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Ultrasound Spectra Relation with Microbubble's Sonodestruction and Molecules Sonotransfer

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Introduction. In the last decades sonoporation is successfully used to transfer the anticancer drugs and genes into the cells and tissues as including tumors [1,2]. Pioneer authors demonstrated that more effective sonotransfer of bioactive molecules can be achieved when contrast agents so called microbubbles (MB) in the sonoporation medium are applied [3]. Further studies demonstrate that sonotransfer efficiency of anticancer drugs is strongly correlated with the MB cavitation and sonodestruction [4,5,6]. In addition the size and resonance frequency of MB are also important. In our studies we used the microbubbles prepared in our laboratory (L-MB) with the diameter of 2.91±0.2 μm [7] and commercial SonoVue MB with the size distributed in the diameter range of 2-4 μm and dimension-depended resonance frequencies of 1.3 – 3 MHz [8].

Here we are aiming to obtain dependence of sonotransfer efficiency of anticancer drug ethidium bromide (EtBr) upon MB sonodestruction rate. Two investigation models were used: 1) the sonodestruction dynamics of L-MB was investigated by analysing spectrograms of ultrasound (US) attenuation and scattering in the excitation duration time (Δt) and negative pressure (P_{neg}) domain; 2) the sonodestruction intensity of commercial SonoVue MB along with EtBr sonotransfer efficiency was studied by analysing US attenuation and scattering spectra recorded in Δt and P_{neg} domain.

Materials and methods. The procedure of L-MB synthesis has been previously described [7]. The SonoVue (SV) MB suspension was prepared from one vial and experiments were performed within first 10 minutes after suspension preparation. Prepared MB concentration was determined by counting samples of MB under light microscope (Motic BA400, Xiamen, China). The hardware setup is presented in Fig. 1a. Three US transducers were used: 1) for excitation, 2) for reception of attenuated waves after transmission in MB suspension and 3) for reception of waves scattered in MB suspension. US excitation was delivered from power US pulser (Totempole, Department of Electronics Engineering, Kaunas University of Technology). Series of excitation waveforms were initiated from computer controlled arbitrary waveform generator. Repetition frequency of excitation pulses was of 1 kHz in burst waveform. Length of burst pulses was

100μs or 100 periods of 1MHz central frequency with duty cycle (DC) 10%. Signals of attenuated and scattered waves were sampled in two channels of computerised oscilloscope PicoScope 5242B (Pico Technology, St Neots, UK). By using the specific fast sampling algorithm the signals were saved in frames, which length was 50 ms and total number of frames 20. Analysing sampled data we assumed that inter-frame gaps, or portions of signal data what we have lost, is of 50 ms. Raw US signals were saved into computer hard-disc for off-line analysis. Examples of signals acquired from attenuation and scattering channels and select times windows for analysis are provided in Fig. 1b.

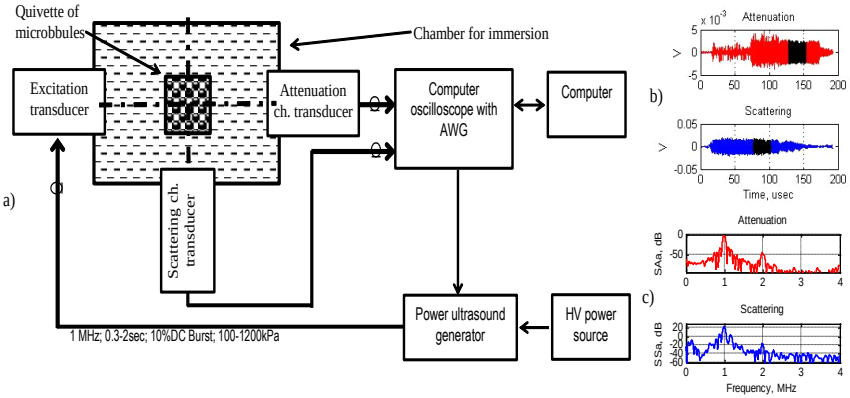

Fig. 1. a) Hardware setup for evaluation of time functions of attenuation and scattering in medium with MB under US excitation *in vitro*; b) An example of US pulses under investigation with time window (Δt_w=30 μs) presented in dark; c) Examples of amplitude spectra of US pulses.

