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Comparison of the effects of the repetition rate between microsecond and nanosecond pulses: Electropermeabilization-induced electro-desensitization?

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ABSTRACT

Background: Applications of cell electropermeabilization are rapidly growing but basic concepts are still unclear. In particular, the impact of electric pulse repetition rate in the efficiency of permeabilization has not yet been understood.
Methods: The impact of electric pulse repetition rate in the efficiency of permeabilization was analyzed in experiments performed on potato tissue and partially transposed on mice liver. On potato tissue, pulses with durations of 100 μs or 10 ns are applied. The intensity of permeabilization was quantified by means of bioimpedance changes and electric current measurements and a new index was defined.
Results: For the two pulse durations tested, very low repetition rates (below 0.1 Hz) are much more efficient to achieve cell permeabilization in potato tissue. In mice liver, using 100 μs pulses, the influence of the repetition rate is more complex. Indeed, repetition rates of 1 Hz and 10 Hz are more efficient than 100 Hz or 1 kHz, but not the repetition rate of 0.1 Hz for which there is an impact of the living mice organism response.
Conclusions: We propose that the effects reported here might be caused by an electroporation-induced cell membrane 'electro-desensitization' which requires seconds to dissipate due to membrane resealing.
General significance: This study not only reinforces previous observations, but moreover it sustains a new concept of 'electro-desensitization' which is the first unifying mechanism enabling to explain all the results obtained until now both *in vitro* and *in vivo*, with long and short pulses.

1. Introduction

Electropermeabilization, or electroporation, names the phenomenon by which cell membrane permeability increases when the cell is exposed to short and intense electric pulses [1]. Depending on the field intensity, the duration of the pulses or the number of pulses applied, the permeabilization can be either transient and reversible (which is useful for drug delivery or gene transfection [2–5]) or irreversible, leading to the death of the cell (which is used among other applications for tissue ablation [6,7] or bacterial inactivation [8–10]). Some standard values of the electric pulses (*e.g.*: 100 μs, 1 kV/cm for reversible electroporation of mammalian cells in suspension) are often used since they were proven to be very efficient. However, many combinations can result in the same final state of the cell even though it is difficult to give an exact rule regarding the relationship between the pulse parameters and the effects on the cells. It has appeared in many reports that a series of short pulses led to higher permeabilization than one single long pulse even if this pulse lasts as long as the cumulated duration of the series of shorter pulses [1]. For example, in almost all the electrochemotherapy treatments, as reported in the Standard Operating Protocols of Electrochemotherapy, eight pulses are applied instead of one [11]. Naturally, this observation has led to study the impact of the repetition rate used when a given number of pulses are delivered. Different studies *in vitro* [1,12–15], *in vivo* [7,16] and in plants [17,18] have indeed shown an impact of the repetition rate. Generally it appears that low rates are more efficient than high ones. However, there are up to now very few clues on whether an optimal rate does exist and on the mechanisms that could explain the impact of the repetition rate.

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More recently, different groups have shown that cell membrane permeabilization can be obtained by the application of pulses of only a few nanoseconds duration. The question of the impact of the repetition rate for such pulses has already been addressed in an *in vitro* study [13]. Authors have shown that for pulse duration ranging from 300 ns to 9 μs, the survival rate of cells exposed in suspension decreases when the repetition rate decreases. The authors proposed a concept of electrosensitation. Other *in vitro* studies have also pointed to an impact of the repetition rate during exposure to nanosecond pulses. They indicate either a higher efficiency of high repetition rate [19] or the existence of an optimal rate [20].

The objective of this study was to test the impact of the repetition rate but on a completely different model. We investigated the permeabilization of tissues by measuring the changes of electrical bioimpedance. We compared, on potato tuber, the permeabilization obtained by two types of pulses with duration of either 100 μs or 10 ns. They will be referred to as microsecond pulses (or micropulses) and nanosecond pulses (or nanopulses) respectively. In addition, the repetition rate impact on permeabilization in mice liver was investigated using micropulses.

2. Materials and methods

2.1. 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 rates ranging from 1 Hz to 7 kHz. To obtain pulse repetition rates lower than 1 Hz, single pulses were manually triggered by the operator at the appropriate rate.

2.2. Nanosecond pulse generator

The generator used to expose the potato samples to nanosecond pulses was 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 outputs that were connected by pairs in series and then globally in parallel.

In order to take into account the distortion of the electric field induced by wave reflection on the potato sample itself, the exact field applied was systematically measured using a D-dot probe directly mounted in the electrodes as described in [21].

2.3. Bioimpedance measurement system

In experiments designed to account for permeabilization, low signal impedance measurements from 100 Hz to 400 kHz were performed with the Bluetooth bioimpedance measurement system custom developed by the Centre Nacional de Microelectrònica (CNM, Barcelona, Spain) [22].

2.4. Potato sample preparation and treatment

Standard potatoes were bought from the local supermarket. Slices of 5 mm homogeneous thickness were first prepared and then small cylinders of 5 mm diameter were punched out from the peripheral part of the potato slice. Impedance measurements were performed with a four needle electrode set-up. The four needle electrodes (diameter 0.3 mm, length 4 mm) are arranged in a row with a 1 mm distance between each other. After the first impedance measurement the sample was placed between two stainless steel plate electrodes separated by a distance of 5 mm that were used to deliver the pulses. Immediately after the last pulse, the potato sample was removed from the plate electrodes and the impedance was measured again with the four needle electrodes at 7 s and 80 s after the last pulse.

2.5. Propidium iodide staining

Potato samples (previously cut at the right dimensions) were dipped for 24 h in a solution of PBS (Life Technologies, ref 14200-083) containing 0.1 mM propidium iodide. Samples were pulsed and, 1 min later, thin slices (thinner than 1 mm) 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 ×10 and images were acquired with a CCD video camera (Zeiss, Axiocam HRc).

2.6. Surgical process for liver treatment

Very young nude Swiss mice were obtained from local production in the animal facility of Gustave Roussy. All the used animals were handled, and then sacrificed in the animal housing facility of Gustave Roussy according to the Experimental Animal Ethics Committees Guidelines and received the agreement of the CEEA26 committee. Mice of age between 8 and 10 days were used for these experiments as the size of their liver allowed to conveniently place it completely between the two plate electrodes separated by a distance of 2 mm. Mice were first anesthetized with a dose of 10 μl per g body weight of a mixture composed of xylazine 12.5 mg/kg (Bayer Pharma, Puteaux, France) and ketamine 125 mg/kg (Parke Davis, Courbevoie, France). The anesthetizing mixture was injected intraperitoneally. An incision was performed in the upper median abdominal (or epigastric) region of the anesthetized animal, and then the liver was pulled out gently and positioned in between the plate electrodes. The setup was performed in such a manner that movements were not possible during the procedure for the liver impedance measurement. A first electrical impedance measurement between the two stainless steel plate electrodes was performed and then, after disconnecting the impedance meter, the pulses were applied using the same electrodes. Approximately 5 s after the last pulse, electrodes were manually connected back to the impedance meter and the impedance was measured again. Voltage and current during pulses were systematically recorded.

