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Monitoring cell monolayers during electroporation: Electrical impedance spectroscopy measurements

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Abstract- Electroporation or electropermeabilization is a phenomenon observed when lipid bilayers, generally cell membranes, are exposed to high electric field pulses becoming transiently permeable to molecules that under regular conditions are not able to penetrate through them. This change in molecular permeability is believed to be produced by transient aqueous pores created in the lipidic structure and can be monitored by changes in the electrical conductivity of these membranes. The aim of this study is to use fast electrical impedance spectroscopy to measure the process of electroporation applied on cell monolayers growing attached to standard multiwell plates. The frequency response of the impedance can provide useful information about the extent of permeabilization in the cell membranes exposed to high electric fields and also the time dynamics of creation and resealing of these pores. For this study we used a microelectrode assembly specifically designed for *in situ* performance of both electroporation and impedance measurements. The design of the microelectrodes is based on a spiral geometry conceived to improve the uniformity of the electric field applied and to perform impedance measurements in a four-electrode configuration.

Keywords: Electrical Impedance Spectroscopy, Electroporation, microelectrode array.

I. Introduction

Membranes of living cells are formed by phospholipid bilayers and constitute the barrier between the intracellular and extracellular medium. One of the main issues presented on biomedical research is the introduction of different molecules through these membranes. Electroporation, also called electropermeabilization, is a phenomenon occurred when cell membranes are exposed to high electric field pulses. When parameters of such electric fields are the appropriate, a transient state of permeability to molecular species is produced [1]. The technique is currently used to insert molecules such as drugs, genetic material, etc. into the cell cytoplasm. Despite the fact that there is still no explanation from a molecular point of view, the most accepted theory is the formation of transient aqueous pores across the cell membranes during exposure to electric field [2]. The interesting characteristic of this phenomenon is the reversibility of it, short time after electric field exposure, pores reseal and cells remain viable. In other situations, and under different pulse conditions cells are not able to reseal after electroporation, thus leading to cell death (irreversible electroporation).

Nowadays, standard methods used to assess the effectiveness of *in vitro* electroporation treatment imply molecular reporters and imaging techniques, moreover, none commercial equipment is able to online monitor the process. In accordance with other studies, a change in the electrical conductivity of cell membranes during electroporation has been observed [3, 4]. In this direction electrical impedance spectroscopy (EIS) is a feasible tool for online label-free monitoring the electroporation process. Current-voltage measurements have been traditionally conducted observing the changes in the applied pulse waveform (dc measurements) [3-5]. These measurements have provided some information about the changes occurred during pulse application, mainly quasi-dc conductivity changes. Other attempts measuring at a single or three frequencies between 0.4 and 40 kHz could only monitor the cell monolayer before and after pulse application but not during the process [6, 7]. Monitoring the evolution of the complete impedance spectrum during electroporation has the advantage of providing additional information of the events produced. Traditional impedance measurement approaches are not feasible due to the long measuring time needed. In this work we make use of a multisine-based approach to perform fast electrical impedance spectroscopy [8] during the interpulse electroporation interval to study pore creation and resealing dynamics and how parameters such as electric field amplitude or pulse number affect the efficiency of the treatment.

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II. Description of the system

A-Microelectrode assembly

In previous studies we developed a microelectrode assembly specially designed for performing *in situ* electroporation to adherent cell monolayers growing in standard multiwell tissue culture plates [9]. The microelectrode assembly is positioned above the cell monolayer during treatment avoiding direct contact between electrodes and cells by means of 10 µm micro-separations patterned on the structure. These microelectrodes are constructed using PCB Class 7 bilayer fabrication technology according to high density integration (HDI) IPC regulation [10](Lab Circuits S.A. 08460 Barcelona, Spain). This fabrication technology involves a drastic reduction in manufacturing costs. This reduction can be translated in the possibility of creating disposable equipment what is desirable for the research laboratory environment. The complete fabrication process, described in [11], is applied to a different electrode configuration in this study.

A spiral geometry was conceived to perform both electroporation and bioimpedance measurements. The design includes six parallel independent lines with dimensions of 75 µm width and 150 µm spacing. A schematic diagram of the structure and connections is depicted in Fig. 1. The idea behind this design is the possibility of performing four-electrode impedance measurements on the whole area of cell growth and applying electroporation pulses with the same set of electrodes.

