Published in final edited form as:

Ultrasonics. 2017 May ; 77: 203–213. doi:10.1016/j.ultras.2017.02.008.

***In vitro* ultrasound experiments: Standing wave and multiple reflections influence on the outcome**

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Abstract

The purpose of this work was to determine the influence of standing waves and possible multiple reflections under the conditions often encountered in examining the effects of ultrasound exposure on the cell cultures *in vitro*. More specifically, the goal was to quantitatively ascertain the influence of ultrasound exposure under free field (FF) and standing waves (SW) and multiple reflections (MR) conditions (SWMR) on the biological endpoint (50% cell necrosis). Such information would help in designing the experiments, in which the geometry of the container with biological tissue may prevent FF conditions to be established and in which the ultrasound generated temperature elevation is undesirable. This goal was accomplished by performing systematic, side-by-side experiments *in vitro* with C6 rat glioma cancer cells using 12 well and 96 well plates. It was determined that to obtain 50% of cell viability using the 12 well plates, the spatial average, temporal average (I_{SATA}) intensities of 0.32 W/cm^2 and 5.89 W/cm^2 were needed under SWMR and FF conditions, respectively. For 96 well plates the results were 0.80 W/cm^2 and 2.86 W/cm^2 respectively. The corresponding, hydrophone measured p_{RMS} maximum pressure amplitude values, were 0.71 MPa, 0.75 MPa, 0.75 MPa and 0.73 MPa, respectively. These results suggest that p_{RMS} pressure amplitude was independent of the measurement set-up geometry and hence could be used to predict the cells' mortality threshold under any *in vitro* experimental conditions or even as a starting point for (pre-clinical) *in vivo* tests. The described procedure of the hydrophone measurements of the p_{RMS} maximum pressure amplitude at the $\lambda/2$ distance (here 0.75 mm) from the cell's level at the bottom of the dish or plate provides the guideline allowing the difference between the FF and SWMR conditions to be determined in any experimental setup. The outcome of the measurements also indicates that SWMR exposure might be useful at any ultrasound assisted therapy experiments as it permits to reduce thermal effects. Although the results presented are valid for the experimental conditions used in this study they can be generalized. The analysis developed provides methodology facilitating independent laboratories to determine their specific ultrasound exposure parameters for a given biological end-point under standing waves and multiple reflections conditions. The analysis also permits verification of the

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outcome of the experiments mimicking pre- and clinical environment between different, unaffiliated teams of researchers.

Keywords

Standing wave; Ultrasound pressure; Ultrasound intensity; C6 glioma; Anticancer therapy; Sonodynamic therapy; Ultrasound bio-effects

1. Introduction

The purpose of this work was to determine the influence of standing waves (SW) and possible multiple reflections (MR) under the conditions often encountered in examining the effects of ultrasound exposure on the cell cultures *in vitro*. There is an ongoing interest in determining the exposure conditions that would be most effective when used in a variety of anticancer therapeutic applications. Thus, synergy between the ultrasound energy and a certain family of drugs referred to as sonosensitizers [2] was proposed to be useful in the treatment of cancer cells (sonodynamic therapy), [26]. In another application the enhancement of the effectiveness of chemotherapeutic drugs (ultrasound-mediated chemotherapy) was investigated [28]. Yet another treatment reported [1] involved interaction of ultrasound and the thrombolytic drug leading to dissolving of clots (sonothrombolysis). As the optimization process of such applications involves *in vitro* experiments to gain some insight into the intricacies of designing such experiments and the associated pitfalls often caused by the geometry of the cell culture containers, below a succinct review of the typical setups is given. The setups described include Petri dish, and multi-well cell culture plates or “OptiCell” culture media along with a brief discussion of their advantages and disadvantages.

Fig. 1. shows a typical [11,18,19,27,29–31] *in vitro* ultrasound exposure configuration, in which the Petri dish, containing the medium and the cells cultured on its bottom, is placed in the ultrasound field. The field is generated by a piezoelectric (PZT) source and coupled to the bottom of the dish through degassed, temperature controlled water bath. The plate is covered with a glass or plastic lid to ensure sterility.

In Fig. 1 setup, the distal water-air boundary (1) is the primary source of the formation of a standing wave. Another source of the wave reflection (3) is due to the mismatch between the transducer’s acoustic impedance and that of water and reflection at this boundary can lead and contribute to constructive interaction with the primary source standing wave. Finally, the reflection (2) occurs between the water and the bottom of the Petri dish.

One way of eliminating the standing waves would be to remove the lid covering the plate or dish with the cells and immerse into the medium an acoustically matched to water ultrasound absorber [13]. An alternative solution would be to immerse ultrasound source (emanating waves downwards) into the medium in well, and position an ultrasound absorbing material below the exposed biological material such as cell colony [15]. However, such a solution makes it difficult to maintain the sterility and there is a risk of overheating of cells, both by the heat emitted at the transducer surface and the heat generated by the

ultrasound absorber, if it is located in the immediate vicinity of the cells. In addition, the diameter of the insonifying transducer must be smaller than the inner diameter of the opening in the plate or dish, which impacts the ultrasound field distribution. Specifically, it reduces intensity of the ultrasound wave at the edges of the well and hence decreases the efficacy of cell destruction.

It would appear that placing cells in a specially designed acoustically transparent container could provide the desirable, standing wave-free experimental conditions. Indeed, such solution was proposed in [8,21]. In the experimental setup described therein the container walls (10 mm × 10 mm plastic bag, in [21] were made of 50 μm thin foil, so that attenuation could be considered negligible and the container itself was placed in a relatively large (1 dm³) water tank with walls covered with acoustic absorber. Yet another solution would be to use an “OptiCell” box with walls made out of a 75 μm thin film [6]. However, here the size of the “OptiCell” box (7 × 7 cm), would require ultrasound source matching this aperture and such sources are seldom, if at all available. It would be worthwhile to note that placing a layer of sponge between the transducer and the Petri dish with the cells would help to eliminate the reflection due to the impedance mismatch between water and ultrasound transducer [4], however this approach would not provide the standing wave free conditions.