3. Detection of permeabilization of tissue by electrical measurements: models and correlations established before data collection

3.1. Impedance of a biological tissue (bioimpedance)

Electrical passive properties of biological tissues have been described with very good precision up to several megahertz [23]. A simple electrical model of a biological tissue, adapted from [24,25], is presented in Fig. 1. It consists in a resistance (R_{ext} [ohm, Ω]) in parallel with a capacitance (C_w [farad, F]) and with a series association of a capacitance (C_m [farad, F]) and a resistance (R_{int} [ohm, Ω]). The resistances R_{ext} and R_{int} are directly linked to the conductive properties of the extracellular and intracellular media respectively which are both ionic solutions. The capacitance C_m represents the dielectric properties of the membranes of cells. In order to have a good representation of the impedance of biological sample, it

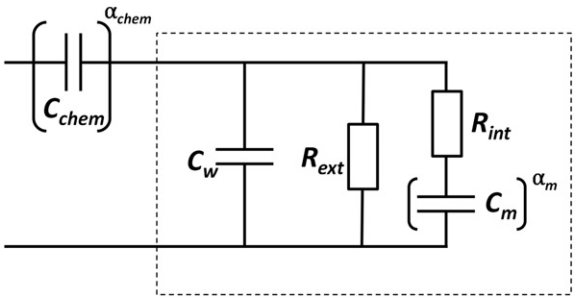

Fig. 1. An electrical model to interpret tissue bioimpedance measurements. The part of the model delimited by dotted lines describes the passive properties of the biological tissue. The additional capacitor C_{chem} is the electrochemical capacitance of the electrodes.

is necessary to modulate the capacitance C_m with a dimensionless coefficient α_m, with a value between 0 and 1 (this parameter is called CPE for Constant Phase Element; it was first introduced by Cole [26] and was further explained in [27]). Finally, the capacitance C_w represents the dielectric properties of intracellular and extracellular media (mainly dielectric properties of water).

With such a model, the inverse of the impedance (Z_{bio} [ohm, Ω]) of the biological tissue is given by Eq. (1) where j is the complex number $(-1)^{1/2}$.

$$\frac{1}{Z_{bio}} = jC_w\omega + \frac{1}{R_{ext}} + \frac{1}{R_{int} + \frac{1}{C_m(j\omega)^{\alpha_m}}} \quad (1)$$

The raw measurements additionally present a capacitive behavior at low frequencies which is due to the ionic double layer facing the electrodes at low frequencies [28,29]. This so-called electrochemical capacitance can be included in the electrical model in the form of a capacitor (C_{chem} [farad, F]) also modulated by a CPE, α_{chem}. The total impedance measurement (Z [ohm, Ω]) can thus be fitted by Eq. (2):

$$Z = Z_{bio} + \frac{1}{C_{chem}(j\omega)^{\alpha_{chem}}}. \quad (2)$$

A bioimpedance measurement of a potato sample, such as those we treated by the micropulses and the nanopulses, as well as its associated electrical model, is represented in the Bode plot (magnitude *versus* frequency) on Fig. 2A and in the Wessel plot (imaginary part of the impedance *versus* real part) on Fig. 2B. The fitting was performed using Matlab. The values of the different components of the model were determined manually and adjusted in order to optimize the fitting (the values obtained from the fitting are given in the legend). The graphical representation of the impedance allows us to bring out R_0 [ohm, Ω] and R_{inf} [ohm, Ω], the resistances at low and high frequencies respectively. On an unaltered tissue, R_0 and R_{inf} can be expressed by (3). Those values will be used to quantify the permeabilization level.

$$R_0 = R_{ext} \text{ and } R_{inf} = \frac{R_{ext}\,R_{int}}{R_{ext} + R_{int}} \quad (3)$$

3.2. Correlation between electrical bioimpedance and electric current measurements during exposition

As mentioned before, the two types of electric pulses used to treat the potatoes and the mice livers had durations of either 100 μs (micropulse) or 10 ns (nanopulse). Their normalized spectra are represented in Fig. 3. Although both spectra have similar shapes, the decrease following the initial plateau begins at a frequency directly dependent on the pulse duration. When looking at the impedance of a potato sample (Fig. 2A), it stands out that the two types of pulses excite the sample on two different frequency bands.

The two theoretical currents that would be obtained when applying a micropulse or nanopulse of 1 V magnitude on the sample presented in Fig. 2 have been calculated. This calculation has been done assuming that the impedance would not change during the exposure to the pulses. The current level reached during the micropulse is approximately 0.6 mA (Fig. 4A) which reveals that the current level is imposed by R_0 (which is around 1.7 kΩ in the sample that is considered). On the other hand, the theoretical current level obtained when considering a nanopulse is around 18 mA (Fig. 4B), as it is imposed by R_{inf} which is approximately 55 Ω in the considered example. Current level during microsecond pulse excitation can thus be considered as an image of the instantaneous R_0 and can thus be used to quantify the permeabilization level (see Section 4.1.2). On the other hand, current level during nanosecond pulses reflects an instantaneous value of R_{inf} which leads to less straightforward interpretation [30]. Measurements of the current values during nanosecond pulse exposure will thus not be exploited in this paper.

3.3. Quantification of permeabilization using the drop of bioimpedance

3.3.1. Normalized impedance drop

A change of membrane conductivity was among the first techniques used to describe the electroporation phenomena [31]. Since then, measurement of passive electrical properties of cells or tissues has proven to be an efficient method to study electroporation [32–39]. In short, permeabilization is an alteration of the membranes and can thus be seen from an electrical point of view as a partial or complete short-circuiting of the capacitor C_m. In consequence, permeabilization

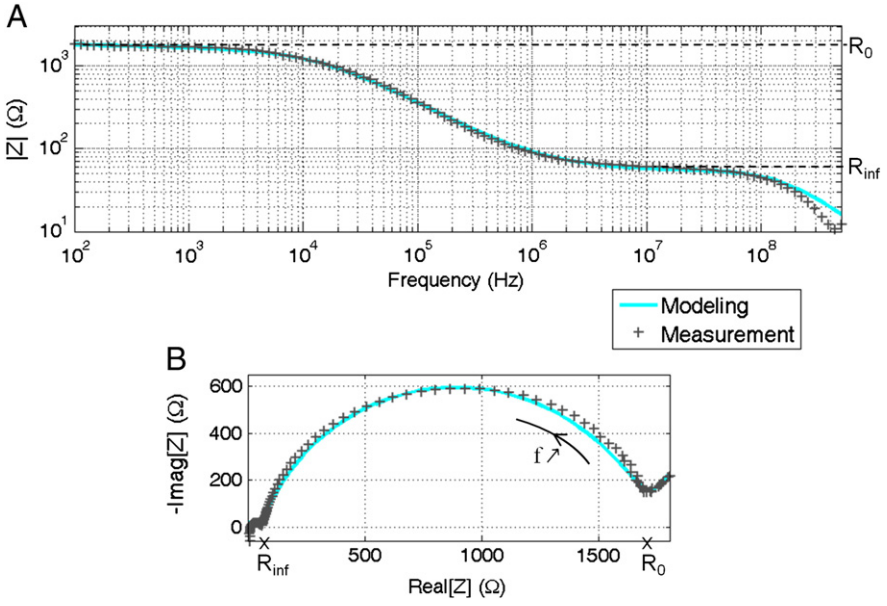

Fig. 2. Example of electrical impedance of a potato sample. Dots indicate experimental measurements and the solid blue line the Cole–Cole model. Measurements were performed with a commercial impedance analyzer (Agilent/HP 4194) from 100 Hz to 40 MHz and with a network analyzer (Agilent E5070B) from 300 kHz to 1 GHz. A — Bode representation of the magnitude of the impedance. B — Wessel representation of the complex impedance. The arrow indicates increasing frequencies. The parameters of the model are the following, R_{int} = 57 Ω, R_{ext} = 1705 Ω, C_m = 74 nF, α_m = 0.79, C_w = 3 pF, C_{chem} = 50 μF, and α_{chem} = 0.7.