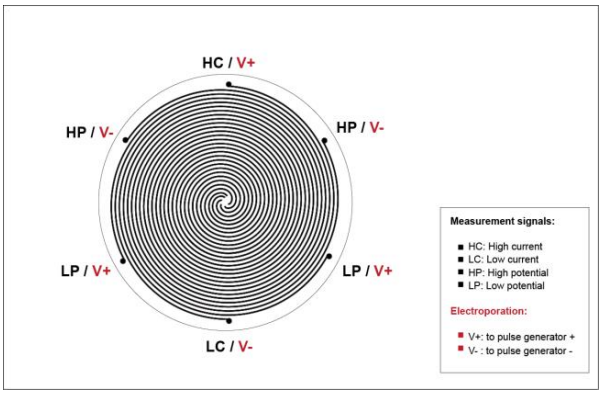

Fig. 1: Diagram of the spiral geometry used in the microelectrode design. Connections for impedance measurements and electroporation are shown.

B- Biphasic pulse generator

Electric field pulses were delivered using a biphasic stimulator developed in our laboratory. The stimulator generates bipolar square pulses acting as a fixed current source and parameters such as pulse amplitude, duration, frequency, etc. are fully programmable by an RS-232 connection to a PC. Additionally, the equipment includes a synchronism output that was used for synchronization with the measurement system.

C- Impedance measurement system

To perform the electrical bioimpedance spectroscopy measurements to monitor the changes in the cell membrane during the interpulse interval of an electroporation treatment, a fast method is necessary. According to [2], expansion and stabilization of pores are in the milliseconds range and resealing can take several seconds. For this purpose we used a (periodic) random-phase multisine excitation as the reference signal. The multisine burst includes 21 frequencies quasi-logarithmically distributed from 5 kHz to 1.313 MHz. The bioimpedance was estimated from measuring and processing an integer number of periods of the bio-system current and voltage response using the direct approach based on cross and autocorrelation [8]. The total measuring time for each multisine burst was 2 ms and the total number of spectra acquired during each interpulse period (1 second) was 230.

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This measuring strategy was implemented on a NI PXIe-1062Q chassis using a NI PXI-5422 card (200 MS/s, 16-bit) for waveform generation, NI PXIe-5122 card (100 MS/s, 14-Bit) as the digitizer/acquisition system and the NI PXI-2530 high-density multiconfiguration multiplexer/matrix for changing between measurement and electroporation signals.

Additionally, a front-end stage was used to apply current and measure both voltage and current signals. A broadband differential amplifier implemented with two operational amplifiers as input buffers was used to measure the voltage signal. Current signal was measured with a transimpedance amplifier. The whole setup has a bandwidth greater than 20 MHz with a CMRR above 60 dB up to 10 MHz.

A block diagram of the complete system and a representation of the signals generated are shown in Fig. 2.

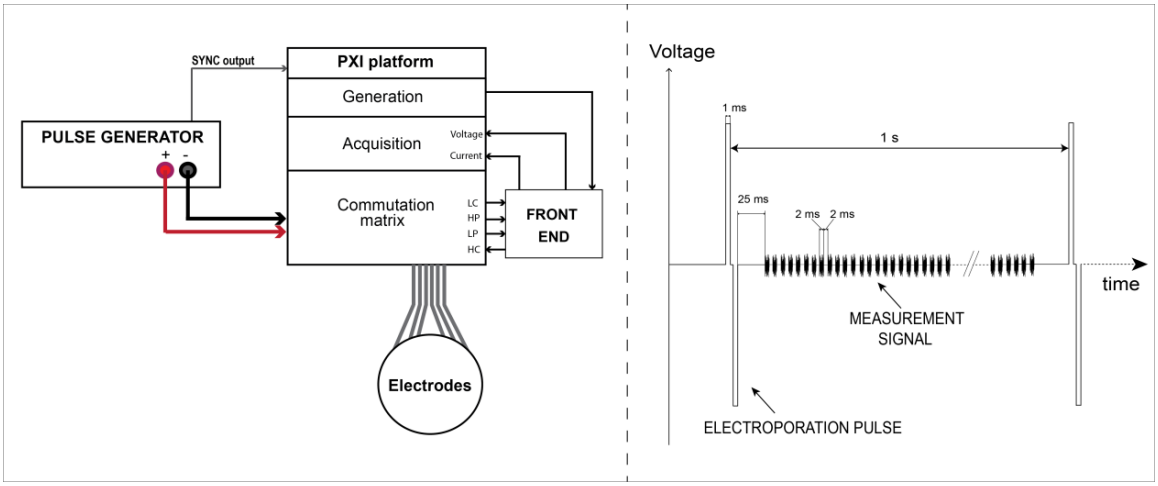

Fig. 2: Block diagram of the complete system and generated signals. Pulse generator creates biphasic electroporation pulses, the SYNC output is used for synchronization with the PXI system. After commutation of connections (25 ms delay) measurement signals are generated and acquired each 2 ms. 230 complete spectra are measured between consecutive pulses.