Analysis of raw US signals was performed in Matlab software environment. In experimental signals we identified pulses received with attenuation and scattering transducers (see Fig. 1b). In these pulses we selected Hanning time window (Δt_w=30μs), which were analysed in joint time - spectra amplitude domain. The amplitude spectra of selected time window was calculated with fast Fourier transform. The examples of amplitude spectra are presented in Fig. 1c.

Results. The attenuation and scattering spectra of US and MB interaction or cavitation are presented in Fig. 2 using so called spectrograms. Spectrograms are provided for these cases: 1) Dulbecco's Modified Eagle's medium (DMEM) only, 2) DMEM medium with cells, and 3) DMEM medium with cells and L-MB.

Analysis of the attenuation spectrograms presented in Fig. 2 and 3 implies that the acoustic transparency (data from attenuation channel) or brightness at the specific frequency ranges is consistent with the amount of US absorption and scattering particles concentration in suspension.

a) P_{neg}=100 kPa

1) DMEM only 2) DMEM and cells 3) DMEM, cells and L-MB

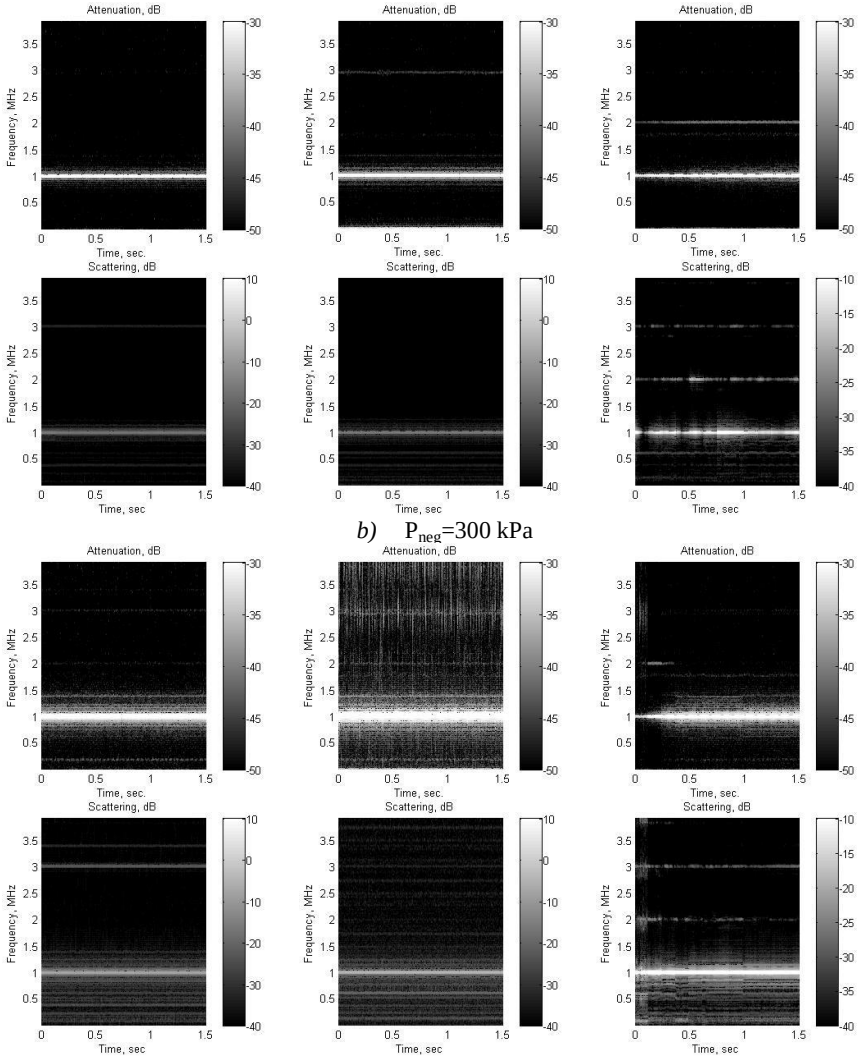

Fig. 2. Attenuation (top row) and scattering (bottom row) spectrograms of suspensions containing: 1) only DMEM, 2) DMEM with cells and 3) DMEM with cells and L-MB. Spectrograms provided for excitations pulses of negative pressure amplitudes P_{neg} 100kPa (a) and 300kPa (b). Abscissa - excitation time duration, Δt = 1.5 s; Ordinates - frequency in the range, 0-4 MHz. Amplitude of spectra is coded in grey scale: the lowest amplitude – black, the highest – white.

In other words the brightness at respective frequency bands in acoustic attenuation spectrograms can be used as a measure of MB concentration. It also

serves as a measure of MB sonodestruction. Initially the low brightness at the band of main frequency of 1MHz as well at higher harmonics of 2 and 3 MHz are consistent with the high concentration of MB. In addition, the notable increase of brightness in the bands of the 1 and 2 MHz (DMEM + cells + L-MB) at pulse strength P_{neg} =100 kPa in the scattering spectrogram comparing with (DMEM + cells) suspension case implies the onset of cavitation as well the start of MB destruction. We believe it is consistent with the phemomenon of so called *stable cavitation* of MB. Continuously with time of excitation the brightness increases, this corresponds to the onset of *inertial cavitation* and more intensive destruction of MB (see Figs. 2 and 3).