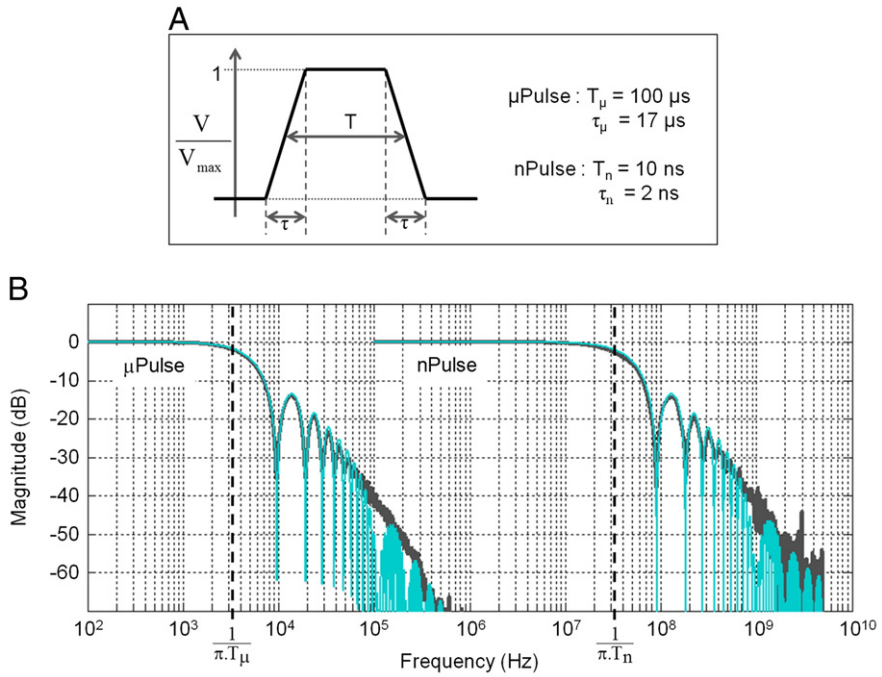

Fig. 3. (A) Time domain features of normalized applied pulses. (B) Associated spectral representation of the pulses. The gray lines correspond to measured pulses and the blue lines to the best fit by a trapezoidal pulse.

increases the conductivity of tissues at low frequencies. Thus, after permeabilization, the resistance of the tissue at low frequencies (R_0^{perm}) should be between the initial values of R_0 and R_{inf}: the more efficient the permeabilization, the more important the drop of the resistance at low frequencies. To quantify the level of permeabilization we defined the normalized impedance drop (NID) (4) and used it to interpret our experimental data. Admitting no change in the impedance other than the drop due to permeabilization, the value of the NID should be between 0 and 1 (1 meaning no permeabilization at all and 0 being the limit reached when going towards a "complete" permeabilizsation).

$$NID = \frac{R_0^{perm} - R_{inf}^{perm}}{R_0 - R_{inf}} \tag{4}$$

The superscript 'perm' refers to the values of R_0 and R_{inf} after the pulses have been applied.

For the sake of simplicity, bioimpedance measurements were not systematically fitted to extract R_0 and R_{inf}. Indeed it turns out that on potato samples R_0 and R_{inf} are approximately equal to the real part of the impedance at 100 Hz ($Z_{100\ Hz}$) and 400 kHz ($Z_{400\ kHz}$) respectively. The normalized impedance drop was thus computed using Eq. (5).

$$NID = \frac{Real(Z_{100Hz}^{perm}) - Real(Z_{400kHz}^{perm})}{Real(Z_{100Hz}) - Real(Z_{400kHz})} \tag{5}$$

Fig. 5 gives a typical example of a change of impedance due to partial permeabilization (NID = 0.77).

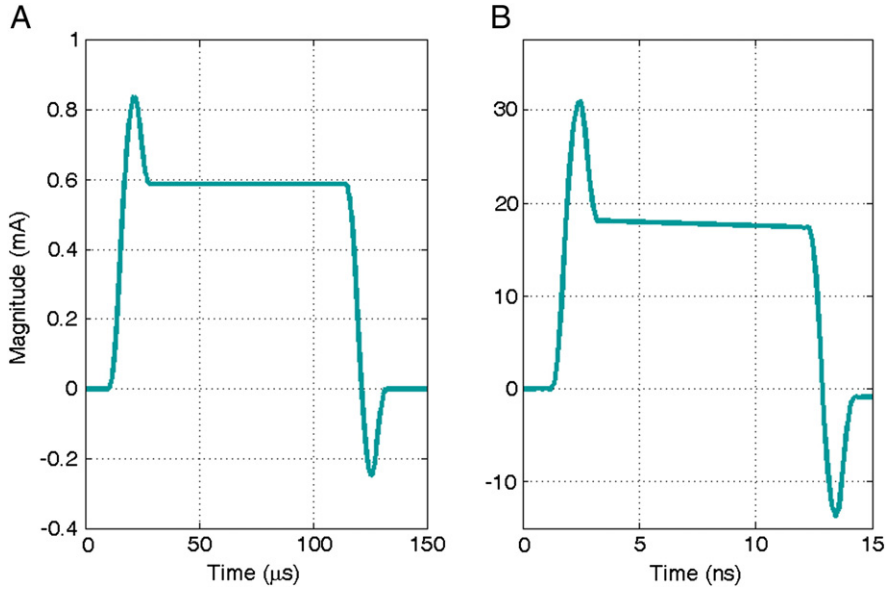

Fig. 4. Modeled current when applying either a micropulse (A) or a nanopulse (B) as defined in Fig. 3, with a 1 V magnitude on the impedance represented in Fig. 2.

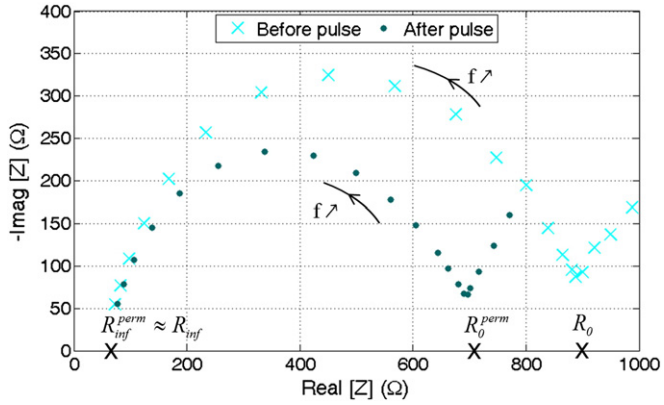

Fig. 5. Impedance of a potato sample shown before and after the exposition to one single microsecond pulse of 100 μs duration and with a magnitude of 120 V/cm. The arrows indicate increasing frequencies.

3.3.2. NID evolution with the number and the magnitude of micropulses

In order to check whether the proposed normalization is relevant, potato samples were submitted to various numbers of micropulses with different magnitudes (Fig. 6). Duration of the pulses was fixed at 100 μs and repetition rate at 1 Hz. This experiment allowed 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). However, when the number of pulses or the field magnitude is increased, the NID can reach almost zero. NID tendencies with field magnitude or number of pulses are thus the expected ones since NID decreases when pulsing parameters become harsher [40].

