Detection of permeabilisation obtained by micropulses and nanopulses by means of bioimpedance of biological tissues

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***Abstract*— In this paper permeabilisation of potato tissue caused by either microsecond electric pulses or nanosecond electric pulses is compared. The intensity of permeabilisation is quantified by means of bio-impedance change. Thanks to this method, the impact of the repetition frequency was investigated. Data show that very low repetition frequencies can be much more efficient to permeabilise.**

I. Introduction

Electropermeabilisation describes the increase of cells membrane permeability after exposure to short and intense electric pulses [1]. Using for example pulses of 100 µs duration is now a standard technique routinely used in laboratories for gene transfer or drug delivery to cells *in vitro* and *in vivo* [2], [3]. It is also used in clinics for cancer treatment: the combination of electric permeabilisation and drug delivery is known as electrochemotherapy. Consensus on the standard electrical parameters is established *in vitro* and *in vivo*. For exemple, to permeabilise mammalian cells in suspension, it is commonly accepted that one should work with pulses of approximatly 100 µs and 100 kV/m using between 2 and 8 pulses (which are refered to as 'micropulses'). Electrical parameters exact impact has been extensively studied *in vitro* and *in vivo* but no exact law has been yet described that could correlate duration, magnitude, number of pulses and repetition frequency. The situation has become even more complex since it was shown that permeabilisation could be obtained with much shorter pulses of only a few nanoseconds duration although a higher amplitude is needed (several MV/m). Those pulses, referred to as 'nanopulses', are of great interest to the biological and medical communities as it appears that they do not only impact on the plasma membrane but also on the membrane of intracellular structures. Moreover, they might be interesting for permeabilisation as an alternative to micropulses in applications like decontamination. Still, there is no evidence yet that the mechanism leading to permeabilisation is the same when cells are exposed to micropulses or to nanopulses. The study that follows compares, on the same biological system, the influence of the repetition frequency for pulses of very different length and magnitude: 100 µs (microsecond pulses) and 10 ns (nanosecond pulses or nsPEF) both square-wave. Typical pulses applied on samples are shown on Fig. 1 both in the time domain and in the frequency domain. Experiments are performed on potato slices which constitute a good tissue model as it is both uniform and homogeneous [4]. Permeabilisation is then quantified by means of bioimpedance measurements [5-7].

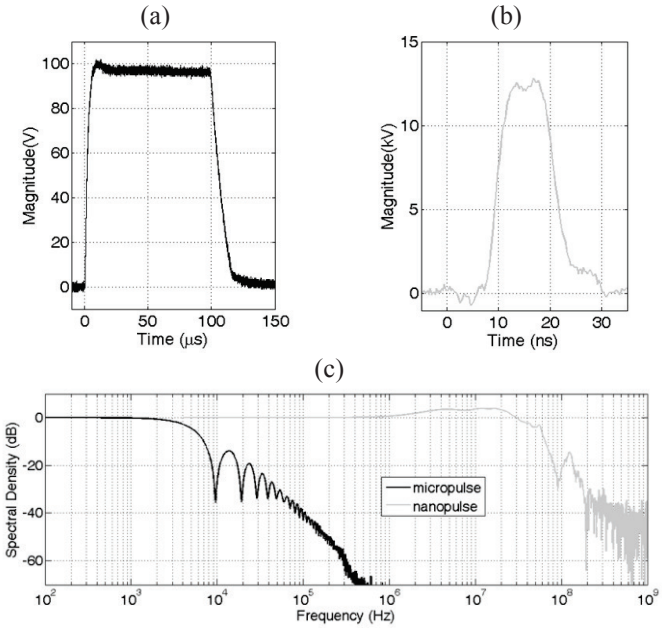

Fig. 1: Typical pulses applied on the samples. (a) the micropulse was measured using a conventional probe (b) nanopulse was measured using our home-made D-dot sensor [8] whose bandwidth was evaluated to 2 GHz (c) Spectrum of both pulses obtained by Fourier Transform

II. Material and Method

A. Microsecond Pulse generator

Square-wave microsecond pulses were delivered by an electroporation power supply (Cliniporator™, Igea, Carpi, Italy) able to apply high-voltage pulses with repetition frequencies ranging from 1 Hz to 7 kHz. To obtain pulse repetition frequencies lower than 1 Hz, single pulses were manually delivered by the operator at the appropriate rate.

B. Nanosecond Pulse Generator

The generator used to expose the potato samples is a commercial generator (FID Technology FPG 10-30MS, Russia) supplied with a DC source (Delta Electronika ES 0.300-0.45). It has four 100 Ω output that were connected by pair in series and then globally in parallel.

