Sonoporation Dosimetric Evaluation from Ultrasound Signals Attenuated by Microbubbles

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Introduction. Sonoporation is a novel method to deliver various molecules into cells and tissues, including drugs, used for anticancer therapy, and DNA, RNA designed for gene therapy. The main principle of molecular sonotranfer employs microbubble (MB) cavitation that can be described as MB oscillation followed by violent collapse occurring as a response to ultrasound (US) irradiation. Cavitating MBs create microstreams and microjets that induce shear stress to cell membranes. This leads to formation of pores in the plasma membrane or/ and activation of endocytotic processes. Pores act as molecule conducting channels, however, if the poration of cell membrane is too extensive, the cell eventually dies [1]. The main task of sonoporation is to minimise cell death and increase molecular transfer. Thus, in order to achieve high molecular delivery and cell viability a specific dosimetry is needed.

MB exposed to US emit broadband ultrasonic signals that can be used for both estimation of molecular delivery and cell death. All approaches involved in this type of dosimetrical investigations have used MB side-scattered signals as the primary metric for sonoporation outcome. Chen et al. (2003) used microbbuble side-scattered signals to calculate inertial cavitation dose (ICD), which correlatated well with erythrocyte hemolysis [2]. Lai et al. (2006) and Qiu et al. (2010) showed that ICD was a suitable metric for both DNA delivery and cell viability [3, 4]. However, there is no data reporting that US attenuation signals passed through the sonoporation cuvette, containing MB and cells, could be used as an estimate for sonoporation bioeffects. Here, we focus on previous work by Chen et al. (2003), on cavitation induced cell death quantification from passive-way received ultrasonic signals. In this study, instead of measuring side-scattered US signal amplitude, we analysed ultrasonic signals, obtained from the receiving transducer, linearly aligned to the transmitting transducer. Scattered US signals and emitted broadband noise originate from exploding MBs, while attenuated US signals directly depend on MBs, acting as an impediment for US passage. So we imply that the parameter, which is able to quantify the amplitude of lineraly transmitted ultrasound signal is able to evaluate MB cavitation induced cell death or irreversible cell sonoporation (current study) and molecular delivery (future studies).

Materials and methods. Chinese hamster ovary cells (CHO) were cultured in DMEM (Sigma Aldrich., USA) growth medium supplemented with 10% foetal bovine serum (Sigma Aldrich., USA), 1% L-glutamine solution

(Invitrogen life technologies) and 100 U./ml penicillin with 100 μg streptomycin (Sigma Aldrich., USA) solution. Cells were grown as monolayers in 20 cm^2 tissue culture flasks (TPP, Switzerland), incubated at 37 °C, in a humidified atmosphere of 5% CO_2, in an incubator (NU-2500E, Nuaire, USA). Cells were harvested using 1 ml of trypsin/EDTA solution (Sigma Aldrich., USA), trypsination was inactivated in 2 min. by adding the same amount of the growth medium. The cell suspension was centrifuged for 2 min at 1000 rpm and prepared in PBS (Lonza, USA) at the final concentration of 2.6×10^6 cells/ml.

The waveform generator/ oscilloscope (Picoscope 5242B, Picotech, UK) was used both to produce and record US signals. The signals were amplified by high frequency signal amplifier (Kaunas University of Technology, Lithuania), powered by high voltage power supply (MCP lab electronics, China). The transmitting transducer (Transducer 1, Medelkom, Lithuania) operating at 1 MHz central frequency was used for MB excitation. Passive US transducer (Transducer 2, Doppler, China) of 5 MHz central frequency aligned 180º to the axis of transmitting transducer was used for US amplitude attenuation measurement. All sonoporation experiments were performed in 1 ml sonoporation cuvette (Plastibrand, Germany). Complete ultrasound exposure system is presented in Fig. 1.

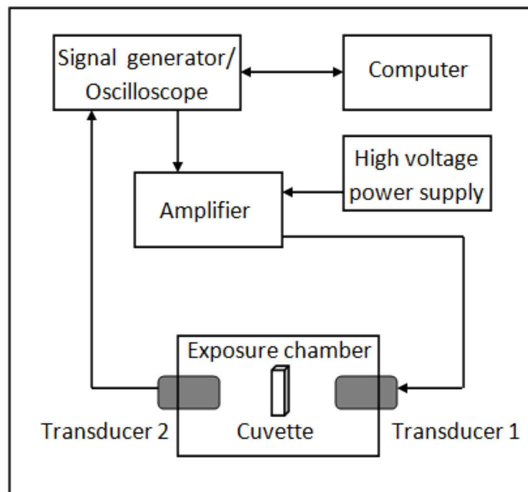

Fig. 1. Ultrasound exposure system for sonoporation experiments

500 μl of prepared cell suspension was supplemented with 400 μl SonoVue® (Bracco diagnostics Inc., Switzerland) MB suspension and 100 μl PBS. The cells in the experimental groups were exposed to 1 MHz central frequency pulsed US at 10 % duty cycle (100 μs ON, 900 μs OFF), 0, 100, 200,

300 and 400 kPa peak negative acoustic pressure (PNP) for 5 s. 10 min after US exposure cell suspension was diluted in MEM (Sigma-Aldrich., USA) and 100 µl of the suspension (~330 of cells) was loaded into 4.1 cm^2 (TPP, Switzerland) tissue culture dishes (2 dishes for each experimental point).