3.3.3. Correlation between NID and propidium iodide staining penetration

In order to validate that the NID quantifies permeabilization, it was compared to a traditional method of permeabilization detection: the propidium iodide (PI) staining of the cells inside, in particular of the cell nucleus [41,42]. Potato samples were submitted to a high number of nanopulses (either 100 or 1000) (see also Section 4). The comparison between PI staining and NID measurements is displayed in Fig. 7. It appears that with 1000 nanopulses of 30 kV/cm magnitude, the high impedance drop can be correlated to a large amount of PI reaching the inside of potato cells and staining the nuclei. Moreover, when 100 nanopulses were applied, no staining was observed whereas the impedance already started to drop. Impedance drop is thus a possible way of quantifying permeabilization even at low levels, before permeabilization can be detected by a more common technique like PI staining.

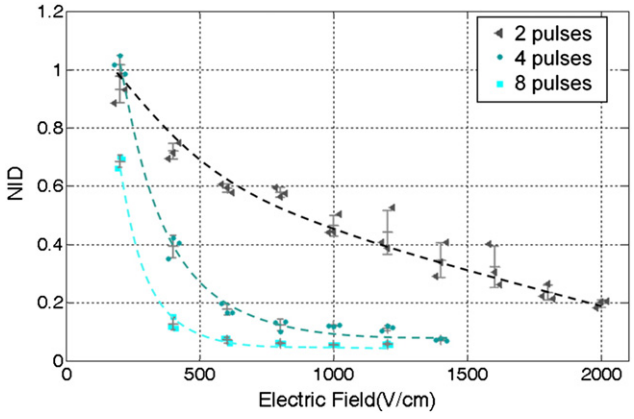

Fig. 6. Normalized impedance drop following the application of 2, 4 or 8 micropulses (100 μs) with different magnitudes applied at a 1 Hz repetition rate. Each marker is a sample; error bars represent the mean ± standard deviation (number of sample n = 3).

4. Impact of the repetition rate: experimental results

4.1. Impact of the repetition rate on permeabilization of potato tissue induced by micropulses

4.1.1. Assessment by bioimpedance measurement

In order to study the impact of the repetition rate on the permeabilization of the potato, samples were submitted to four micropulses with an electric field magnitude of 800 V/cm. The maximum repetition rate that could be reached with the generator was 7 kHz. Starting from this value the repetition rate was then gradually reduced to 1 Hz (Fig. 8). For each condition, eight samples were treated. The impedance was measured before the pulses delivery and approximately 7 s after the last pulse. The computation of the impedance drop shows a very low change in impedance for repetition rates between 300 Hz and 7 kHz. Below 300 Hz, NID then gradually decreases with the decrease of the repetition rate, the same pulses provoking therefore a very important change of impedance at 1 Hz. Other samples were submitted to only one micropulse keeping the magnitude at 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.

In order to investigate even lower rates, it seemed appropriate to lower the magnitude of the pulse so that a further increase in permeabilization could be measured. Four micropulses of 300 V/cm were thus applied to the potato samples. Repetition rates 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 (Fig. 9A) and 3 min after the first one (Fig. 9B) 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. It appears that repetition rates below 1 Hz are even more efficient in permeabilizing the potato tissue. At 0.1 Hz and below, saturation appears. Results are similar whether measurements were made 7 s after the last pulse or 3 min after the first one. Rates below 0.02 Hz were not tested in order to limit the duration of the treatment.

4.1.2. Correlation with current measurements during pulse application

As shown in Section 3.2, the current level during micropulse is representative of the instantaneous value of R_0 (which is the value neither before the pulse nor just after). During experiments with micropulses, the current value delivered through the sample was recorded for the four delivered pulses. For all samples, the current value at the end of each pulse was divided by the current value at the end of the first pulse, and the relative current increase in pulses 2, 3 and 4 was then analyzed. The pulse parameters used were the same as in previous experiments where bioimpedance changes were followed. At 800 V/cm, five different repetition rates were tested (Fig. 10A). At 10 Hz, 100 Hz and 1 kHz, the current increase from one pulse to the next one was identical. This increase became more important at 1 Hz and even more at 0.1 Hz (Fig. 10A). The same experiment performed using lower rates and a field magnitude of only 300 V/cm showed saturation in the conductivity increase for rates below 0.1 Hz (Fig. 10B) which is consistent with the observations in bioimpedance changes.

It should be noted that the instantaneous R_0 computed from the voltage over current ratio is very close to R_{inf} (data not shown) even in conditions in which R_0 goes back close to its original value in less than a few seconds after the pulse. Such observation was already previously described in the literature [34]. The fact that R_0 can recover so quickly is consistent with the fact that some recovery of the membrane is happening extremely rapidly after the pulse with a time constant smaller than 1 s [33,34].

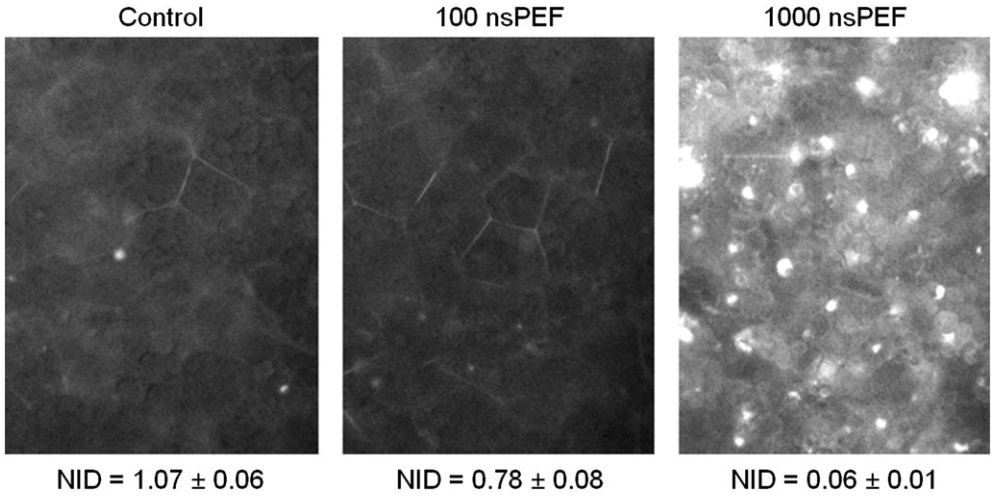

Fig. 7. Permeabilization of potato tissue assessed by penetration of propidium iodide and by the computation of the NID. The samples were submitted to either 100 or 1000 nsPEF with a 30 kV/cm magnitude, applied at 10 Hz.

4.2. Impact of the repetition rate on permeabilization of potato tissue induced by nanopulses

Permeabilization on potato tissue was then tested using the 10 ns duration electric pulses. During the first experiments, the repetition rate was fixed at 100 Hz and the impact of pulse magnitude and number of pulse were tested. Because of reflection phenomena, the exact voltage depends on the initial global impedance of the sample. Thanks to the regularity in the calibration and to the good homogeneity in the electrical properties of all samples, the global impedance was not too different from one sample to another. The fluctuation of the voltage applied was thus of less than 10% between all the samples treated with a given condition.

