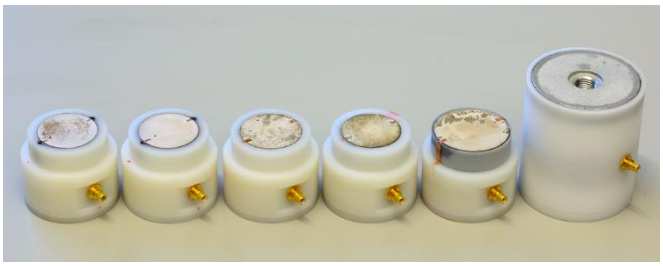

Fig. 2. Transducers designed for the experiment.

TABLE I.

Centre frequency MHz	*Transducer material*	*Chirp frequency MHz*	*Chirp pulse length ms*	*Pulse repetition ms*	*Driving voltage Vpp*
40.9 kHz	Langevin	burst 40.9 kHz	burst 24.45	61.12	447
0.149	Pz26	.141-.156	6.7	16.75	400
0.209	Pz26	.198-.219	4.8	12	371
0.538	Pz26	.511-.565	1.8	4.5	262
1.02	Pz28	0.97-1.07	1	2.5	107
2.10	Pz28	1.99-2.21	0.7	1.75	150
3.34	Pz28	3.17-3.51	0.5	1.5	257
6.63	Pz28	6.29-6.96	0.5	1.5	308

B. *Measurements*

A diagram of the measuring system used in the experiment is shown in Fig. 3. The ultrasonic transducer was excited by the ar500A250B power amplifier (Amplifier Research, Souderton, USA) controlled by the 81150A pulse function arbitrary generator (Agilent Technologies, Loveland, USA). The transducer was excited with a chirp matched to the transducer bandwidth. The chirp time was 1000 sine periods with the centre frequency of the transducer, and the duty ratio was t/T = 0.4.

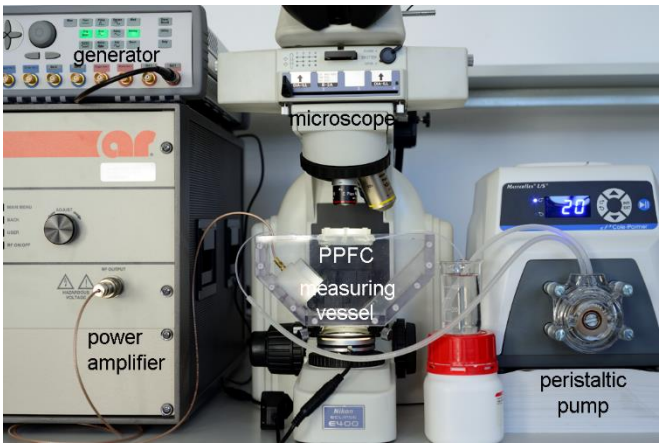

Fig. 3. Set-up for sonothrombolysis experiments.

The 2 W/cm^2 I_{SATA} spatial averaged, temporal averaged ultrasound intensity was determined by measuring the acoustical power using an ultrasound power meter UPM-DT-1E (Ohmic Instruments, Easton, USA). Measurements were made with the chirp driving the transducer as during the experiment. Intensity losses inside PPFC were verified by measuring the sound pressure with a PAFOH48 fiber optic hydrophone (Precision Acoustics, Dorchester, UK).

The pulsating flow through the PPFC was controlled by the Masterflex L/S peristaltic pump with the 96410-14 tube (Cole-Parmer, Vernon Hills, USA). The pump was rotating at 20 rpm, which caused a 1 Hz pulsation. The pump forced an average flow rate of 3.8 ml/min. The flowing liquid was a DMEM solution (Dulbecco's Modified Eagle's Medium, 51449C, Sigma-Aldrich, Germany) with the addition of 10µg/l tissue plasminogen activator tPA (Actilyse, Boehringer Ingelheim,

Ingelheim am Rhein, Germany). The solution temperature was 37°C.

The thrombus was formed from 5 µl ± 1 µl of the human blood collected from the patient by finger prick. Blood volume was measured by pipette. The blood drop was placed in the center of the PPFC base and allowed to clot for 15 min. The PPFCs were then sealed and quenched with DMEM. After another 15 min, tPA was added, pump and ultrasound were turned on. Pictures of the blood clots were taken every 30 s for 20 min. The similarity of the thrombolysis process obtained from 5 µl of blood with a similar volume fragment cut from a larger thrombus was experimentally verified. The research was conducted with the consent of the Ethics Committee at the Military Medical Institute in Warsaw.

Thrombus photographs were analyzed with the Matlab® program. The initial area occupied by the thrombus was determined, and then changes in the brightness of the selected area were recorded. Alignment of the area brightness with the background brightness was treated as 100% dissolution of the thrombus.

III. Results

The results of the measurements of ultrasonic losses inside PPFC are shown in Table 2. Not measured at 40.9 kHz, as it was below the frequency range of the fiber optic hydrophone.

TABLE II.

Frequency	*149 kHz*	*209 kHz*	*538 kHz*	*1.02 MHz*	*2.10 MHz*	*3.34 MHz*	*6.63 MHz*
Intensity loss (dB)	*-4.8*	*-4.3*	*-9.7*	*-4.0*	*-6.7*	*-4.5*	*-5.2*

The time and effectiveness of thrombus dissolution at various ultrasonic frequencies are presented in Fig. 4. and Table 3. Fig. 5. shows the sequence of photos taken every 2 min of dissolved clot at 1 MHz

TABLE III.