D- Experimental conditions

Experiments were performed using CHO (Chinese Hamster Ovary) cell line cultured as a monolayer in 24 multiwell plates. Cells were cultured in DMEM/Ham's F12 with glutamine medium (PAA: The Cell Culture Company) supplemented with 10% FBS and 1% PSF at 7 x 10^4/well. Plates were cultured at 37 ºC in a humidified 5 % CO_2 incubator for approximately 24 h, to reach 50–60 % confluence. Low-conductivity electroporation buffer (LCE) consisting of 10 mM Na_2HPO_4 (pH 7.4), 1 mM $MgCl_2$ and 250 mM sucrose was used [12].

Before pulse application, cells were incubated during 5 minutes with LCE at room temperature for stabilization, also microelectrode assembly was positioned above cell monolayer 1 minute before initiating the measurements. 230 spectra where measured immediately before first electric field pulse and also between consecutive pulses. Eight electric field pulses were applied with 1ms duration, 1 Hz frequency repetition (time interval of 1 second for measurements) and electric field intensities of 200, 600, 1000 and 1400 V/cm.

III. Results and Discussion

Results of electrical impedance measurements during the electroporation procedure are shown in Fig. 3. Normalized module and phase time evolution at 3 frequencies is shown for four different electric field intensities. Each vertical line marked in the figure corresponds with the application of an electroporation pulse. It can be clearly observed a dose-response relationship between the electric field intensity applied and the relative

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impedance change measured. This is in accordance with the fact that a higher permeabilized area of the membrane is achieved with higher electric fields.

A- Long term evolution

Analyzing these results two different dynamics are observed. First, we can observe an impedance time evolution along the complete process. These long term changes are accumulative and show an asymptotic behavior with the number of pulses applied. Comparing the three different frequencies shown, it is noticeable that long term dynamics are similar for the three frequencies but the magnitude of the relative change observed is different. In the lowest frequency, corresponding with the impedance at 7 kHz, the change observed is a decrease of more than 20 % of the initial state. However, for the highest frequency of 1,122 MHz less than 10 % of change is measured. According to bioimpedance basic theory, low frequency impedance of living structures is increased by the presence of cell membranes behaving as capacitors. When these membranes are permeabilized, low frequency signals can find new current paths, thus decreasing the impedance in the range of low frequencies. At high frequency, current flows freely across the membranes and no significant change should be observed due to electroporation.