Bioimpedance measurements were made before the pulses as well as 7 s after. Fig. 11 shows the NID obtained for three different field magnitudes and for various numbers of pulses. Even for the highest magnitude that could be achieved (40 kV/cm) an important number of pulses was necessary to achieve NID values similar to the ones obtained with micropulses. Nevertheless, like for micropulses, the drop of impedance increased with the field magnitude or with the number of pulses applied.

The impact of the repetition rate was studied with a magnitude fixed at 40 kV/cm. First, 300 nsPEF were applied and the repetition rate was varied between 2 Hz and 300 Hz (rates above 300 Hz could not be

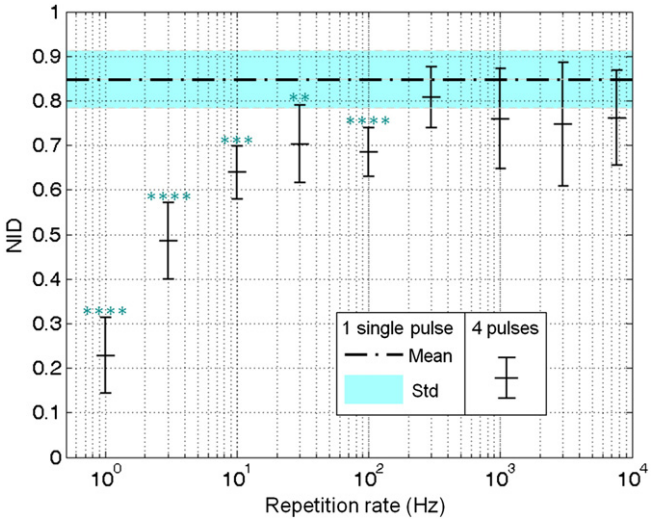

Fig. 8. Impact of the pulse repetition rate. The blue zone represents the effect of one single micropulse of 100 μs and 800 V/cm. Error bars represent the mean ± standard deviation (n = 7) obtained when applying four identical micropulses at different repetition rates. Statistical analyses compare the effects of four pulses at different repetition rates *versus* only one pulse. (****p < 0.0001; ***p = 0.0001; **p < 0.01 with unpaired *t*-test).

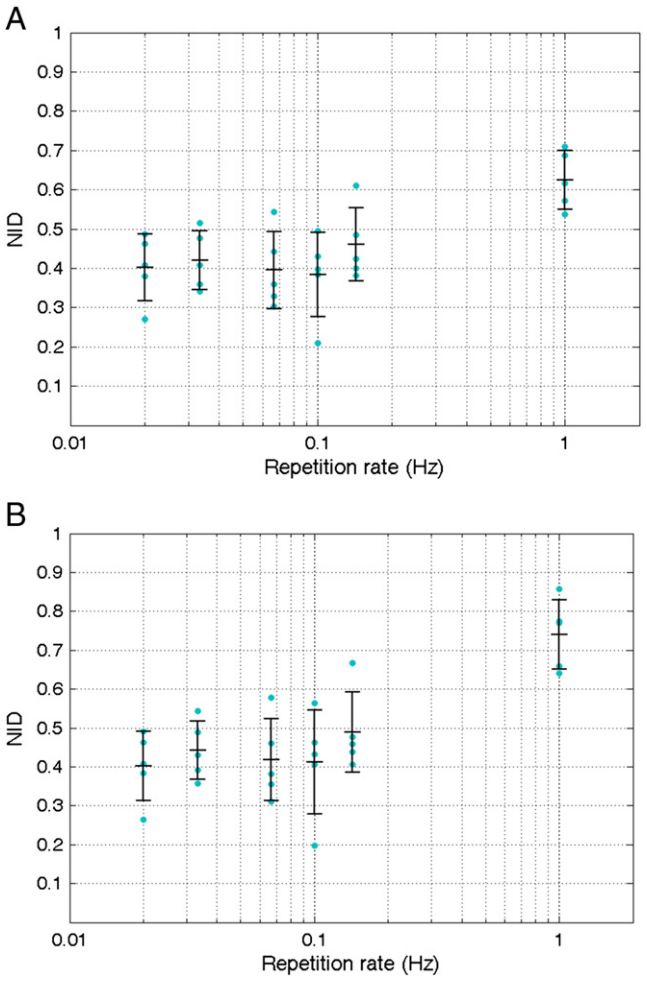

Fig. 9. NID at very low pulse repetition rates. Samples were exposed to four micropulses of 100 μs and 300 V/cm. Each individual sample is represented with a gray dot. Error bars indicate mean value ± standard deviation (n = 5). NID is computed either according to measurements: A — 7 s after the last pulse. B — 3 min after the first pulse.

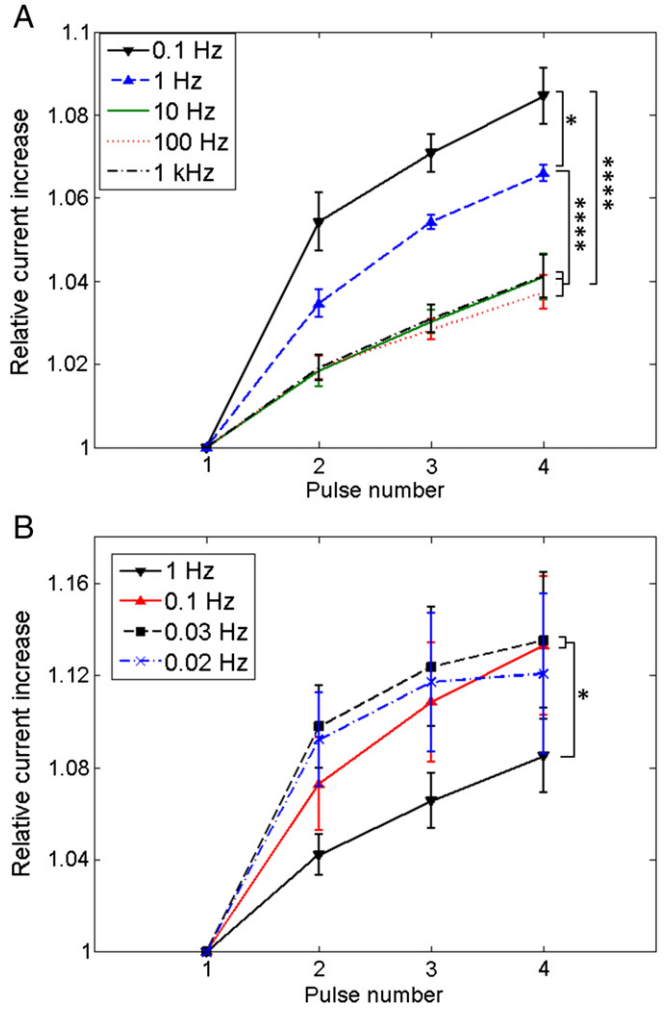

Fig. 10. Relative current increase for each pulse referenced on the first pulse. Samples were exposed to four micropulses of 100 μs of (A) 800 V/cm and (B) 300 V/cm. Error bars represent the mean ± standard deviation (n = 5). Statistical analysis: ****$p < 0.0001$; *$p < 0.05$ with unpaired *t*-test.

delivered because of technological restrictions of the nanopulse generator). In this range, NID evolved from about 0.7 at 300 Hz to almost 0 at 2 Hz (Fig. 12A). In order to explore lower repetition rates, between 0.1 Hz and 10 Hz, a lower number of pulses (only 50) was applied to limit experiment durations (Fig. 12B). The lowest repetition rates had again the largest impact on the bioimpedance change and the saturation of this effect could not be reached as the testing of still lower rates would imply too long experiments. It was nevertheless decided to challenge an extremely low repetition rate, 0.01 Hz. This implied that one pulse was applied every 100 s. The number of pulses was thus limited to five. In the same experiment, other samples were submitted to five pulses applied at 100 Hz (Fig. 12C). 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, which allows us to conclude that even a very low number of nanopulses can produce large biological effects if they are applied at a sufficiently low repetition rate.