Centre frequency	*Results of sonothrombolysis*
no ultrasound	*6% volume reduction in 20 min*
40.9 kHz	*complete dissolution in 4 min*
149 kHz	*complete dissolution in 8 min*
209 kHz	*complete dissolution in 9 min*
538 kHz	*91% volume reduction in 20 min*
1.02 MHz	*complete dissolution in 12 min*
2.10 MHz	*82% volume reduction in 20 min*
3.34 MHz	*69% volume reduction in 20 min*
6.63 MHz	*27% volume reduction in 20 min*

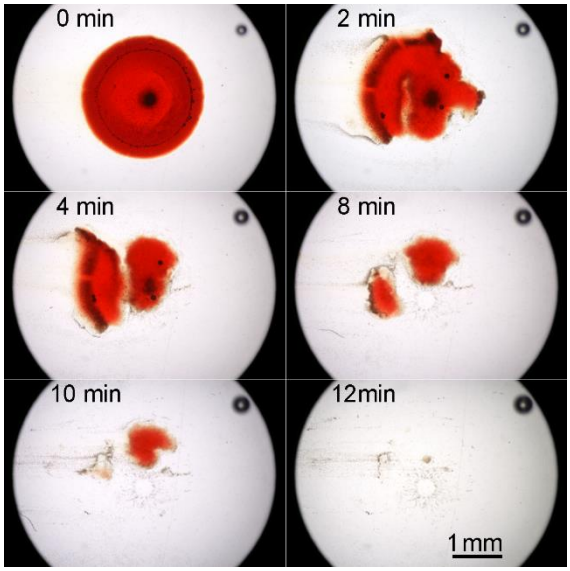

Fig. 5. Sequence of sonothrombolysis process taken every 2 min at 1 MHz.

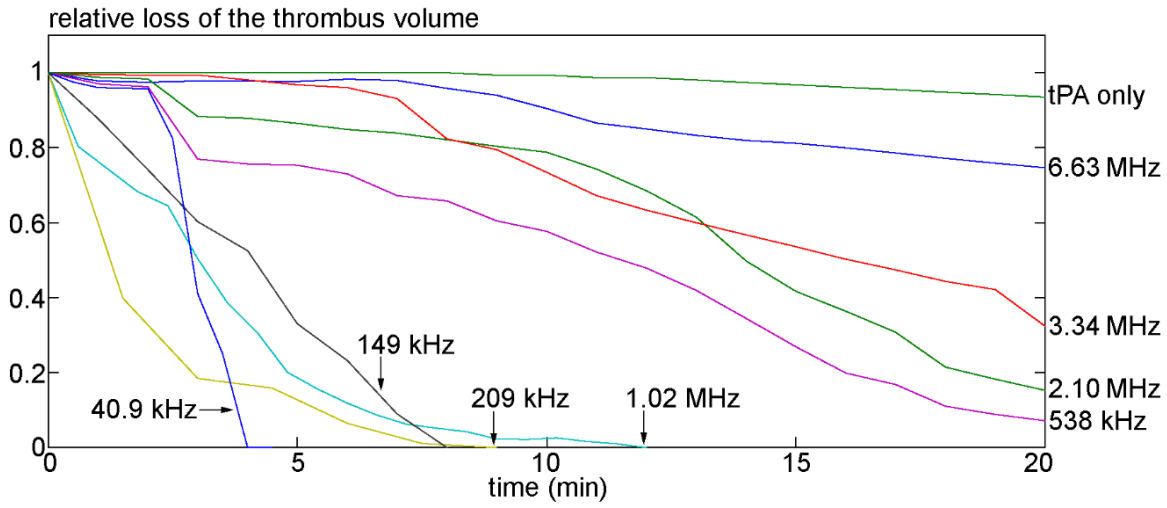

Fig. 4. Efficacy of the thrombus dissolution in the sonotrombolysis process at ultrasonic intensity I_{SATA} = 2W/cm^2 and frequencies of 40.9 kHz - 6.63 MHz.

IV. Conclusions

The designed PPFC was suitable for research on the interaction of ultrasound and the drug that dissolves the thrombus. Blood clots formed from 5µl of blood guaranteed the repeatability of measurements, and the analysis of microscopic images with the help of Matlab® software gave quantitative results in the form of instantaneous thrombus volume. The effect of the standing wave was eliminated by ultrasound propagated at 45 degrees, and the effect of surface wave by the chirp driven transducer.

The sonothrombolysis process was studied in a wide frequency range of 40.9 kHz - 6.63 MHz. This covered the range of the acoustic wavelength of 37 mm - 230 µm. The average diameter of the thrombus was 2.4 mm, so the wavelengths were both many times larger than the size of the thrombus and many times smaller. The thrombus dissolution efficiency was inversely proportional to the ultrasonic frequency. It was the smallest at 6.63 MHz and the largest at 40.9 kHz. The thrombus was completely dissolved for 4, 8, 9 and 12 minutes for the ultrasound frequencies of 40.9, 149, 209 kHz and 1.02 MHz, respectively. For higher frequencies, for 20 minutes, the thrombus only reduced its volume by 82%, 69% and 27% for the frequencies 2.10, 3.34 and 6.63 MHz, respectively. Sonication for 20 - 60 minutes did not cause further dissolution of thrombi. An exception occurred at 538 kHz, when the performance was similar to 2.10 MHz. The supposed cause was the -9.7 dB reduction of the sound pressure inside the PPFC for this frequency.

For other frequencies, the intensity losses inside PPFC ranged from -4 dB to -5 dB, which corresponds to the reduction of I_{SPTA} spatial peak, temporal average intensity to 0.8 - 0.6 W/cm^2, respectively.

Acknowledgment

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