The attenuation and scattering spectrograms in case of SonoVue MB has been analysed in alternative way to extract scalar parameters of US and MB interaction. The peak to peak amplitudes of pulses received in attenuation (AApp) and scattering (ASpp) channels were evaluated to obtain time functions. The time functions of harmonic power of attenuation and scattering (PAh and PSh) and non-harmonic power (PAnh and PSnh) were evaluated after spectral filtering of attenuated and scattered pulses. Harmonic power was integrated in 0,5 MHz frequency bands centered at frequencies 1, 2, 3 and 4MHz. Non-harmonic power was integrated in 0,5 MHz frequency bands centered at frequencies 0.5, 1.5, 2.5 and 3.5 MHz. For the excitation of suspension with MB and for analysis of stored US signals the same mode of excitation has been used: 10% DC, pulse repetition frequency – 6.7 kHz, period of excitation – 150 μs, excitation time duration Δt = 300 ms, length of excitation burst as well the Hanning time window – 15 μs.

Signals for analysis were taken in single frame of computerised osciloscope. Doing this way no inter-frame data has been lost. Results are presented in Fig.4. The graphs expose that initially after US excitation is started, there exists the short delay time Δt_S before the AApp starts to rise. This delay time is dependent reciprocally on P_{neg}. In case of P_{neg} = 400 kPa Δt_S =100 ms; P_{neg} =800 kPa - Δt_S=60 ms; P_{neg}=1200 kPa - Δt_S=30 ms (see AApp time functions in top row of Fig. 4). Noteworthy, while pulses from attenuation channel "keep silent" the ASpp show the significant increase of amplitude (see ASpp time functions in forth row of Fig. 4). Thus the „silence" of pulses from attenuation channel argue that no MB destruction occur, whereas rising amplitude of pulses in scattering channel implies the beginning of MB cavitation. We assume that both above events are consistent with the phenomenon which is called as *stable cavitation* of MB. With excitation span the AApp increase up till the asymptotic its value is achieved.

