Sonoporation-mediated intracellular delivery dependence on molecule size

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Introduction. Sonoporation is a method for drug, gene or other molecule delivery into the cells with the aid of ultrasound (US) and microbubbles (MB) [1]. It is a promising method with a possible application in human therapy, since US can penetrate tissues non-invasively. However, sonoporation is still not used in therapy, because its efficiency remains relatively low. There are many factors, influencing sonoporation efficiency. Some studies show that sonoporation efficiency depends on US parameters [2, 3], MB type [4] or cell culture type [4]. Some studies showed that sonoporation efficiency depends on MB concentration [5, 6, 7] or cell concentration [7]. We have also recently investigated sonoporation efficiency and cell viability dependence on MB and/or cell concentration and concluded that both sonoporation effectiveness and cell viability were directly related to the distance between cells and MBs [8]. Besides above mentioned factors, bioactive molecule delivery could depend on molecule size. It has been showed that sonoporation could be used to deliver small molecules [9], macromolecules [10 - 12] including plasmids [13, 14]. However most studies use only one type of bioactive molecule, thus intracellular delivery dependence on molecule size could not be done. Intracellular delivery varies noticeably in these studies. Only few studies were done to evaluate sonoporation efficiency depending on molecule size [15, 16]. Karshafian et al. study states that intracellular delivery does not depend on molecule size. Afadzi et al. [16] work supports such statement only for big molecules, while smaller molecules show increased delivery at the same conditions. Thus it remains unclear if molecule size influences sonoporation efficiency, especially when molecules are small.

Thus the aim of this study was to determine sonoporation assisted intracellular delivery of two different size molecules: propidium iodide (PI) and FITC dextrane. We showed that intracellular delivery of these molecules was practically identical, even if molecular size was different. Our data supports previous data that intracellular delivery using sonoporation does not depend on molecular size, at least at the range of tested molecules.

Methods. 1 MHz unfocused 19 mm diameter US transducer was connected to US generator (UAB Medelkom, Vilnius, Lithuania), which delivered 1 ms US signal of 300 kPa peak negative pressure, with 10% duty cycle and 100 Hz repetition frequency.

MBs were produced in our laboratory as describe previously (Lama et al., 2013). In short, they were made of 2 mg/ml polyethyleneglycol-40 stearate

(Sigma-Aldrich, St. Louis, MO, USA) and 2 mg/ml DL-alpha-phosphatidylcholine (Sigma-Aldrich, St. Louis, MO, USA), dissolved in 1 mL sterile 0.9% NaCl solution, saturated by 5 ml of sulphur hexafluoride gas (Linde Gas AGA, Sundbyberg, Sweden) and sonicated with 20 kHz power with 150 W and 300 W.

Chinese ovary cancer (CHO) cell culture at the concentration of 3.3×10^6 cells/ml was used. Cell to MB ration was 1:20 and 1:40, as previously we have showed that this is the most optimal MB concentration at our conditions [8]. 1 mM propidium iodide (PI) and 250 nM 4 kDa FITC dextran were used to determine sonoporation efficiency. Both compounds can enter the cell only when pores are formed, and they can be seen under fluorescent microscopy. In addition, PI also stains dead cells, which lost their membrane integrity. Thus, PI was added into cell suspension containing MB and FITC dextran solution 15 min after US application, when all pores were closed. In such a way only dead cells were stained, and they were subtracted from sonoporation induced delivery. Thus, after sonoporation, dividing PI or FITC dextran stained cells by the total number of cells, reversible sonoporation could be calculated. Experiments were repeated 2 - 5 times, the data are presented as mean±SEM.

Results. We performed sonoporation experiments using these parameters: 1 MHz central frequency, 300 kPa peak negative pressure, 10% duty cycle and 1 s exposure time. CHO cell concentration in a cuvette was 3.3×10^6 cell/mL. We used laboratory made bubbles, which concentration was 20 times or 40 times higher than cell concentration. Previously we have found that these are the most optimal parameters for sonoporation [8]. First, we tried to compare sonoporation efficiency using two different molecules: PI and FITC dextrane. Molecular weight of PI is 668.4 Da, while size of FITC dextrane is 4 kDa. Results show that intracellular delivery of PI or FITC dextrane was very similar (Fig. 1). When cell to MB ratio was 1:20, sonoporation efficiency was 11.35±4.4% (n = 2) and 12.4±3.3% (n = 5) for FITC dextran and PI, relatively. When MB concentration was increased up to 1:40, it was 14.95±3.2% (n = 2) and 15.9±1.5% (n = 5) for FITC dextran and PI, relatively. The data shows that even the molecular weight between these two molecules differs 6 times, intracellular delivery is practically identical. It should be stressed, that our data showed reversible sonoporation: after US application the pores closed, PI could not enter the cell any more, but cells remained alive.