An alternative method to eliminate standing waves was proposed in [7], where broadband (300 kHz centered around 1.5 MHz) ultrasound source was used to generate chirp insonification to measure local viscoelastic properties of tissue. It was effective at low duty factor (6.5 ms/15 s = 4 · 10⁻⁴), however, this approach was not effective to eliminate standing waves during the blood-brain barrier disruption [23] and was not tested for the anticancer therapy applications.

As already noted above, in practice, the lossless propagation of ultrasound waves is attained only for the thin film or foil (ideally, two orders of magnitude thinner than the wavelength). This is unachievable in *in vitro* exposure arrangement where the bottom of the Petri dish or multi well plate attenuates the intensity of the acoustic wave, and converts it into heat, increasing the temperature of the dish bottom.

The importance of the analysis presented here is further corroborated by the recent comprehensive review [28] where the variety of exposure conditions of anticancer treatment were reviewed. For instance, Table 1 therein shows that the *in vitro* experiments with cell cultures and suspensions reported in the literature used treatment times ranging from 1 to 5 min whereas the ultrasound energy was delivered either as continuous wave or relatively long (1000 cycles at 1 MHz) pulses delivered with the duty factor between 0.2 and 0.4 and pulse repetition frequency varying between 1 Hz and 1 kHz [28].

In summary, the review of the literature indicates that the *in vitro* experiments are difficult to reproduce because the exposure setups are not “standardized” and in general, each user develops an individual approach to eliminate or minimize the influence of the standing wave in the experiment. Typically, in the experiments performed under standing wave conditions the actual pressure amplitude or intensity level are not determined and the efficacy of the examined therapy is related to a given incident spatial average, temporal average ultrasonic

intensity I_{SATA} , calculated from the measured ultrasound power [11,18,19,27,29–31]. Furthermore, the *in vitro* conditions are in general difficult to extrapolate to those encountered *in vivo* because in clinical situation standing wave-free conditions are rarely, if at all achievable. Indeed, as indicated in [23], where the transcranial focused ultrasound (0.27–2.53 MHz, 0.58–0.71 MPa) was used to disrupt the blood-brain barrier, the wave reflection from the surface of the skin or bone was present.

At this juncture, prior to moving on to the description of the details of this study, it is appropriate to note that the analysis of ultrasound fields in cell culture wells during *in vitro* experiments was reported in [12]. However, in these experiments the acoustic pressure amplitude was limited to 200 kPa_{p-p} (= 71 kPa_{RMS}) delivered as 5 periods, 1 MHz pulses. As the corresponding wavelength was 1.5 mm, the interaction between incident and reflected wave was present at limited (3.75 mm) distance from the air and water boundary, far away (10 mm) from the cells. Thus neither standing wave nor multiple reflections were recorded at the cells' level.

In contrast to experimental design of [12], in this study (see next section) the glioma cells were insonified by the relatively long, 1 MHz ultrasound bursts (1000 cycles) with pressure amplitudes (and corresponding intensity levels) sufficient to cause cells' death. In agreement with the outcome of the experiments described below, such relatively long bursts might have been necessary to induce and to sustain the sono-chemical reaction generating cytostatic substances such as free radicals suggested to be present in the similar experiments originally proposed in [30]. The reason for selection of 1 MHz frequency and these (glioma) cells is discussed further in the following.

Overall, as already noted, the primary motivation of this study was to determine the influence of the standing wave (including possible multiple reflections) on the results of *in vitro* experiment and to compare the efficacy of the ultrasound assisted treatment under standing waves and the free field conditions with concurrent monitoring of cells viability, key ultrasound field parameters (see below) and temperature effects. Such side-by-side comparison was not reported yet and as evidenced below the experimental results unveiled that standing wave environment could be advantageous, especially if minimizing temperature elevation within the insonified volume is needed. The work examines tumor cell viability after exposure to ultrasound of known pressure and intensity, and discusses the measurement of temperature rise caused by the ultrasound. In the next section the materials and methods along with the approach are carefully described. Section 3 outlines the acoustic measurements performed to link the biological endpoint with the relevant parameters of 1 MHz (unfocused) ultrasound field such as pressure amplitude, intensity and power. In Section 4 the results obtained using 12 and 96-well plates under free field (FF) and standing wave (SW) conditions are presented and in Section 5 conclusions, including those that might be applicable to *in vivo* exposure are summarized.

2. Materials and methods

To facilitate the discussion of the results a brief summary of the formulas used here is given. It is recognized that all of them can be found in the textbooks [14], however, they are reproduced here for convenience.

2.1. Standing wave coefficients to be considered

The reflection coefficients R and transmission coefficient T (both for intensity and assuming plane wave and lossless medium) at the interface of two media with different acoustic impedances are given by formulas [14]:

$$R = \left(\frac{Z_2 - Z_1}{Z_1 + Z_2} \right)^2 \quad (1)$$

$$T = \frac{4Z_1 Z_2}{(Z_1 + Z_2)^2} \quad (2)$$

where Z_1 is acoustic impedance of the medium where ultrasound source is placed and Z_2 is acoustic impedance of the second medium. The power standing wave ratio can be expressed as [25]:

$$\text{PSWR} = \frac{I_m}{I_0} \quad (3)$$

where I_m is maximum standing wave intensity and I_0 is the intensity of incident wave. Acoustic properties of the air, water, Pz28 piezoceramic (Meggitt, Denmark) and multi-well cell culture plate polystyrene (PS), [16] are presented in Table 1.