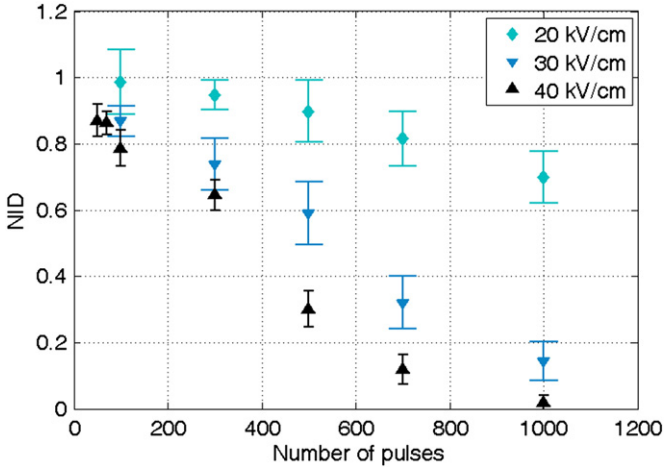

Fig. 11. NID obtained by applying nanopulses (10 ns) of about 20 kV/cm, 30 kV/cm or 40 kV/cm with a repetition rate of 100 Hz. Error bars represent mean value ± standard deviation.

4.3. Impact of the repetition rate on permeabilization of mice liver caused by micropulses

In order to test the impact of the repetition rate on a different tissue, experiments were repeated on mice liver. Liver was chosen since it is one of the most homogeneous organs and has already been proven to be an efficient model to detect permeabilization from the measurement of passive electrical properties [33]. Since the death of the animal will affect the electrical properties of all tissues very quickly, experiments were performed on mice under anesthesia [27]. Bio-impedance measurements on the liver could not be done using the previous four needle electrodes mainly because the blood leaking on the needles caused short-circuit. The measurements were thus done between the two plate electrodes used to apply the electric pulses. This way of measuring increases considerably the impact of the electrochemical capacitance C_{chem}. An example of fitting is given in Fig. 13. The model without the electrochemical capacitor is also drawn on the figure. It appears that the distortion induced by the electrochemical capacitor is important. However, the low frequency value of the impedance of the liver could be easily extracted since it corresponds exactly to the interception of the tangent to the low frequency measurements with the real axes. For the sake of simplicity, the high frequency impedance was considered as equal to the real part of the measurement at 400 kHz.

During experiments, it was noted that after the delivery of pulses, impedance was not stable over time. An example of evolution of R_0 and of the NID is given in Fig. 14. This behavior is very different from that of the potato tissue which on the contrary has very stable impedance over time after permeabilization. This impedance evolution of liver after pulses had already been reported in [33]. Authors mentioned that the evolution is very different depending on the intensity of reached permeabilization. Since the measurement device did not allow the monitoring of bioimpedance over time with a millisecond dynamic, only relative current increase was analyzed in order to determine the dependency to repetition rate. Reasons for such evolution will be discussed in Section 5.3.

Five different repetition rates were tested. Results presented in Fig. 15 are the concatenation of four independent experiments (representing a total of 120 mice). The data present some large scattering typical of *in vivo* experiments. However a general trend can be observed: the two repetition rates 1 and 10 Hz were almost always significantly more efficient than 100 Hz and 1 kHz. It must be noted also that, even though almost no difference was observed between 100 Hz and 1 kHz, in pulse 4 100 Hz appeared slightly more efficient than 1 kHz. Similarly, if the repetition rates of 1 Hz and 10 Hz appeared to be both equally efficient in permeabilizing, the net average value of the relative current increase was nevertheless always higher at 1 Hz compared to 10 Hz, but this difference was not significant according to statistical analysis. The mice liver behavior follows thus to a certain extent the same tendency as the one observed on potato tissue. On the contrary, data at 0.1 Hz display a different trend. Indeed, the relative current increase at 0.1 Hz is much lower compared to the value at 1 Hz.

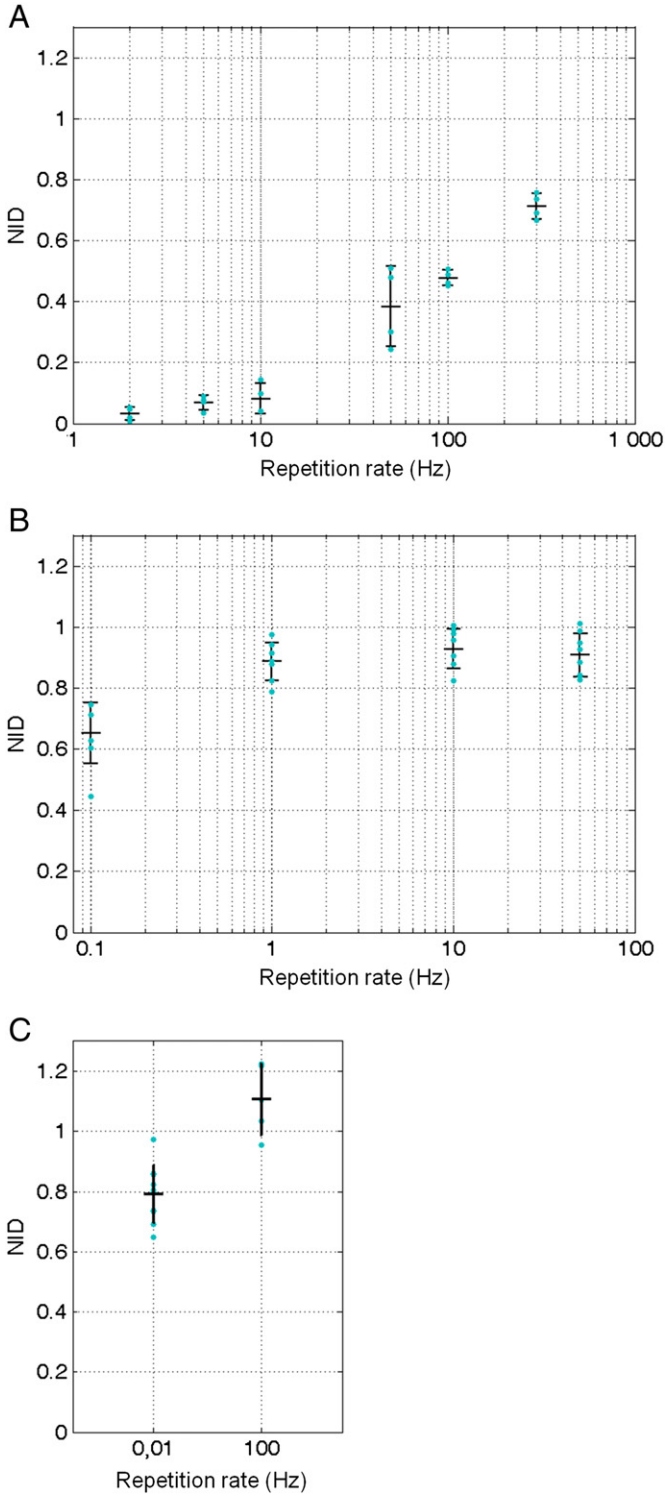

Fig. 12. Pulse repetition rate impact during the exposure to 10 ns nanopulses of 40 kV/cm. A — 300 pulses applied. B — 50 pulses applied. C — 5 pulses applied at either 0.01 Hz or 100 Hz. Dots represents each individual samples. Error bars are mean values ± standard deviation.

