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High frequency electroporation efficiency is under control of membrane capacitive charging and voltage potential relaxation

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ABSTRACT

The study presents the proof of concept for a possibility to achieve a better electroporation in the MHz pulse repetition frequency (PRF) region compared to the conventional low frequency protocols. The 200 ns × 10 pulses bursts of 10–14 kV/cm have been used to permeabilize Chinese hamster ovary (CHO) cells in a wide range (1 Hz–1 MHz) of PRF. The permeabilization efficiency was evaluated using fluorescent dye assay (propidium iodide) and flow cytometry. It was determined that a threshold PRF exists when the relaxation of the cell transmembrane potential is longer than the delay between the consequent pulses, which results in accumulation of the charge on the membrane. For the CHO cells and 0.1 S/m electroporation medium, this phenomenon is detectable in the 0.5–1 MHz range. It was shown that the PRF is an important parameter that could be used for flexible control of electroporation efficiency in the high frequency range.

1. Introduction

Application of high pulsed electric field (PEF) to cells induces electroporation. This phenomenon is driven by the reorientation of lipids and formation of pores in the cell membrane, causing changes in membrane permeability and the increase of molecular transport [1,2]. The surface changes in the membrane structure occur due to the charge accumulation and transmembrane voltage induction during the electric pulse. Electroporation takes place when the increased transmembrane voltage reaches a threshold (typically >200 mV) [3,4]. Therefore, electroporation is a pulse (voltage or transmembrane voltage) dependent phenomenon [5,6], and a cell can be approximated as a simple equivalent electrical RC circuit, where the charging of the membrane depends on the electrical parameters of both the cell and the suspension [7]. Subsequently, if a steady state induced transmembrane voltage (ITV) is not reached (i.e. the pulse is shorter than the cell RC charging constant or PEF amplitude is low), the efficiency of electroporation is reduced dramatically [8–10]. Usually a trade-off between the PEF amplitude and the pulse duration is made [11–13]. For mammalian cells a well-established protocol is to apply a burst of microsecond pulses (e.g. 100 μs × 8) in the kV/cm PEF range [14–16]. However, during recent years, a number of papers focusing on shorter duration, but higher intensity PEF pulses were published [17–20]. The rationale of the sub-microsecond protocols is derived from the capability to bypass the plasma membrane of the cell and affect the inner organelles using energy efficient and non-thermal methodology [7,21,22]. Additional potential of theses protocols lies in the possibility to induce cell apoptosis [23,24] and in the area of biomedical applications (i.e. improvement of electrochemotherapy or tissue ablation protocols).

At the same time, by finite element method (FEM) and theoretical considerations it was proven that high-frequency fields ranging from 500 kHz to 1 MHz are best suited to penetrate epithelial layers without the induction of significant Joule heating [25]. However, the available experimental contributions dealing with the high frequency electroporation (>0.5 MHz range) are focusing only on the bipolar pulsing protocols, presumably due to the technological challenges of generating high power and high voltage monopolar pulses in MHz range. As a result, the coverage of research on the influence of PRF during monopolar electroporation is limited to the kHz range [26–29], while MHz range remains theoretical [30,31]. However, even taking into account the technological limitations and moderate frequency range coverage, new frequency dependent electroporation phenomena have been recently established (i.e. cell sensitization and de-sensitization) [32–35] indicating high potential of frequency manipulation to achieve specific goals in electroporation protocols.

According to the established theory, the reciprocal of the pulse duration lays within the low-frequency plateau for the microsecond and millisecond pulses [30], while the amplification of the external electric field in the cell membrane is frequency dependent and decreases in the sub-megahertz range [32,30,31]. It implies that, in order to induce the same value of ITV using the nanosecond pulses, the applied voltage (using same setup) can be hundreds of times higher compared to conventional micro-millisecond protocols. High PRF (kHz range) further results in the reduction of the electroporation efficiency [29,36]. However, the

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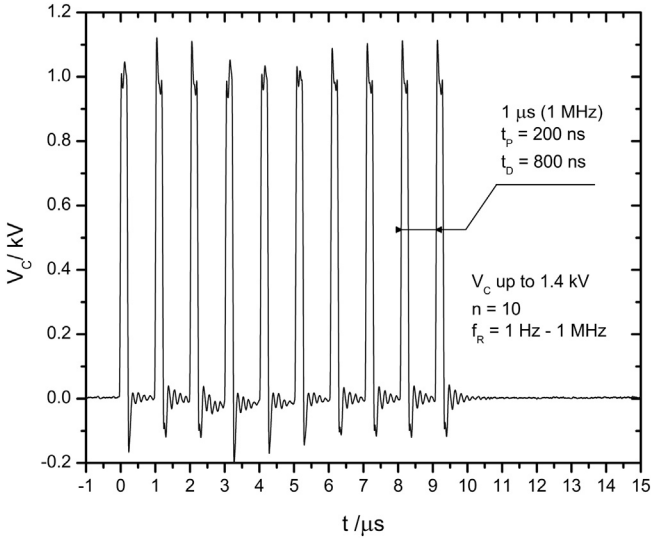

Fig. 1. The waveform of the applied electrical pulses. Acquired using DPO4034 digital oscilloscope (Tektronix, Oregon, USA), post-processed using OriginPro software (OriginLab, Northampton, MA, USA).

currently available literature on either electroporation theory or experimental applications does not take the speed of charging and discharging of the cell membrane into the account, when the ITV amplitude is analyzed in the sub-megahertz PRF range. Therefore, we have speculated that it is possible to generate a high frequency pulse burst to achieve a threshold PRF (presumably unique for each cell type and electroporation medium), when the discharging (ITV relaxation) time of the membrane is higher than the delay time between the pulses.