For the water-air interface $R = 1$ and $\text{PSWR} = 4$. As mentioned in the introduction there is also additional reflection at the water-piezoceramic interface that needs to be considered. For Pz28 piezoceramic used here, according to the energy conservation law the total value of $\text{PSWR} = 5.7$. For the polystyrene, the intensity reflection coefficient was deemed to be negligible and was omitted in the following considerations. The acoustic pressure amplitude was considered to be proportional to the square root of the acoustic intensity. Accordingly, the expected increase in the ultrasound pressure amplitude was calculated as the square root of the corresponding PSWR ratios, i.e. 2 and 2.4, respectively.

2.2. Cell culture – glioma cells

The rat C6 glioma cells obtained from American Type Culture Collection (Manassas, VA, USA) were used in the experiments described in the next section. These cells were chosen because they are well susceptible to ultrasound treatment and were reported as well responsive to non-invasive ultrasound assisted brain cancer therapy [17]. The cell

preparation procedure was the same as described in [15] and similar to those reported in [4,29]. Briefly, the cells were grown on a Petri dish after addition of Dulbecco's Modified Eagle's Medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were grown at 37 °C in a humidified atmosphere containing 5% carbon dioxide. DMEM medium was changed every 3–4 days. Then, the cells were sown in the multi well cell culture plates. The two most widely used sizes of the plates (12 and 96 Well Cell Culture Plates (Cellstar, USA)) were selected for the experiment. The cells were sown either in every second well of 12 Well Cell Culture Plate 665-180 (Cellstar, USA), or in four neighboring wells of 96 Well Cell Culture Plate 655-180 (Cellstar, USA). The filled wells were separated by the empty wells to prevent the interaction of ultrasound in the adjacent well during insonification. Arrangement of the filled wells in the cell culture plates is shown in Fig. 2.

The concentration of $2 \cdot 10^5$ cells/ml and DMEM medium supplemented again with 10% FBS and penicillin and streptomycin were put into one well. 2 ml into 12 well plate, 22.2 mm bottom diameter, 5.2 mm deep and 0.2 ml into 96 well plate, 6.6 mm diameter, 5.8 mm deep. After 24 h incubation at 37 °C, the medium was replaced with a new DMEM but without FBS. The cells were then exposed to ultrasound for 3 min (180 s). It was observed that cell mortality was proportional to exposure time but leveled off after approximately 180 s [3]. Accordingly, in the experiments reported 180 s exposure was chosen. After the experiment, the cells were replaced in the incubator for another 24 h and then 20 µl solution of MTT viability test assay 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Sigma-Aldrich, USA) was added to each well containing cells. After 3 h incubation the DMEM medium was removed and 200 µl DMSO (dimethylsulfoxide, Sigma-Aldrich, USA) lysis buffer was added. Immediately after, the plates with cells were placed on a shaker (MS3D, IKA, Germany) for 15 min. The percentage of viable cells was determined by measuring the absorption of 570 nm light in a spectrophotometer Epoch Microplate Reader (BioTek, USA) and compared to not insonified cells [9,10].

2.3. Measurement setups

The polycarbonate cuboid container (wall thickness 10 mm) designed for the ultrasound insonification of the cells is shown in Fig. 3.

The 1.4 l vessel ($230 \times 160 \times 70$ mm external size) had a builtin electronic thermostat stabilizing water temperature at $37 \text{ °C} \pm 1 \text{ °C}$ and a magnetic stirrer. The distilled (conductivity $X \approx 3 \text{ µS}$), degassed water was used. The top cover of the vessel had a 40 mm diameter hole and machined 5 mm deep recess. Two different covers were prepared separately for 12 and 96 well plates. The covers were carefully designed in such way that both 12 and 96 well plates could be placed symmetrically over the ultrasound transducer or absorber. The principle of shifting 12 and 96 Well Cell Culture Plates over the cover with milled, T-shaped recess is shown in more detail in Fig. 4.

Four measurements sets were performed: two for 12 Well Cell Culture Plate and two for 96 Well Cell Culture Plate. In the first two, 20 mm diameter, unfocused, 1 MHz ultrasound transducer, (Pz28, Meggitt, Denmark) was used. Pz28 material was chosen because it is best

suitable for relatively high total acoustic power (up to 22 W) applications expected in the experiment.

The 1 MHz transducer was placed either in the polycarbonate chamber in order to intentionally introduce the standing wave (Fig. 5a), or was inserted into the well in the plate, with the absorber placed in the chamber to prevent the formation of standing waves (Fig. 5b). The cone silicone rubber absorber had a diameter of 50 mm and a height of 30 mm (AD-1, silikon polskie, Poland).

For a 96 Well Cell Culture Plate another, 25 mm dia, unfocused 1 MHz transducer (Pz28, Meggitt, Denmark) was used so that the four adjacent wells of 6.6 mm diameter each could be exposed to ultrasound simultaneously (Fig. 6a). For the free field mode exposure, transducer was placed above the multi well plate (Fig. 6b). Because the diameter of the transducer was larger than the diameter of the well the cell medium (Fig. 6) was poured into the well with some excess and the transducer was placed 0.5 mm above the plate. The medium excess was necessary to ensure transducer contact with the fluid without the air bubbles. The distance of 0.5 mm was determined by the thickness of a ring attached to the transducer surface preventing the mechanical contact of the transducer with the plate. This is because the direct propagation of ultrasound from the transducer to polystyrene plate could change the amplitude or distribution of the acoustic field at the bottom of the well.