However at such a low repetition rate, the pulses are applied every 10 s, which gives sufficient time for tissue physiological responses to be activated. This implies that additional physiological responses (local or global) of the mice can be superimposed on the direct response of cell membrane. This might explain the deviation from the general trend and it will be further developed in Discussion section.

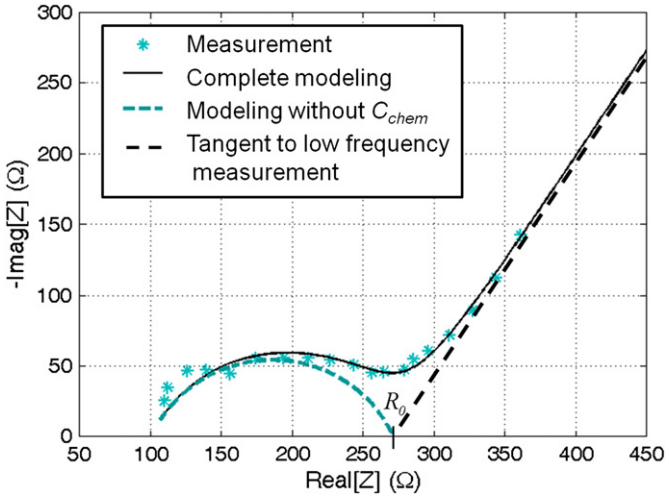

Fig. 13. Example of a bioimpedance measurement on a rat liver. The fit of the model is presented as well as the fit of the same model without the electrochemical capacitance. The parameters of the model are the following, $R_{int} = 159\ \Omega$, $R_{ext} = 270\ \Omega$, $C_m = 0.7\ \mu F$, $\alpha_m = 0.67$, $C_{chem} = 80\ \mu F$, and $\alpha_{chem} = 0.63$.

5. Discussion

5.1. Methodological aspects

Evaluating the effect of repetition rate is not a trivial task, especially because several biases may influence the results. All biases do not appear in all types of experiments and depending on the experimental approach, different artifacts must be considered.

The most obvious bias is a thermal effect. For a given pulse protocol, increasing repetition rate implies that the same amount of energy is delivered to the biological sample under test in a shorter amount of time which may lead to a higher transient temperature increase. This however is limited to studies requiring very high number of pulses and high energy per pulse. Since it is such an obvious bias, it is usually anticipated and avoided in the experiments.

The second bias introduced by a change in the repetition rate is due to the fact that the total exposure time is not constant. When experiments are conducted on cells in suspension, this has at least three major consequences that can influence the outcome of an experiment: sedimentation of cells, cell osmotic swelling between pulses and cell rotation. Sedimentation of cells is relatively slow and depends of the height of the solution and on medium density. In a standard electroporation cuvette and medium with low viscosity, time scale for this phenomenon is in the range of a few minutes. However this is not extraordinarily long for a pulse treatment and in some experiments, total treatment time ranges from 100 ms to 40 min [20]. Sedimentation of cells will most probably diminish the impact of electric pulses since it is known that if cells are too close to each other, an external electric field is less efficient than if the same cells are dispersed [43]. In that case, a low repetition rate would thus appear artificially less efficient. Cell swelling on the contrary can most probably improve the efficiency of low repetition rates. Indeed, the increase of cell diameter after a first pulse implies that a higher transmembrane voltage is induced by the next pulse which in turn makes electroporation more efficient [44]. Given the dynamics of cell swelling after electroporation [45], this should be a concern as soon as intervals between pulses are in the range of a few seconds. Finally, if total treatment duration is increased, cells might have more opportunity to rotate between pulses. In [46] the authors estimated that the rotational coefficient time of CHO cells in suspension is 200 s. Pulse protocols longer or with duration in the same order of magnitude might therefore be additionally influenced by this aspect. However, it is not easy to predict how rotation will

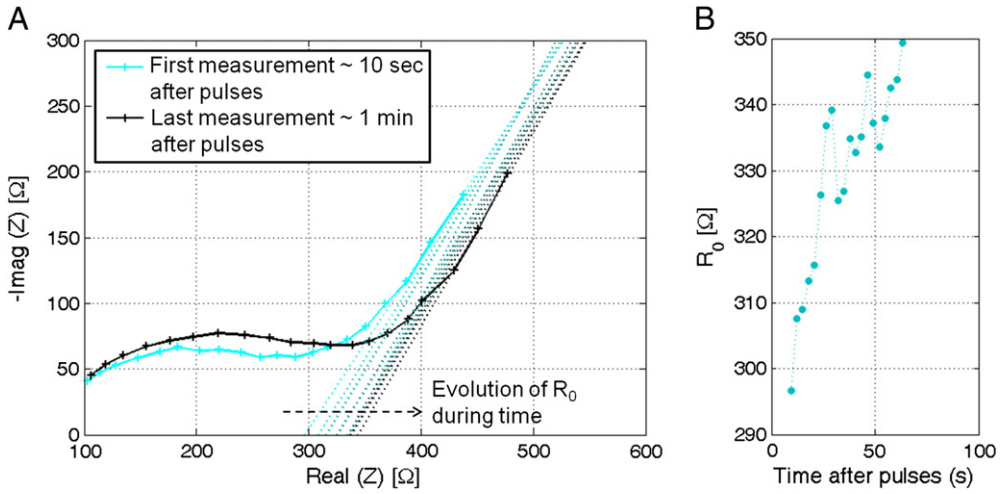

Fig. 14. Example of the evolution of R_0 in time after application of four micropulses of 100 μs of 1000 V/cm.