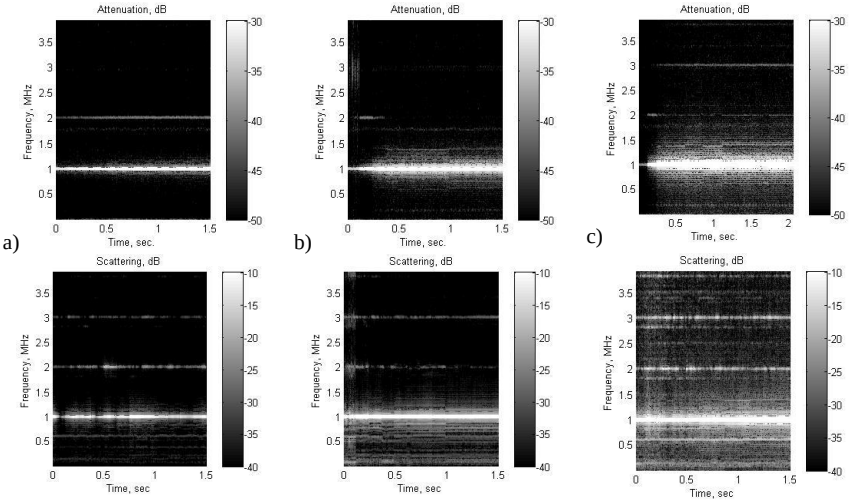

Fig. 3. Attenuation (top row) and scattering (bottom row) spectrograms of MB cavitation in DMEM with L-MB and cells suspension *versus* increasing of US pulses strength, P_{neg}: a) 100kPa; b) 300 kPa and c) 400 kPa.

Time function of amplitude of pulses received from attenuation and scattering channels represents the measure of scattering centres in suspension. In other words the significant increase in amplitude of attenuation and scattered pulses is related with the MB destruction in the whole volume of suspension. The following pattern can be related to the phase of US and MB interaction called *inertial cavitation*. Given dynamics of AApp and ASpp time functions is in line with rising acoustic transparency in spectrograms discussed above. The further increase of amplitude in attenuation and scattered channels argue the increasing cavitation activity following to the final sonodestruction of MB. The maximal asymptotic amplitude of pulses received in attenuation channel implies that sonodestruction of the main part of MB took place. Noteworthy the delay time duration Δt_d needed to achieve the total MB destruction is reciprocal to the value of the US excitation strength. Accordingly graphs in Fig.4 argue that at P_{neg}=400 kPa, Δt_d= 200 ms; P_{neg}=800 kPa, Δt_d=100 ms; P_{neg}=1200 kPa, Δt_d = 100 ms. However it should be noted that above discussed $P_{neg} - \Delta t_d$ relations are valid at the definite value of excitation time duration. For the study case it is 300 ms.

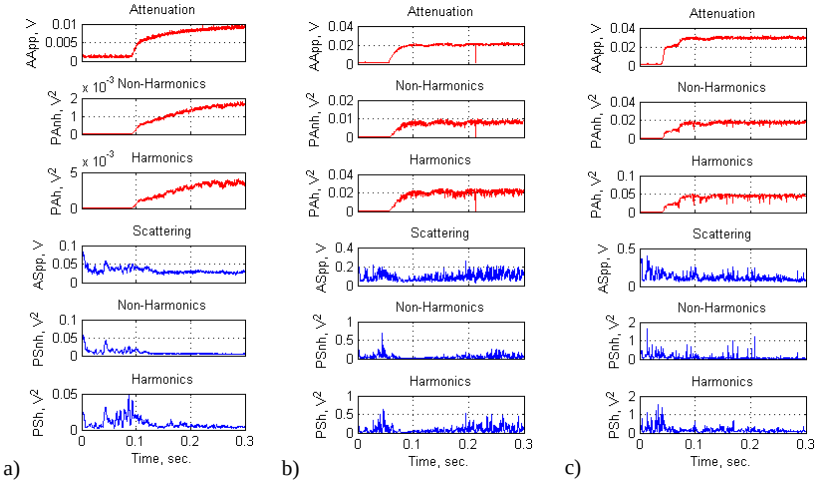

Fig. 4. Sonovue MB attenuation and scattering dynamics as a function of excitation pulse pressures P_{neg}: a) 100kPa; b) 300 kPa and c) 400 kPa respectively. The excitation time duration Δt = 300 ms in all cases is constant. Top three stripes represent time functions of pulse amplitude (AApp) and non-harmonic (PAnh) and harmonic (PAh) power in attenuation channel; the lower three rows represent time functions of pulse amplitude (ASpp), and non-harmonic (PSnh) and harmonic (PSh) power in scattering channel.