The 12 Well Cell Culture Plate was insonified from the bottom (Fig. 5a), with acoustic wave intensities $I_{SATA} = 0.16, 0.32, 0.48$ and 0.64 W/cm^2 in the presence of standing wave (Fig. 5a). These intensities corresponded to the acoustic powers $P_A = 0.5, 1.0, 1.5$ and 2.0 W , measured by radiation force balance UPM-DT-1A (Ohmic Instruments, USA). The intensities were selected to obtain comparable results for two different diameter transducers and to facilitate comparison with the literature. Insonification from the top was performed using intensities of $4.46, 5.09, 5.73, 6.37$ and 7.00 W/cm^2 (corresponding to 14, 16, 18, 20 and 22W of acoustic power, respectively) under free field conditions (Fig. 5b). Cells were sown in every second well, and together six wells were used. Of these, four wells were insonified with the same intensities, leaving two not insonified wells as a reference (Fig. 2).

Similarly, in the 96 Well Cell Culture Plate, the cells were seeded in six groups of four wells lying side by side. Four groups, namely 16 wells were insonified, leaving eight wells as a reference. The 96 Well Cell Culture Plate was insonified from the bottom with intensities $I_{SATA} = 0.61, 0.81, 1.02, 1.22, 1.43$ and 1.63 W/cm^2 (again, corresponding to 3, 4, 5, 6, 7 and 8W, respectively) in the presence of standing wave (Fig. 6a) and from the top with $I_{SATA} = 1.83, 2.44, 3.06, 3.67 \text{ W/cm}^2$ (9, 12, 15, 18 W) without the standing waves present (Fig. 6b).

2.4. Driving electronics

Both ultrasound transducers were excited by the voltage signal delivered via 3100LA RF power amplifier (ENI, USA). The amplifier was controlled by a 33,250 arbitrary waveform generator (Agilent, USA). The transducers were excited by 1 MHz sine wave burst, 1000 periods long, repeated every 2.5 ms (duty factor = 0.4). Frequency, tone burst length and duty factor were chosen to facilitate comparison with the results reported earlier

[11,18,27,29]. Selection of frequency was dictated by considering the possible clinical relevance of the designed experiments. More specifically, 1 MHz frequency was reported earlier [22] as an acceptable choice for creation of focused volume within a brain glioma after passing through a highly attenuating skull (13 dB at 1 MHz) [24] during ultrasound treatment *in vivo*. Acoustic power was adjusted by changing the voltage amplitude at the output of the generator. The generator output voltage was varied from 45 mV_{pp} to 339 mV_{pp} to result in acoustic power of 0.5–22W for 20 mm 1 MHz source and from 65 mV_{pp} to 306 mV_{pp} for 1–18W to be generated by 25 mm transducer. These voltages were adjusted via generator's knob within 1 mV resolution.

The total acoustic power generated by the given source was measured with the overall uncertainty on the order of 3% (manufacturer data) using the above mentioned radiation force balance Ultrasound Power Meter (UPM) UPM-DT-1A, (Ohmic Instruments, USA). The acoustic wave intensity I_{SATA} (space averaged, time averaged) was calculated according to the formula [32]:

$$I_{SATA} = \frac{P_A}{s} \quad (4)$$

where P_A is the total acoustic power measured by the UPM and S is the transducer active aperture measured by AMS (Acoustic Measurement System, Sonora, USA - now Acertara Acoustics Lab, USA) and was 3.1 cm² for 20 mm transducer and 4.9 cm² for 25 mm one.

Table 2 summarizes the relevant exposure parameters defined according to the guidelines described in [5]. The table also contains additional information on acoustic properties of the suspending medium and geometry of the exposure chamber [5].

2.5. Acoustic pressure amplitude measurements

To determine the influence of the standing waves the measurements of acoustic pressure amplitudes were performed in the free field and under standing wave conditions at the distance of 0.75 mm above the bottom of 12 and 96 Well Cell Culture Plates. This distance was chosen because at 1 MHz frequency it corresponds to the half wavelength ($\lambda/2$) in the cell solution. As the amplitude of the standing wave is repeated every $\lambda/2$, the intensity of the wave at a distance of 0.75 mm from the bottom is identical with that experienced at the bottom of the well, that is at the level of cells. Such approach ensured that the fragile needle hydrophone (see vendor information below) was protected from mechanical contact with the bottom well. To obtain distance accuracy to within 0.1 mm, the depth of the well was measured by the depth micrometer, hydrophone was aligned with the upper surface of the well and lowered to the depth of the well minus 0.75 mm. The free field measurements were done in the same hydrophone-transducer setup inside the large (>100 l) water tank 20 cm below the water level. All measurements within the exposure chamber were performed using calibrated 0.2 mm dia needle hydrophone, (S/N 1661, Precision Acoustics, UK), preamplifier and DC coupler with power supply DCPS142 (Precision Acoustics, UK). The output signal from the hydrophone was recorded using 62Xi Waverunner digital oscilloscope (LeCroy, USA). Although during glioma cell insonification the transducer was excited by 1

MHz sine wave bursts, 1000 periods long, repeated every 2.5 ms, during free field measurements the pulses were repeated every 100 ms in order to eliminate multiple reflections in the container. However, the standing wave intensity was measured with identical, 2.5 ms pulse repetition rate, the very same as the one used in the cell experiments.