The cells were cultured for 7 days, afterwards the colony formation test was performed. Cells were dehydrated as the growth medium was substituted with 1 ml of 96% ethanol and incubated for 10 min. Then cells were stained with crystal violet (Sigma Aldrich., USA), ~ 1 ml per dish, the incubation time was 2 min. The number of colonies formed was assessed using the light microscope МБС-9 (Russia), at the magnification of ×8.

MB attenuated signals were recorded in subsequent experiment, performed without cells, to avoid MB interference with cells. Therefore, the signal alterations originate only due to the MB concentration decrease. 600 µl of MB suspension and 400 µl PBS were added to sonoporation cuvette and the signals were recorded at 0, 100, 200, 300 and 400 kPa PNP. Overall signal duration of 5 s was aquired in 25 frames (time segments) and the length of each frame was 10 ms. At the initial stage of signal processing the mean value of recorded signal amplitude (AMP) was calculated and plotted vs. US exposure time. Then the integral of AMP vs. time was estimated and defined as "AMP dose" for each PNP value.

Results and discussion. In this study microbubble attenuated US signal was recorded and quantified as AMP dose (Fig. 2a). The results show that this dosimetric parameter increases linearly in 100 - 500 PNP range.

The dependence of AMP dose on PNP is similar to ICD. The only difference arises considering the origin of these two parameters: ICD is obtained from MB side-scattered signals, while AMP dose – from attenuated US signals due to MB presence. ICD quantification needs more mathematical operations (Fourier transformation, root mean square (RMS) calculation and RMS integration in time domain), while AMP dose estimation is simpler, being only the absolute value of recorded discrete signal. Moreover, different groups have evaluated ICD at different frequency ranges ranging from 2.3 - 2.5 MHz [5] to 10 MHz [4]. The latter reason makes difficulties to compare the results of different groups. Thus, AMP dose parameter is easier to calculate as well as it is more general being independent on the frequency range.

Cell death due to MB cavitation revealed logarithmic growth in 0-500 kPa PNP range. The highest rate of viability decrease was in 0 - 200 kPa PNP range and then tended to saturate (Fig. 2b).

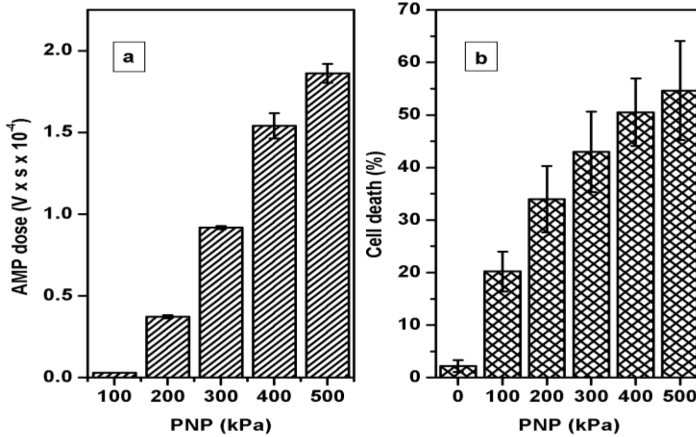

Fig. 2. Microbubble attenuated ultrasound signals quantified as AMP dose (a); MB cavitation induced cell death in dependence on PNP (b)

To evealuate, whether AMP dose could be used as a metric for cell death estimation, we plotted the level of cell death in logarithmic AMP dose scale (Fig. 3).

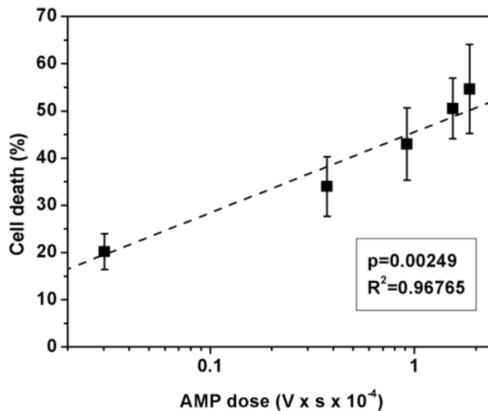

Fig. 3. Dependence on MB cavitation induced cell death on AMP dose

As it is seen from the figure the percentage of MB induced cell death increases linearly in the logarithmic scale of AMP dose. The latter result indicates that cavitation impact on cell viability can be accurately (R^2 = 0,97) predicted from AMP dose and used as dosimetric estimate for microbubble and ultrasound induced irreversible sonoporation.

Conclusion. In the range of our parameters tested, MB attenuated ultrasound signals are sufficient to accurately prognose cell irreversible sonoporation induced by MB cavitation. Our study revealed new easy-to-calculate MB cavitation dose metric, AMP dose, which is sufficient to prognose microbubble and ultrasound induced cell death in PNP range up to 500 kPa.

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New dosimetric estimate for ultrasound and microbubble assissted molecular delivery method (sonoporation) is presented in current study. Ultrasound signals, attenuated by cavitating microbubbles, were quantified as amplitude vs. time integral – „AMP dose“. The latter metric accurately (R^2=0.97) predicts irreversible sonoporation to CHO cells, induced by microbubble cavitation in vitro, and holds promising prospects for future applications to dosimetric evaluation of molecular delivery.