In order to take into account the distortion of the electric field induced by the potato sample itself, the exact field applied was systematically measured using a D-dot probe directly mounted in the electrodes [8].

C. Bioimpedance measurement system

In experiments to account for permeabilisation, low signal impedance measurements from 100 Hz to 400 kHz were performed with the Bluetooth bioimpedance measurement system provided by the Centre Nacional de Microelectrònica (CNM, Barcelona, Spain).

D. Potato samples preparation

Standard potatoes were bought from the local supermarket. Slices of 5 mm were cut and then small cylinders of various diameters were stamped in the peripheral part of the potato. Impedance measurement was performed with a four needles electrode. Immediately after, the sample was placed between two stain-less steel plate electrodes separated by a distance of 5mm. Immediately after the pulse, the potato sample was removed from the plate electrodes and the impedance was measured again with the four needles electrodes 7 s and 80 s after the last pulse.

E. Propidium Iodide staining:

Potato samples already dimensioned were dipped during 24h in a solution of PBS containing 0.1 mM propidium iodide. Samples were pulsed and then one minute after, thin slices (less than one millimeter) were manually cut with a razor blade and placed on a microscope slide. Observations were made under an inverted microscope (Zeiss, Axiovert S100) at a magnification of x10 and images were acquired with a CCD video camera (Zeiss, Axiocam HRc)[9].

III. Detection of permeabilisation with bio-impedance measurements

Permeabilisation results on an important modification of the impedance between 100 Hz and 1 kHz whereas there is no change above 100 kHz (Fig. 2). The drop of impedance at low frequencies can thus be used as a quantitative method to evaluate the permeabilisation. The formula that has been used in all experiments to compute the normalised impedance drop (NID) is given by (1).

$$NID = \frac{R_{eal}\left(Z_{100Hz}^{perm}\right) - R_{eal}\left(Z_{400kHz}^{perm}\right)}{R_{eal}\left(Z_{100Hz}\right) - R_{eal}\left(Z_{400kHz}\right)} \qquad (1)$$

According to many tests, it seemed to us as the best parameter to quantify permeabilisation (details of this choice are not given in this paper).

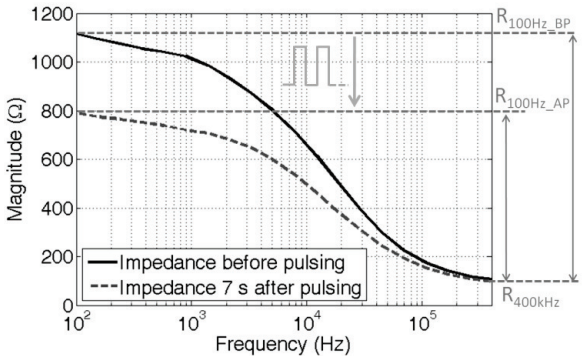

Fig. 2: Typical aspect of the impedance of a sample before and after pulsing (In this case 4 pulses of 100 µs, 300 V/cm were applied at 1 Hz).

Figure 3 shows results from an experiment using various numbers of microsecond pulses with different amplitudes. In this preliminary experiment the duration of the pulses was fixed at 100 µs and the repetition frequency at 1 Hz. It allows to verify that for a low number of pulses and a low electric field magnitude, the impedance is not much affected (the NID is around 1) whereas when the number of pulses or the field magnitude is increased the NID can reach almost zero. The tendency observed here is thus the one expected.

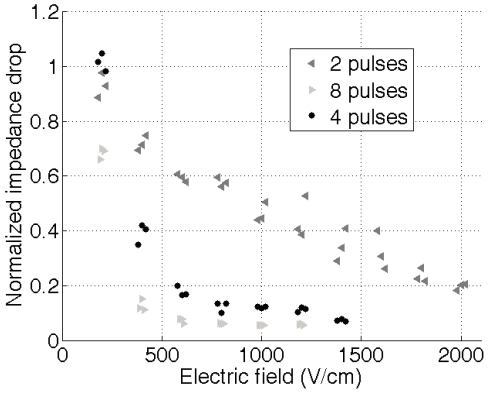

Fig. 3: Normalised impedance drop following the application of 2,4 or 8 pulses of 100 µs duration and different amplitudes applied at a 1 Hz repetition frequency. Each marker is a sample.

In order to check whether this normalization was relevant and could be correlated to standard criteria of permeabilisation, we confronted NID measurements to more traditional experiments of propidium iodide uptake. Some potato samples were submitted to a high number of nanosecond pulses (either 100 or 1000) and the permeabilisation of those samples was

checked by measuring the NID and by observation of propidium iodide staining. Fig. 4 shows that the impedance drop can indeed be correlated to the amount of propidium iodide reaching the inside of potato cells and thus to permeabilisation.