During the 3 min exposure time, $N = 72,000$ pulses were generated, each 1000 periods long. In order to take into account pressure amplitude variations during the pulse duration, expected in standing wave conditions, the root-mean-square pressure value p_{RMS} was calculated as:

$$p_{RMS} = \frac{1}{t_d} \sqrt{\int_{t_d} p^2(t) dt} = \frac{V_{RMS}}{s} \quad (5)$$

where t_d denotes pulse duration, $p(t)$ – acoustic pressure, V_{RMS} – RMS voltage value at the hydrophone output at time t_d and s is hydrophone sensitivity (V/Pa). The relationship between p_{RMS} pressure amplitude and the I_{SPPA} (spatial peak pulse averaged) intensity can be expressed as [32]:

$$I_{SPPA} = \frac{1}{z_0 t_d} \int_{t_d} p^2(t) dt = \frac{1}{Z_0} p_{RMS}^2 \quad (6)$$

where Z_0 is the acoustic impedance of the medium.

To analyze pressure amplitude variations under the standing wave conditions, mean pressure $p_{RMS}(mean)$ and maximum pressure $p_{RMS}(max)$ were calculated for 3 min period:

$$p_{RMS}(mean) = \frac{1}{N} \sum_N p_{RMS}(n) \quad (7)$$

$$p_{RMS}(max) = \max(p_{RMS}(n)) \quad (8)$$

where $p_{RMS}(n)$ – pressure RMS value for n-th pulse, N – number of pulses ($N = 72,000$).

The ultrasound pressure amplitude loss due to the attenuation of the wave within the well bottom wall was also measured by Precision Acoustics needle hydrophone using substitution method (see next section for details).

2.6. Temperature measurement

As mentioned in the introduction, ultrasound attenuation in the bottom of the well may increase the temperature within the cell medium. Similarly, the heat radiated by the transmitter can also cause temperature rise, so the temperature was carefully monitored during the experiments. The laboratory set-up used for measurement of the temperature

inside the well of the 12 Well Cell Culture Plate was the same as for the cells insonification, shown in Fig. 5a and b. The 0.5 mm diameter thermocouple TP-201 (thermo-product, Czaki, Poland), connected to the USB-TEMP module (Measurement Computing, USA) was placed at the center of the well bottom. The temperature was measured after 3 min, time period identical to cells' ultrasound exposure time.

3. Results

The results of the experiments are presented below. For clarity, the results obtained with the 12 and 96 well cell plates are discussed separately. To obtain comparable results for two different diameter transducers and to facilitate comparison with the literature, I_{SATA} intensities were calculated from equation (4). Accordingly, all results below are presented as cell viability versus ultrasound intensity I_{SATA} .

3.1. 12 well cell culture plate exposure

The efficacy of cancer cells destruction as a function of applied ultrasound intensity I_{SATA} is shown in Fig. 7. The 50% lethal intensity levels were determined as intensity values at which the cell viability was 50%. The mid-scale value was set to minimize possible errors caused by living cells left at the edge of the well bottom and the unpredictable cell death at low ultrasound intensity. For the 12 well plate this level was determined to be equal to $I_1 = 0.32 \text{ W/cm}^2$ for the standing wave and $I_2 = 5.89 \text{ W/cm}^2$ under free field exposure conditions (Fig. 7).

The p_{RMS} pressure (Eq. (5)) distribution across the ultrasound beam was measured in both cases; in free field above the bottom plate without standing waves and with standing waves reflected from both surfaces, i.e. water and water–transducer interface. Histogram of the 72,000 pressure values p_{RMS} calculated from all pulses emitted during 3 min of insonification under the standing wave conditions is shown in Fig. 8.

The transmission measured pressure amplitude loss due to attenuation of the 1 MHz wave within the polystyrene well bottom was determined to be $0.15 \text{ dB} \pm 0.05 \text{ dB}$ (1.7%) both for the 12 well 96 well plates, under both free field and standing wave conditions.

The measurement results of the ultrasound pressure amplitudes for 12 well plate are shown in Figs. 9 and 10.

Fig. 9 shows the transverse distribution of p_{RMS} pressure in a free field with multi well plate removed at a distance of 10 mm from the transducer surface, the same as during cells insonification (Fig. 5b). Hydrophone was positioned opposite to the 1 MHz source in exact place of the removed plate. The measurement was performed at spatial average, temporal average intensity I_{SATA} equal to 5.89 W/cm^2 at which 50% cell viability was observed. The measured maximum pressure value on the center of the well ($x = 0$) was $p_{RMS}(\text{max}) = p_{RMS}(\text{mean}) = 0.75 \text{ MPa}$ (Eqs. (7) and (8)).

Fig. 10 shows six plots of the transverse distribution of p_{RMS} in terms of standing wave at a distance of 10.75 mm from the transducer, measured 0.75 mm above the bottom of the well in the plate. Measurements were made for intensities $I_{SATA} = 0.32 \text{ W/cm}^2$ (50% lethal

intensity level), 0.48 and 0.64 W/cm², identical with those at which the cell viability was determined. The upper graphs show the peak values of pressure amplitudes, equal to the maximum value of $p_{RMS}(\max)$ (Eq. (8)), which occurred during the 3 min (180 s) exposure of ultrasound. The bottom graphs show the average $p_{RMS}(\text{mean})$ (Eq. (7)) values, during the same, 3 min exposure. For $I_{SATA} = 0.32$ W/cm² intensity, corresponding to 50% cell viability, the $p_{RMS}(\max)$ pressure amplitude on the axis of the transducer was 0.71 MPa and its mean value was 0.26 MPa.

Fig. 11 shows photographs of the 12 well plate during insonification at these four spatial average, temporal average intensity levels.

The measurements of the temperature elevation after 3 min ultrasound exposure are shown in Fig. 12. As noted earlier, these measurements were made using thermocouple placed at the bottom of the 12 well plate; three distances (5, 10 and 15 mm, see below) were selected to examine the impact of the heat emitted by transducer. The measurements were taken with the 1 MHz source inserted into one well of the 12 well plate, placed at a distance of 5 mm, 10 mm and 15 mm from the bottom of the well (Fig. 5a).