Since we used different cell/MB ratio we also aimed to evaluate cell viability. It was done using PI, which is able to enter dead cells, as described in methods. The data shows, that cell viability was not compromised due to sonoporation, and it was also very similar for these two bioactive compounds (Fig. 2). When cell to MB ratio was 1:20, cell viability for FITC dextran experiments was 87±6% (n = 2); for PI experiments: 85.8±1.7% (n = 5). Even when MB concentration increased up to 1:40, cell viability also remained similar: 83.5±5.5% (n = 2) for FITC dextran experiments and 84±1.6% (n = 5)

for PI experiments. At control condition, when no US was applied, cell viability was 93.4±0.5 (Fig. 2, right).

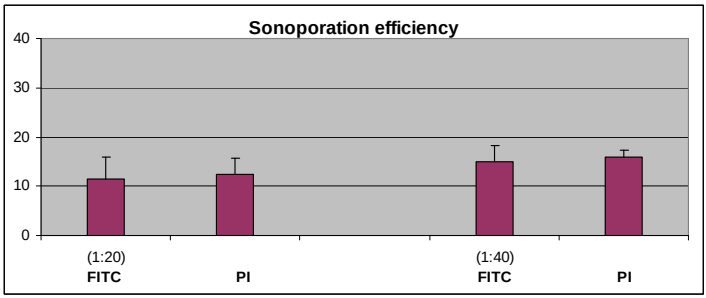

Fig. 1. Sonoporation efficiency comparison for FITC dextrane and PI, using 1:20 and 1:40 cell to MB concentrations. US parameters: 1 MHz central frequency, 300 kPa peak negative pressure, 10% duty cycle, 1 s exposure time. n = 2 for FITC dextran (both 1:20 and 1:40) and n = 5 for PI (both 1:20 and 1:40) experiments.

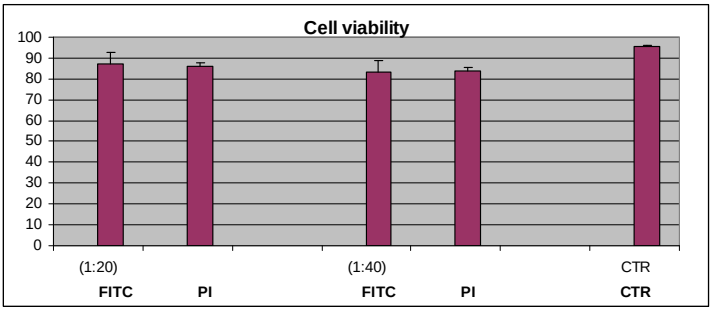

Fig. 2. Cell concentration influence on sonoporation efficiency and cell viability, using 1:20 and 1:40 cell to MB concentrations. US parameters: 1 MHz central frequency, 300 kPa peak negative pressure, 10% duty cycle, 1 s exposure time. n = 2 for FITC dextran (both 1:20 and 1:40) and n = 5 for PI (both 1:20 and 1:40) experiments.

Discussion. The intracellular delivery of bioactive molecules, aided by sonoporation, is compatible with pore formation in the plasma membrane. The pore formation can proceed by different scenarios. According to the first scenario, large pores could be produced after US application. In such a way, the molecular size of bioactive compounds, which are delivered into the cells, would not influence sonoporation efficiency. A second scenario could also happen, when pores of different sizes are formed, depending on US parameters or other factors. In such a way molecular size of intracellular delivered compounds would play a crucial role. Literature data is confusing about this issue. Some authors showed that in vitro sonoporation efficiency was dependent on molecular size [17]. This study suggested that during

sonoporation pores as large as 56 nm were formed on cell membrane, since molecules up to 2 MDa could enter the cell. Others found that sonoporation efficiency did not depend on the size of FITC dextrans, with the exception of the smallest (4 kDa) dextrans, when transfer was increased [16]. These authors also stated that sonoporation was aided by endocytosis. Other groups found no dependence at all on molecular size for intracellular delivery after sonoporation [2, 10]. Therefore we have chosen small (0.7 kDa PI) and intermediate (4 kDa FITC dextran) size molecule delivery to compare after sonoporation. Our results showed that intracellular delivery by sonoporation was practically identical for these two molecules. It is in agreement with Guzman and Karshafian publications [2, 10]. Guzman et al used calcein (0.6 kDa), bovine serum albumine (66 kDa), and two dextrans (42 and 464 kDa), while Karshafian et al used four different dextranes (10 kDa, 70 kDa, 500 kDa and 2 Mda). We used PI (0.7 kDa) and 4 kDa FITC dextrane. This wide range of molecules supports the idea that after US application in the presence of MBs, large pores are formed at once and the size of delivered molecule in the range up to 4 kDa does not play a crucial role.

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Few experiments were done to investigate intracellular delivery by sonoporation dependence on molecular size, and the data are confusing. Some authors state that molecule size is important for intracellular delivery, while others found no dependence on molecular size. To solve this issue, we investigated two different molecule intracellular delivery under the same sonoporation parameters. One is small, 0.7 kDa propidium iodide; other is medium sized, 4 kDa FITC dextrane. Our results show that it was no difference between these two molecules intracellular delivery under the same sonoporation parameters.