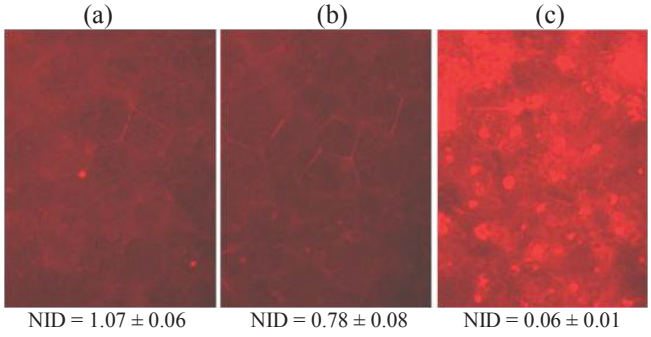

Fig. 4: Permeabilisation of potato tissue assessed by penetration of propidium iodide and by the computation of the NID. The samples received either 100 or 1000 nsPEF with a 30 kV/cm magnitude, applied at 10 Hz.

IV. Impact of the Repetition Frequency on Permeabilisation

A. Impact of repetition frequency when micropulses are applied

In order to study the impact of the repetition frequency on the permeabilisation of a potato, a first series of experiments consisted in the delivery of four pulses of 100 µs duration with an electric field magnitude of 800 V/cm (400 V were applied on the 5 mm thick samples). The maximum repetition frequency that could be reached with our generator was 7 kHz. Starting from this value the repetition frequency was then gradually reduced to 1 Hz (Fig. 5). For each condition, eight samples were treated. The impedance was measured before the pulses delivery and as much close as possible to 7 seconds after the last pulse. The computation of the impedance drop showed a very low change in impedance for repetition frequencies between 300 Hz and 7 kHz. Below 300 Hz, NID then gradually decreases with the decrease of the repetition frequency, the same pulses provoking therefore a very important change of impedance at 1 Hz.

Other samples were submitted to only one pulse keeping the duration and magnitude of the pulse at 100 µs and 800 V/cm. It appears that the NID drop obtained with four pulses at high repetition rate is surprisingly close to the one reached with only one pulse, as if the three following pulses were inefficient when applied extremely quickly after the first one (Fig. 5).

In order to investigate even lower frequencies, it seemed more appropriate to lower the magnitude of the pulse so that a further increase in permeabilisation could be measured. Four pulses of 100 µs duration and 300 V/cm were thus applied on the potato samples. Repetition frequencies between 1 Hz and 0.02 Hz were investigated. This resulted in treatments lasting between 3 s and 150 s. Because of the important difference of treatment duration, the impedance of the samples after treatment was checked both 7 s after the last pulse and 3 minutes after the first one in order to eliminate possible artifacts due to the samples drying or to an evolution of the bioimpedance due to other metabolic changes occurring after the delivery of the first pulse.

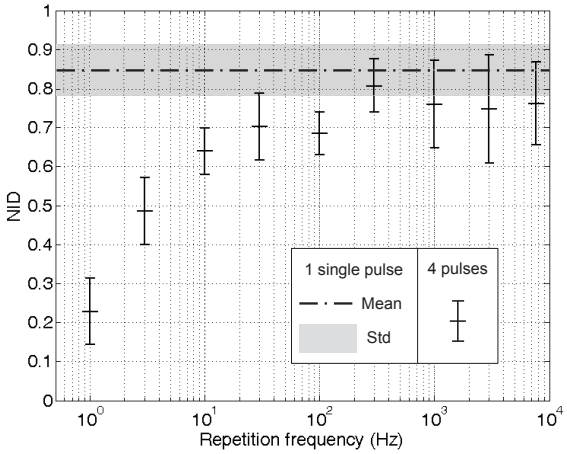

Fig. 5: Pulse repetition frequency impact. The grey zone represents the effect of one single microsecond pulse of 100 µs and 800 V/cm. The crosses represent the mean and standard deviation obtained when applying four identical microsecond pulses at different repetition rates.

No significant difference was observed between the two measurements (data not shown). It appears that repetition frequencies below 1 Hz are even more efficient in permeabilising the potato tissue (Fig. 6). For 0.1 Hz and below, saturation appears. Frequencies below 0.02 Hz were not tested in order to limit the duration of the treatment.