At a distance of 15 mm, in order to eliminate the effects of heat emitted by the transducer, the cooling chamber was inserted between the transducer and the well and separated from the well by a layer of 0.5 mm thick polycarbonate. The 5 mm thick, 20 mm diameter chamber was filled with 37 °C cooling water circulated at 1 l/min with the help of an external pump. The I_{SATA} values measured at the distance of $z = 15$ mm include acoustic energy losses in this 0.5 mm thick plate. The free field results of the temperature elevation were compared with those obtained under the standing wave conditions, where the ultrasound source was placed under the multi well plate (Fig. 5b) at the distance of 10 mm from the upper surface of the bottom (the 10 mm distance and ultrasound parameters were identical with those under which the cells were insonified).

It is acknowledged that the maximum temperature of the cells should not exceed 43 °C which is known to be lethal [15]. This temperature is 6 °C higher than that at which the glioma cells were exposed. The temperature of 43 °C was attained here during free field exposure at the I_{SATA} intensities of 1.87, 2.65 and 2.43 W/cm² when the transducer was positioned above the plate at a distance of 5, 10 and 15 mm. Under the standing wave conditions (transducer positioned 10 mm below the plate), the 43 °C temperature was reached at the intensity level of 0.55 W/cm². The free field measurements performed at $z = 10$ mm and 15 mm indicated similar pattern of the temperature rise. Thus, for axial distances equal to 10 mm and above, the effect of the heat emitted by the transducer under FF conditions appears to be negligible.

3.2. 96 well cell culture plate exposure

The efficacy of cancer cells obliteration as the function of applied ultrasound intensity I_{SATA} is shown in Fig. 13. The 50% lethal intensity levels were determined as the intensity values at which the cell viability was 50%.

For a 96 well plate exposure these 50% lethal intensity values were determined to be $I_1 = 0.80 \text{ W/cm}^2$ and $I_2 = 2.86 \text{ W/cm}^2$, respectively (Fig. 13).

The measurement results of the ultrasound pressure for 96 well plate are shown in Figs. 14 and 15. Again, Fig. 14 shows the distribution of transverse pressure amplitude p_{RMS} in a free field with multi well plate removed at a distance of 10 mm from the transducer surface.

The measurements were performed at the intensity I_{SATA} equal to 2.86 W/cm^2 , corresponding to 50% cell viability for 96 well plate. I_{SPTA} intensity was measured twice: under the free field conditions (Fig. 14b) and under standing wave conditions inside the well of the 96 well plate, 0.75 mm above the bottom (Fig. 14a). It was noted that in the free field, the 50% cell viability intensity was different for 96 well plate than that observed when using 12 well plate. To analyze this phenomenon, two additional measurements were performed. The first one in free field, the second, inside the single well of the 96 well plate with the wall bottom removed.

Measurement in a well with removed bottom unveiled an increase of the pressure amplitude within the well. Transverse field distributions were asymmetrical, because the center of the well ($x = 0$) was shifted by 6.4 mm from the axis of the 1 MHz (25 mm dia) source used to insonify four adjacent wells (Fig. 6). The measured maximum value of $p_{RMS}(\text{max})$ pressure amplitude within the well was equal to 0.73 MPa.

In Fig. 15 six plots of the transverse pressure amplitude distribution p_{RMS} under standing wave conditions at a distance of 10.75 mm from the transducer are shown. These measurements performed at the distance of 0.75 mm above the bottom of the well in the plate were made for I_{SATA} intensities equal to 0.40, 0.60 and 0.80 W/cm^2 ; they all correspond to the experiments, in which the glioma cells viability was ascertained.

The upper graphs of Fig. 15 show the peak pressure amplitude values $p_{RMS}(\text{max})$, equal to the maximum value of p_{RMS} , which occurred during 3 min exposure of ultrasound. The bottom graphs show the average $p_{RMS}(\text{mean})$ values, encountered during 3 min exposure of ultrasound. For $I_{SATA} = 0.80 \text{ W/cm}^2$ intensity, corresponding to 50% cell viability, the $p_{RMS}(\text{max})$ pressure amplitude on the axis of the transducer was 0.75 MPa, and its mean value $p_{RMS}(\text{mean})$ was 0.42 MPa. Fig. 16 shows photographs of the 96 well plate during insonification.

The measurement set ups used along with the corresponding spatial average, temporal average intensities and peak And mean RMS values of pressure amplitudes that led to 50% necrosis of the glioma cells exposed are listed in Table 3.

4. Discussion

The results presented above indicate that standing wave field effectively increased the pressure of the ultrasound exposure at the cells' level (see Fig. 10). Hence, the existence of the SW field allows the ultrasound intensity generated by the transducer to be reduced while achieving the same outcome.

In the following, the results presented above are interpreted separately for 12 well- and 96 well plates because of the observed differences in the I_{SATA} 50% lethal intensity.

Specifically, under the SWMR conditions I_{SATA} 50% lethal intensity for a 96 well plate was higher than that observed in 12 well plate because higher intensity level was needed to create the disturbed pattern surface of the water in the 96 well plate (Fig. 16) under standing wave conditions. The free-field (FF) I_{SATA} 50% lethal intensity was lower in the 96 well plate due to an increase of the ultrasound pressure amplitude within the well under free field conditions (Fig. 14) compared to the 12 well plate.

