The Investigation of Calcein Sonoextraction from Cells *in vitro*

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**Introduction.** Sonoporation is a novel technique to deliver bioactive molecules, drugs, DNA and RNA, into cells and tissues. The process of sonoporation is associated to microbubble (MB) cavitation. Cavitating MBs mechanically create microstreaming and/or microjetting, which are capable to modify cell membrane impermeability to exogenous biocompounds. There are two hypotheses concerning molecular transfer at the membrane level: i) pore formation hypothesis and ii) endocytosis hypothesis [1].

Cavitating MBs are capable to induce transient nonspecific pores that automatically reseal after a period of time. During the opening period molecules pass to the interior of the cell mainly due to passive diffusion [2]. However, if the cell membrane pores are too large to be repaired, the cell eventually dies. Endocytosis hypothesis states molecular entry within membrane vesicles, which after degradation within the cytoplasm liberate the cargo inside the cell. The latter process is not associated with diffusion.

The dominant mechanism of sonoporation remains unresolved so far. After extracellulal ultrasonic trigger is applied to the cells, the outflux of small molecules is expected to occur mainly through sonoporation induced pores via passive diffusion. Our previous studies have shown that by controlling electroporation parameters one can control extraction efficiency of calcein from calcein-preloaded cells [3]. Since we had already proved sonoporation to be an effective technique for the efficient delivery of biomolecules [4, 5], the aim of this study was to test whether sonoporation can be exploited for the extraction of small exogenous molecules from cells *in vitro*.

**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. The cells were grown in monolayers in 10 cm Petri dish (TPP, Switzerland), incubated at 37 °C, in 5% CO₂ atmosphere. Cell loading with calcein AM was performed in growth medium by adding calcein AM to 1 μM final concentration and incubating for 1 h. Membrane permeable calcein AM is processed by the cells resulting in production of fluorescent nonpermeant calcein. The cell suspension for experiment was prepared in 1x PBS (Lonza, USA).
The arbitrary waveform generator (Picoscope 5242B, Picotech, UK) was used to produce US signals. The signals were amplified by signal amplifier (Kaunas University of Technology, Lithuania), powered by high voltage power supply (MCP lab electronics, China). The US transducer (Medelkom, Lithuania) operating at 1 MHz central frequency was used for MB excitation. All sonoporation experiments were performed in the cuvette (Plastibrand, Germany). The ultrasound exposure system is presented in Fig. 1.

![Experimental system](image)

**Fig. 1.** Experimental system

For sonoporation experiments the cell suspension was supplemented with SonoVue® MBs (Bracco diagnostics Inc., Switzerland) to the final 450 µl volume with final 0.8 mln/ml cell and 10% vol MB concentrations, respectively.

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 - 200 kPa acoustic peak negative pressures for 2 s.

After sonoporation the cell suspension was centrifuged and the fluorescence spectra of the supernant were evaluated using optical spectrofluorimeter (Avantes, Netherlands) with bifurcational optical cable. 473 nm wavelength was used for calcein excitation, while fluorescence was measured at 515 nm wavelength to quantify calcein release. The measurement was performed in 100 µl cuvette (Beckman coulter, USA). Cell viability was evaluated using cell colony test. After cell suspension exposure to the US, the cells were incubated for 10 minutes at 37°C. Then diluted in growth medium (~330 cells) and cultured for 7 days. After that cells were fixed using 96% ethanol and stained with 5% crystal violet (Sigma-Aldrich, USA). The number of cell colonies was evaluated using light microscope (MBS-9; LOMO, Russia).

**Results and discussion.** In this study the efficiency of calcein release was evaluated altogether with cell viability.

Calcein release graph (Fig. 2 left) shows that calcein release efficiency increases with increasing acoustic pressure. However, the cell viability (Fig. 2 right) remains rather high (>80%). This data suggests calcein release to occur through transient membrane pores induced by extracellular MB cavitation. These findings are in line with the results obtained by other groups, where there
had been stated small molecule delivery/release to occur mainly through sonopores [1, 2, 6]. In addition, these findings go in line with our electroporation results showing that electroextraction can be compatible with reversible electroporation and preservation of cell viability [3, 7]. These data imply that transition of small molecules through cell membrane is a bidirectional process associated with diffusion which occurs mainly through sonopores or electropores, respectively.

**Fig. 2.** Calcein release efficiency (left) and cell viability (right) dependence on US acoustic pressure

**Conclusion.** MB cavitation induced calcein release from cells states pore presence and importance for small molecule delivery/release from cells.

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**References**


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In current study sonoporation induced calcein release efficiency was evaluated altogether with cell viability. The results show that the efficiency of the extraction of small molecules from cells occurs at relatively high cell viability. These results imply transient pores to be the dominant pathway for small molecules to cross cell membrane during sonoporation.