The results of EtBr sonotransfer efficiency dependence on MB sonodestruction dynamics are presented in Fig. 5. and Fig. 6.

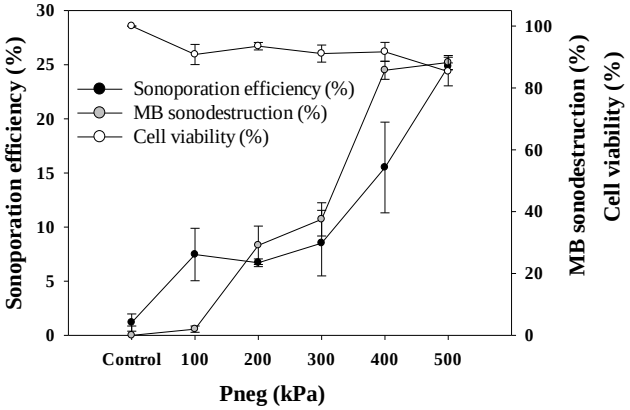

Fig. 5. EtBr sonotransfer efficiency, CHO cells viability and of L-MB sonodestruction intensity as the function of P_{neg} in vitro. Excitation frequency 1 MHz, excitation time duration Δt=2 s, burst time 1 ms, 10% DC.

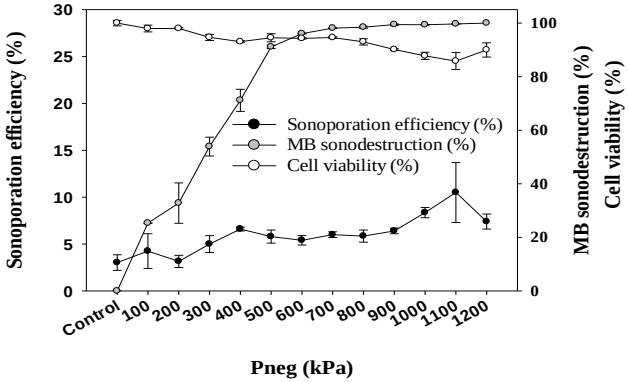

Fig. 6. EtBr sonoporation efficiency, CHO cells viability and of SonoVue MB sonodestruction intensity as the function of P_{neg} in vitro. Excitation frequency 1 MHz, excitation time duration Δt=300 ms, 10% DC, burst time - 150 μs.

Discussion and conclusions. The analysis of US attenuation and scattering spectra images, MB sonodestruction graphs as well as intracellular sonotransfer efficiency of ethidium bromide, presented in Figs. 2 - 6 implies:

1. There exist three phases of MB sonodestruction, which are strongly dependent on US strength P_{neg} and excitation duration time Δt: i) the phase consistent with stable cavitation at the range of 0-200 kPa, ii) the phase respective to the inertial cavitation at the range 200-500 kPa and iii) the phase of total sonodestruction of MB at P_{neg}>500 kPa.
2. The sonotransfer efficiency of anticancer drug etidium bromide into the cells in vitro show the consistent pattern with the above phases of MB sonodestruction dynamics, however this *nonlinear* relation is rather dependent on MB sonodestruction rate than of MB concentration.
3. The correlation of the MB sonodestruction intensity with the sonotransfer efficiency of small molecules is dependent on the characteristics of applied US as well as on the type of used MB (compare graphs in Fig. 5 and Fig. 6).

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The dependence of sonotransfer efficiency of anticancer drug etidium bromide into the cells upon microbubbles (MB) sonodestruction rate was studied. The analysis of ultrasound (US) attenuation and scattering spectra, MB sonodestruction rate as well as sonotransfer efficiency implies the following:1) The MB sonodestruction dynamics is strongly dependent on US strength and pulse duration; 2) Sonotransfer efficiency show the consistent pattern with MB sonodestruction dynamics; 3) The correlation of the MB sonodestruction intensity with the sonotransfer efficiency is dependent on the characteristics of applied US as well on type of used MB.