In the case of 12 well plate (see Fig. 7) the desirable 50% cell viability level was achieved at the spatial average, temporal average intensity of 320 mW/cm^2 only. This intensity level (proportional to the total ultrasound power emitted from the 1 MHz source) is 18.4 times less than the 5.89 W/cm^2 level needed to achieve the same result under (FF) exposure. This result is somewhat surprising because the theoretical estimate (cf. Eq. (3)) indicated factor of 5.7 to be anticipated. However, it is conceivable that the ultrasound introduced water surface disturbance (see Figs. 11 and 16) aided in creating transient focusing field conditions. Under FF conditions, at the I_{SATA} intensity of 5.89 W/cm^2 , the corresponding root mean square p_{RMS} pressure amplitude was determined to be equal to 0.75 MPa (Fig. 9). A similar level of maximum pressure amplitude was measured in the standing wave field $p_{RMS}(\text{max}) = 0.71 \text{ MPa}$ (Fig. 10).

In this context it should be noted that the mean pressure $p_{RMS}(\text{-mean})$ averaged over 180 s of insonification time was 7.5 times lower and determined to be equal to 0.26 MPa. Based on the histogram in Fig. 8 it could be noted that during the 3 min of insonification, only a few of 1000 period bursts delivered the pressure amplitudes (0.75 MPa) were sufficient to induce 50% necrosis of the glioma cells.

This outcome should be compared with the results obtained under SWMR conditions of 12 well plate. There, the cell viability was maintained at 12% and 15% for the 0.48 and 0.64 W/cm^2 intensities, respectively. It is plausible that these results were due to the reduced (insufficient to destroy the cells) ultrasound intensity level near the edges of the well as the diameter of the 1 MHz transducer (20 mm) was smaller than the diameter of the well in the plate (22 mm).

Analyzing (based on temperature measurements, see Fig. 12) the possibility of the thermally induced cell necrosis it was determined that this took place under FF conditions at the intensity level of 7 W/cm^2 ; all cells were destroyed due to the excessive temperature rise from 37°C to over 55°C .

For the 96 well cell culture plate exposure, the 50% cell viability was obtained at the I_{SATA} intensity level equal to 2.85 W/cm^2 for the free field exposure $p_{RMS}(\text{max}) = 0.73$ and under standing wave conditions $p_{RMS}(\text{max}) = 0.75$, respectively.

In summary, in all four exposure arrangements (Figs. 5a, b, 6a, and b), for a 50% cell viability, similar levels of $p_{RMS}(\text{max})$ pressures ranging from 0.71 to 0.75 MPa were identified. This indicates that $p_{RMS}(\text{max}) = 0.73 \pm 0.02 \text{ MPa}$ (mean $\pm$ std dev) pressure

amplitude value was independent of measurement set-up and could be used to predict I_{SPTA} intensity for 50% C6 glioma cells mortality under either FF or SW exposure conditions in any *in vitro* experiments. The *in vitro* results may also serve as a starting point for *in vivo* therapy.

This $p_{RMS}(\max)$ pressure value corresponds to $I_{SPPA} = 36.0 \pm 2.0$ - W/cm² spatial peak, pulse averaged intensity (Eq. (6)). The most likely explanation of the phenomena is that the measured pressure corresponds to the cavitation threshold, as reported in [20].

Concurrently, the careful temperature measurements showed that only under standing wave conditions, the temperature within the exposure chamber did not exceed 43 °C, which prevented cell mortality.

5. Conclusions

A set of *in vitro* experiments was carried out under carefully characterized and controlled conditions. The outcome of these experiments indicated that substantially lower (18 times) spatial average, temporal average ultrasound intensity is needed to destroy the cells under the standing wave/multiple reflections (SWMR) conditions in comparison with the intensity level needed to achieve the same 50% cell viability under free field exposure. As evidenced above, in the SWMR field the reproducible and controllable destruction of the cells can be implemented at the tissue temperature level. This indicates the potential of intentionally using such environment in *in vivo* tissue exposure, in which the cells' response to different stimuli (such as ultrasound) can be monitored in reproducible way without the risk of overheating of the cells and causing thermal necrosis. The outcome of the measurements also indicates that SWMR exposure might be useful at any ultrasound assisted therapy experiments as it permits to reduce thermal effects.

The analysis presented provides methodology facilitating independent laboratories to determine their specific ultrasound exposure parameters for a given biological end-point under standing waves and multiple reflections conditions. Moreover, the analysis also permits verification of the outcome of the experiments mimicking pre- and clinical environment between different, unaffiliated teams of researchers. However, it should be noted that the results presented in this work are not universal; therefore the specific experimental conditions have to be accounted for to determine the difference between the FF and SWMR conditions, allowing the clinically relevant exposure conditions to be mimicked. For example, the materials and dimensions of various well plate products vary as do the applied acoustic pulses and the rate of their delivery. Hence, shorter (than the ones used here) pulses may result in much lower level of standing waves. These specific conditions can be determined using the described procedure of the calibrated hydrophone measurements of the p_{RMS} maximum pressure amplitude at the $\lambda/2$ distance from the cell's level at the bottom of the dish or plate. Overall, the study presented provides useful guidelines permitting to quantitatively link the "stimulus" (ultrasound exposure) and the desirable biological end-point.

Acknowledgments

This study was supported by the National Science Centre of Poland grant 2014/15/B/ST8/04345. Partial support for one of the authors (Peter A. Lewin) was sponsored by the National Institute of Health (NIH) grant R01NR015995.

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Fig. 1.
Typical *in vitro* ultrasound exposure setup. See text for details.

Fig. 2.

Arrangement of the filled wells. (a) 12 Well Cell Culture Plate. (b) 96 Well Cell Culture Plate. The wells with cells are marked red. Sonfied wells marked US, other were left as a reference REF. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 3.

Experimental setup for 1 MHz ultrasound insonification of glioma cells.