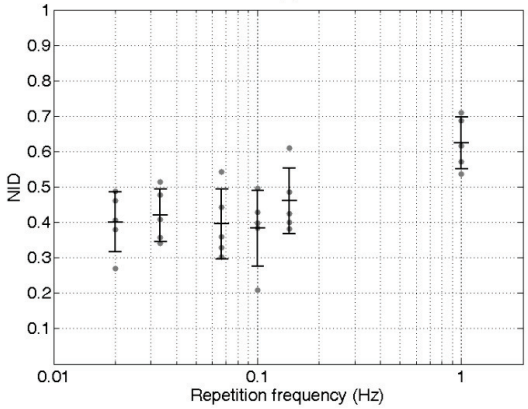

Fig. 6: NID at very low pulse repetition frequencies. Samples were exposed to four pulses of 100 µs and 300 V/cm. Each individual sample is represented with a gray dot. The bars indicate mean value ± standard deviation.

B. Impact of repetition frequency when nanopulses are applied

The impact of the repetition frequency was studied with a magnitude fixed at 40 kV/cm. The number of pulses had however to be adjusted depending of the repetition frequency explored, in order to have reasonable durations of the experiments. First, 300 nsPEF were applied and the repetition frequency was varied between 2 Hz and 300 Hz (frequencies

above 300 Hz could not be delivered because of technological restrictions of the nsPEF generator). In this range, a huge variation of the NID with the variation of the repetition frequency was already observed (Fig. 7). In order to explore lower repetition frequencies, between 0.1 Hz and 10 Hz, a lower number of pulses (50 nsPEF) was applied. Lower repetition frequencies again impacted more bioimpedance changes and saturation of this effect could not be reached as the testing of lower frequencies would have implied too long experiments. It was nevertheless decided to challenge an extremely low frequency, 0.01 Hz. This implied that one pulses was applied every 100 s. The number of pulse was thus limited to five. In this experiment, other samples were submitted to five pulses applied at 100 Hz. In both cases the post-treatment impedance was measured 100 s after the last pulse. A remarkable significant change in bioimpedance was actually detected at 0.01 Hz (data not shown), which allows to conclude that even a very low number of nsPEF can produce large biological effects if they are applied at a sufficiently low repetition rate.

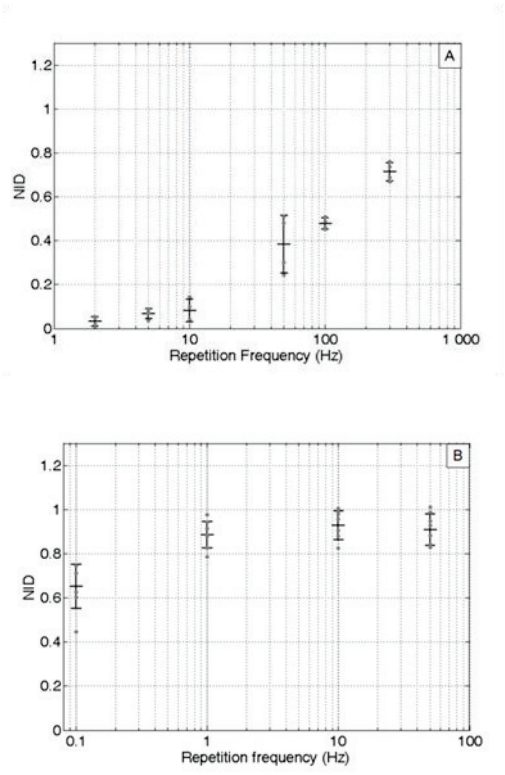

Fig. 7: Pulse repetition frequency impact during the exposure to 10 ns nanopulses of 40 kV/cm. (A) 300 pulses applied (B) 50 pulses applied

V. Discussion

In this article, the correlation between NID and permeabilisation has been experimentally validated by comparing impedance measurement to penetration of propidium iodide in potato tissue. Moreover, electrical modelling of permeabilisation also predicts a drop of impedance at low frequencies when a tissue is permeabilised. We thus believe that permeabilisation intensity in potato can be directly related to the value of the NID.

In view of this result, it was possible to study the repetition frequency impact on permeabilisation. The permeabilisation induced on potato tissue both by microsecond pulses and nanosecond pulses appears to be much more effective at very low frequencies. Some previous work both *in vitro* and *in vivo* had shown similar tendencies although repetition frequencies below 1 Hz were rarely challenged. Moreover this study is to our knowledge the first attempt to compare microsecond pulses and nanopulse impact on the same system even though the comparison made here is limited to the repetition frequency impact. Relaxation and repair of damages induced could be responsible for a better efficiency of very low repetition frequencies. The similarities that have been observed in the time constant involved suggest that the type of damage is similar when induced by both type of pulses. In the near future, the same comparison will be extended to other tissues.

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