Therefore, in this study we expand the experimental coverage of the electroporation frequency range (1 Hz–1 MHz) by presenting experimental and FEM modeling data on the influence of the PRF during sub-microsecond electroporation of mammalian cells.

2. Materials and methods

2.1. Electroporation setup

For electroporation, the 0–3 kV, 60 A square wave pulse generator (100 ns–1 ms) that was developed in Institute of High Magnetic Fields (VGTU, Vilnius, Lithuania) was applied [37]. The commercially available electroporation cuvette with 1 mm gap between the electrodes (VWR International, Radnor, PA, USA) was used as a load. Bursts of 10–14 kV/cm, 200 ns have been generated at PRF of 1 Hz–1 MHz. The 10-pulse protocol was used. As a reference, single 2 µs electric pulse with equivalent energy (200 ns × 10) was applied. The waveform of the 1 MHz pulse burst is shown in Fig. 1.

The 1–1.4 kV pulses were generated in the cuvette, corresponding to the 10–14 kV/cm electric field amplitudes. The waveform had clear spikes at the beginning and end of each pulse due to a shunting resistance in parallel to the load, which is present in part of the electroporator [37]. It might be compensated by an exact matching of the load for each experiment, however due to the short duration of the oscillation and relatively low amplitude of the spike (<200 V), the influence on the transmembrane potential was considered negligible and, therefore a fixed value of the shunting resistance was used.

2.2. Biological cells

Chinese Hamster Ovary (CHO) cells were used as an electroporation object. Cells where grown in Dulbecco's modified Eagle's medium (supplemented with FBS (10%), L-Glutamine and penicillin/streptomycin (1%) Sigma, St. Louis, MO, USA) at 37 °C in humidified 5% CO_2 atmosphere in incubator. CHO cells were passed every 3 days and 24 h before experiment.

2.3. Flow cytometry and cell permeabilization assay

After trypsinization CHO cells where suspended at concentration of 2×10^6 cells/ml in 270 mOsm and 7.1 pH electroporation medium. Electroporation medium was composed of sucrose (242 mM), Na_2HPO_4 (5.5 mM), NaH_2PO_4 (3 mM) and $MgCl_2$ (1.7 mM). The measured specific conductivity of electroporation medium was 0.1 S/m at 25 °C.

During the experiments 36 µl of the cell suspension (2×10^6 cells/ml) and 4 µl of propidium iodide (PI) (40 µM) was mixed and placed in the electroporation cuvette. After electroporation, the cells were transferred to 1.5 ml tube (Eppendorf, Hamburg, Germany) and incubated for 10 min. Afterwards, the evaluation of PI electrotransfer was performed using flow cytometer BD Accuri C6 (BD Biosciences, Ann Arbor, MI, USA).

For appropriate gating strategy for PI fluorescence evaluation several additional experimental points were included in the study. Firstly, to determine the PI induced fluorescence in dead cells, CHO cells were incubated at −80 °C for 30 min. Results are presented in Fig. 2B. Consequently, PI positive cells with fluorescence intensity above 1.5×10^4 RLU were defined as dead cells. Those cells that had fluorescence intensity below 1.4×10^5 were considered as viable cells (Fig. 2A).

Cell electroporation (positive control; 1.4 kV/cm, 100 µs × 8) in the presence of PI was performed to determine gating for "PI permeable cells". As seen in Fig. 2C, fluorescence intensity of PI positive cells is between fluorescence intensity thresholds of viable and dead cells. Mean fluorescence intensity of gated PI permeable cell population was also analyzed in the study.

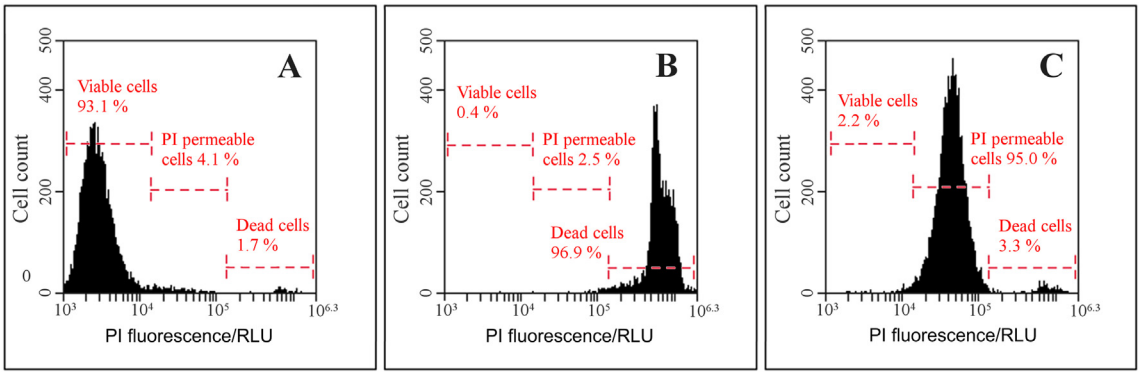

Fig. 2. Cell gating strategy during flow cytometry analysis. Three gates were set (for details see the text): viable cells, PI permeable cells and dead cells, where A – untreated cell fluorescence after cell's incubation with PI for 10 min; B – PI fluorescence of cells that were incubated at −80 °C degrees for 30 min and afterwards incubated with PI for 10 min; C – PI fluorescence of cells 10 min after PI electrotransfer (1.4 kV/cm, 100 µs × 8).