Fig. 4. Four positions ensuring the same geometry of the insonification exposure of the (a) 12 well plate and (b) 96 well plate on the test bench in Fig. 3.

Fig. 5. Positioning of the 20 mm dia, plane 1 MHz ultrasound transducer for the 12 well plate insonification. (a) Under the plate – intentionally induced standing wave conditions. (b) Over the plate - standing waves eliminated. All dimensions are in millimeters.

Fig. 6. Positioning of the 25 mm dia, 1 MHz ultrasound transducer for the 96 well plate insonification; (a) under the plate – intentionally induced standing wave conditions. (b) Over the plate standing waves eliminated. All dimensions are in millimeters.

Fig. 7.

Degree of C6 glioma cell viability (%) after insonification in the standing wave field (a) and under free field conditions (b) for the 12 Well Cell Culture Plate. Mean value (circle), standard deviation (vertical post) and polynomial fit curve are shown.

Fig. 8.
p_{RMS} pressure distribution histogram (standing wave conditions) for 3 min measurement at
 $I_{SATA} = 0.32 \text{ W/cm}^2$ (12 well plate).

Fig. 9. Needle hydrophone measured free field transverse distribution of p_{RMS} pressure amplitude for the 12 well plate at the intensity level of $I_{SATA} = 5.89 \text{ W/cm}^2$.

Fig. 10. Needle hydrophone measured transverse distribution of p_{RMS} pressure amplitude under standing wave conditions for the 12 well plate. Mean values (mean) and maximum (max) for $I_{SATA} = 0.32, 0.48$ and 0.64 W/cm^2 .

Fig. 11.

Photographs of one well of the 12 well plate during ultrasound exposure at standing wave conditions for the intensities $I_{SATA} = 0$ (a), 0.16 (b), 0.32 (c) and 0.48 mW/cm² (d). The distortion of the fluid surface is visible in (b–d). The level of distortion increases with increasing intensity. At 0.48 mW/cm² (d), variation of the water-air surface height was 3 mm, equal to 2 wavelengths.

Fig. 12.

Temperature at the bottom of the 12 well plate after 3 min insonification as a function of the ultrasound intensity I_{SATA} . 1 MHz ultrasound transducer was placed over the multi well plate at distances of 5, 10 and 15 mm under free field conditions and 10 mm below the multi well plate in the standing waves field.

Fig. 13. Degree of C6 glioma cell viability after the insonification in the standing wave field (a) and under free field conditions (b) for the 96 Well Cell Culture Plate. Mean value (circle), standard deviation (vertical post) and polynomial fit curve are shown.

Fig. 14. Needle hydrophone measured transverse p_{RMS} pressure amplitude distribution for the 96 well plate under free field conditions for $I_{SATA} = 2.86 \text{ W/cm}^2$. (a) Measurements inside the well of the 96 well plate, with removed bottom, (b) free-field measurements.

Fig. 15. Needle hydrophone measured transverse p_{RMS} pressure amplitude distribution for the 96 well plate under the standing wave conditions. Mean values (mean) and maximum (max) for $I_{SATA} = 0.40, 0.60$ and 0.80 W/cm^2 .

Fig. 16.

Photographs of one well of the 96 well plate during ultrasound exposure at standing wave conditions for the intensities $I_{SATA} = 0$ (a), 0.40 (b), 0.60 (c) and 0.80 W/cm² (d). The distortion of the fluid surface is visible in (b–d). The level of distortion increases with increasing intensity. At 0.80 mW/cm² (d), variation of the water-air surface height was 1.5 mm, equal to one wavelength.

Table 1

Acoustic impedance Z, intensity reflection coefficient R and intensity transmission coefficient T of selected materials at the water boundary.

Medium	Acoustic impedance	R	T
Air	$4.3 \cdot 10^2$ Rayl	1	0
Water	$1.5 \cdot 10^6$ Rayl	–	–
Piezoceramic	$3.1 \cdot 10^7$ Rayl	0.82	0.18
Polystyrene	$2.5 \cdot 10^6$ Rayl	0.06	0.94

Table 2

Exposure parameters for 12 well and 96 well cell culture plates following the guidelines on measurements and reporting on acoustic output and exposure [5].

<i>Exposure parameters</i>	
Acoustic power output at transducer	0.5–22 W
Mode of operation	PW
Pulse center frequency	1.0 MHz
Pulse duration	1.0 ms
Duty factor	0.4
Pulse repetition frequency	400 Hz
<i>Chamber</i>	
Chamber shape	Circular
Chamber diameter (12 well plate)	22.2 mm
Chamber diameter (96 well plate)	6.6 mm
Construction material	Polystyrene
Construction material density	1.10 g/cm ³
Construction material speed of sound	2800 m/s
Chamber standing wave assessment	Described in text
<i>Suspending medium</i>	
Suspending medium speed of sound	1520 m/s
Suspending medium temperature	37 °C
Suspending medium depth (12 well plate)	5.2 mm(2.0 ml)
Suspending medium depth (96 well plate)	5.8 mm(0.2 ml)
Suspending medium density	1.05 g/cm ³

Table 3

Exposure parameters for 12 well and 96 well plates. I_{SATA} , $p_{\text{RMS}}(\text{mean})$ and $p_{\text{RMS}}(\text{max})$ correspond to 50% cell viability level.

Measurement setup	I_{SATA} (W/cm ²)	$p_{\text{RMS}}(\text{mean})$ (MPa _{RMS})	$p_{\text{RMS}}(\text{max})$ (MPa _{RMS})
12 well free field	5.89	0.75	0.75
12 well standing wave	0.32	0.26	0.71
96 well free field	2.86	0.73	0.73
96 well standing wave	0.80	0.42	0.75