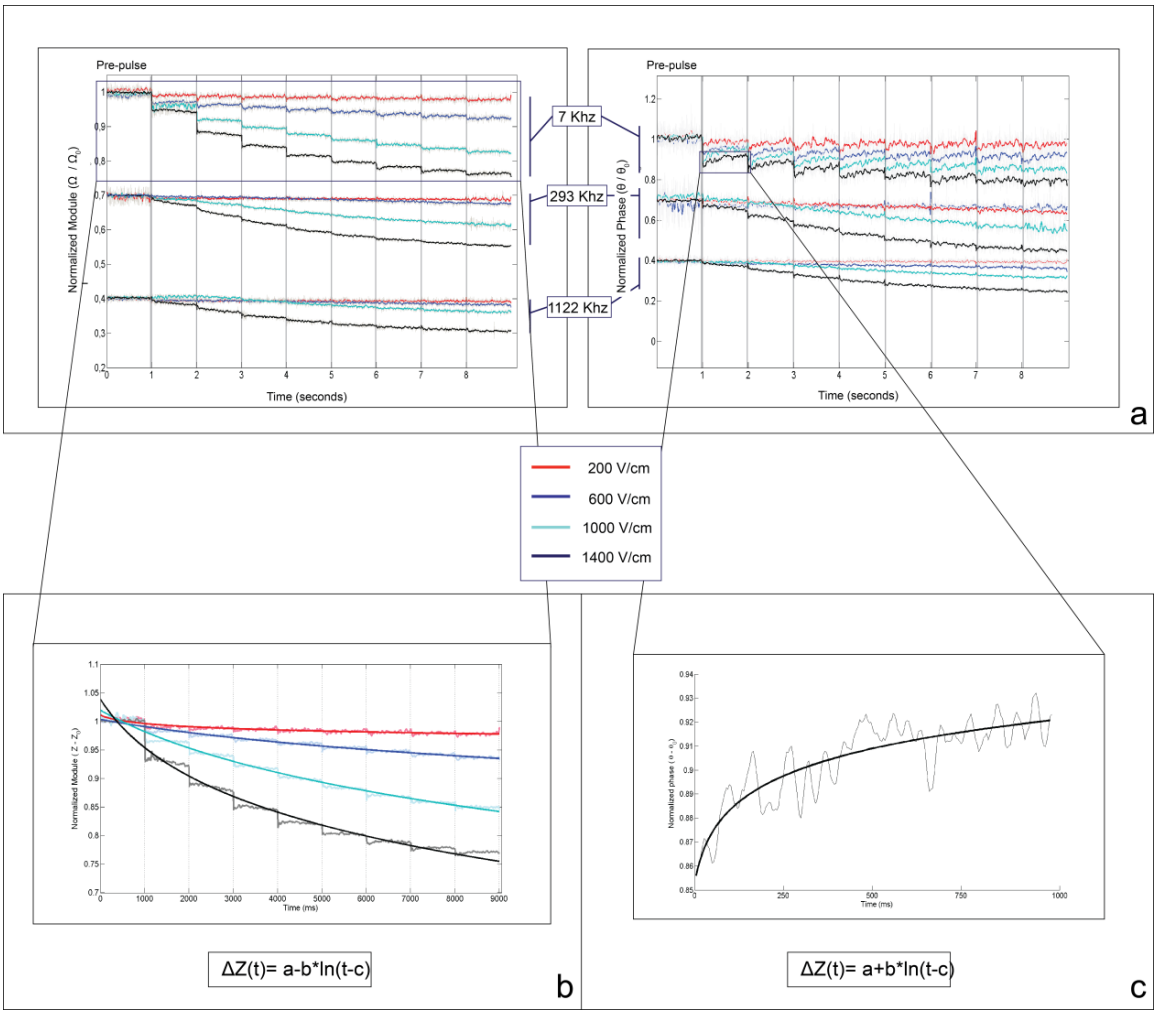

Fig. 3: Impedance measurements during electroporation at 4 different electric field intensities. a; Evolution of normalized module and phase at three different frequencies. Measurements during the first second correspond to the pre-electroporation state. Vertical lines mark each electroporation pulse. b; Zoom on the long-term module evolution at 7 kHz and fitted data to a logarithmic function. c; Zoom on the short-term phase evolution at 7 kHz after the first electroporation pulse and fitted data to a logarithmic function.

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Our explanation for the observed decrease in impedance at high frequency is that two basic events affecting the impedance recordings are occurring at the same time; first, the resulting creation of pores in the membranes and second, a change in conductivity of the extracellular medium caused by ion exchange. Unbalanced concentration of ions can produce leakage of these ions across the pores by passive diffusion from the intracellular space to the extracellular. This can explain the different magnitude of the relative change observed at low and high frequency and supports the idea that acquiring complete impedance spectra different effects can be separated.

In Fig. 3c the evolution of module at 7 kHz is shown in detail. We observed that this long term evolution follows a logarithmic model as already reported in [13], where this behavior is attributed to the existence of a wide range of resealing time constants. Our data was fitted to a function with the expression:

$$\Delta Z(t) = a - b \cdot \ln(t - c) \quad (1)$$

These long term changes can be related with the creation of stable pores in the cell membrane that are not able to reseal in the interval between pulses and that, according to [14], are called long-lived pores.

B- Short term evolution

Another type of pores is believed to form during the electroporation procedure called short-lived pores[14]. These changes in the lipidic bilayer begin to create immediately after the initiation of the electric field and start to reseal immediately after the electric field ceases. Resealing time of these pores is considerably shorter than that of the long-lived ones and can only be monitored in the time between pulses. In Fig. 3d a zoom in the evolution of the phase in the time gap between two pulses is shown. A logarithmic behavior is also observed in this case but with a faster time constant than that of long-lived pores.

We observed that low frequency phase measurements are less sensitive to conductivity changes than module measurements and are more suitable for monitoring these fast events. In the case of module measurements the dynamics of ionic diffusion masks the resealing of the membrane. According to these measurements, the majority of short lived pores reseal before the next pulse is applied and the number of short lived pores created decreases with the pulse number. This can be explained by the fact that when the number of stable pores or long-lived pores increases, the polarization of the membrane is more difficult and thus to reach the transmembrane voltage threshold required for electroporation.

IV. Conclusions

In this work our objective was to study the dynamics of electroporation of cell monolayers by means of electrical impedance spectroscopy and study if by acquiring complete spectra instead of single frequency measurements more information can be extracted from the process.

According to the first results shown here, we were able to monitor the accumulation of stable pores across the process as a decline in the impedance module and phase and the resealing of the short lived pores occurred in the time gap between pulses. We detected significant changes with increasing electric field amplitude and pulse number. Also we observed logarithmic behavior in the dynamics of pore resealing both for long and short term evolution. We conclude that acquiring complete spectral response different effects associated with the electroporation process can be separated.

Further work will comprise improving time resolution of measurements, adjustment to impedance spectrum models in order to extract more information about the membrane behavior and extend the study to more cell lines.

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