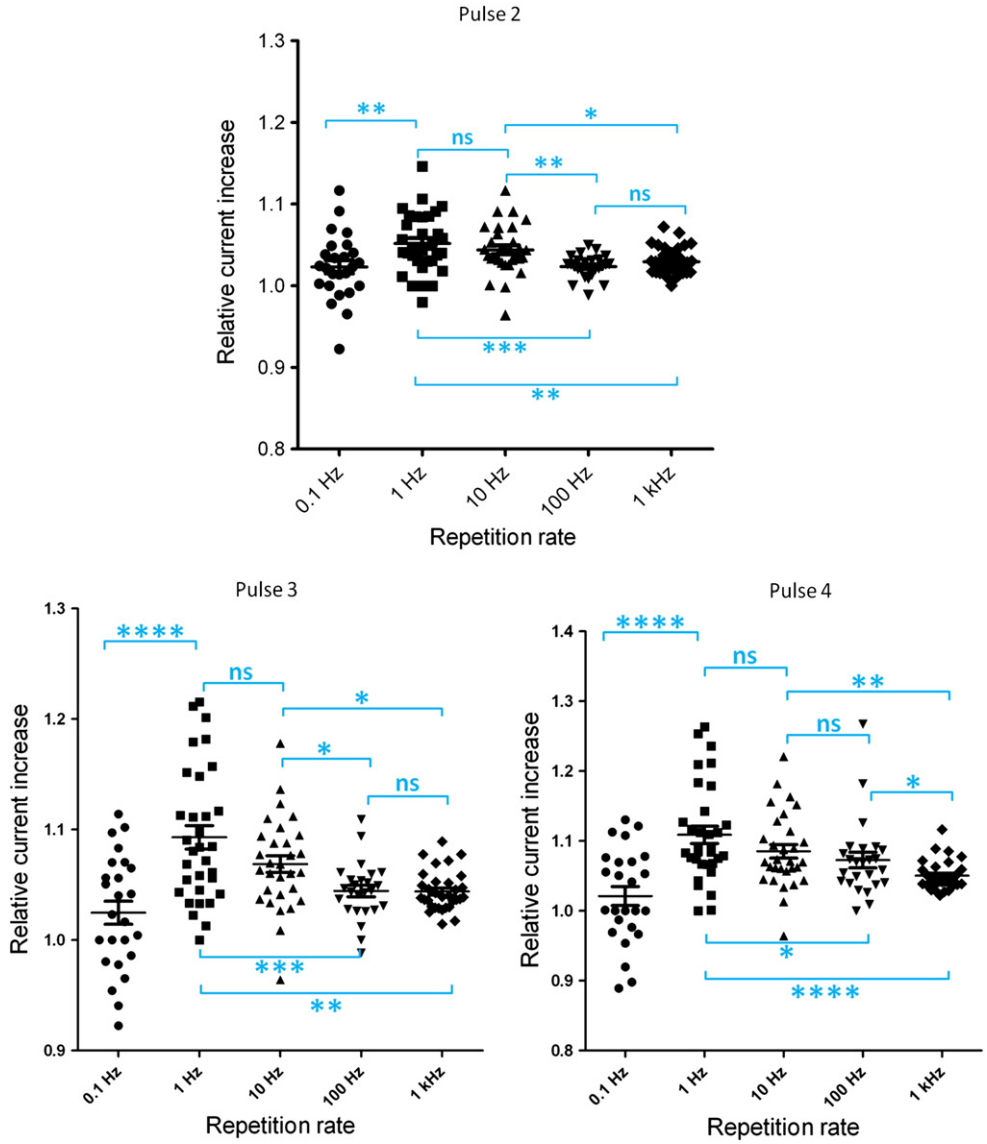

Fig. 15. Relative current increase during exposure to four micropulses of 100 μs of 1000 V/cm. Each graph represents the current increase normalized to the first pulse. Mean values ± standard errors are represented as well as individual samples. Statistical analyses are performed with unpaired *t*-test (****p < 0.0001; ***p < 0.001; **p < 0.01; *p < 0.05; ns non-significant statistical difference).

influence the treatment. In order to keep the time constant between the first pulse and the last pulse as well as the exposure cumulated duration, it has been suggested to adjust the number of pulses and their duration [1]. This approach however is also delicate since parameters other than repetition rate are modified. In particular, if electroporation efficacy is evaluated by the electrotransfer of a reporter plasmid, pulse number and duration directly impact on electrotransfer efficacy irrespectively of the electroporation level [4,47].

Increase of total treatment duration can also have a major influence during *in vivo* experiments. One of the well documented biases is related to the fact that electric pulses induce not only electropermeabilization of the exposed cells but also some physiological responses like a vascular lock [48,49]. Very low repetition rates, which imply longer durations of the pulse protocol, are likely to be more influenced by such overall physiological response, as in our experiments reported here in mice livers.

Other biases might come from the fact that the techniques used to monitor cell permeabilization are indirect. In most cases, only the consequences of permeabilization are observed. This can be for example cell death which can be due to a combination of effects: not only permeabilization but also cell swelling or toxicity of compounds that reach the cell intracellular space (as it was suggested in [13]). In many studies, permeabilization is assessed by the uptake of some normally impermeable markers (bleomycin, Lucifer Yellow, Propidium, Tallium, Yo-Pro, Calcein *etc.*) occurring only across the electropermeabilized membranes. The choice of the technique to evaluate cell membrane permeabilization is not without consequences since the outcome can be quite different from method to method. As a matter of fact, Faurie et al. [12] described an improvement of electroporation al low repetition rate in experiments dealing with the uptake of a permeabilization marker and the opposite tendency in experiments measuring the cell survival. It is interesting to note that Vernhes and colleagues [15] also assessed cell permeabilization by two techniques (PI uptake or cell viability) yielding slightly different results. Indeed, in their experimental conditions, two repetition rates (0.5 Hz and 50 Hz) induced similar viability rate (80% in both cases) but permeabilization rates are significantly different: 50% and 80%.

Additionally, in case an impermeable molecule is chosen as a marker, the overall result is due both to the level of permeabilization and to the efficiency of the transport of this molecule. Transport is known to happen through diffusion and through electrophoresis in case the markers are charged molecules. The latter might become predominant in sub-optimal conditions where diffusion hardly can happen. Moreover, it is known that the permeabilization markers first interact with the membrane of permeabilized cells before they reach the inside of the cell. This has been extensively shown for PI and was even used to assess the fraction of the membrane permeabilized by millisecond pulses [50,51]. Similar interaction of siRNA with a permeabilized membrane was also recently shown after delivery of a 10 ns pulse and transport across the membrane was found electrophoretically driven [52]. If after the first pulse, charged markers are still barely interacting with the membrane, applying additional pulses very rapidly will favor their electrophoretic translocation inside the cell. Such configuration would then favor high repetition rates. This aspect might also explain the results from Vernier et al., obtained with 4 ns pulses in conditions where only very low permeabilization was detected using the calcium ions as permeabilization marker (therefore a very small product highly charged since it contains 2 net charges per 40 Da) [19].

We have chosen in this study to study the influence of repetition rate by monitoring the changes in electrical properties of potato and liver tissues. We believe that this approach facilitates the data interpretation as far as the previously mentioned biases are concerned. Moreover, changes of conductivity in cells and biological tissues are commonly used to detect permeabilization and have already been proven to be efficient [32–37]. In this article we use a similar approach and define a parameter, the NID, to quantify the change of conductivity. The qualitative correlation between NID and permeabilization has been experimentally validated here by comparing impedance measurements to penetration of propidium iodide in potato tissue. We thus believe that permeabilization intensity in potato can be directly related to the value of the NID. The NID criterion was then used to study the impact of the repetition rate on permeabilization of potato tissue. Moreover, the correlation between impedance drop and relative current increase from pulse to pulse allowed transposing this study *in vivo* on mice liver.

5.2. Impact of the repetition rate on cell permeabilization by microsecond pulses

In the case of potato, data show that for pulses of 100 μs, very low repetition rates are more efficient in permeabilizing the cells. Rates from 0.02 Hz to 7 kHz were tested and a saturation was observed below 0.1 Hz. Similar tendencies have been reported using other plant tissues and in particular apple [18] and onion [17]. Moreover, several studies have drawn similar conclusions for single cells *in vitro* [1,14] even if others only partially agree with this tendency [12,15] under the probable influence of one of the biases discussed here above.

Our experiments on mice liver tend to show that the lowest rates are not necessarily the most efficient in reaching permeabilization. It seems that an optimal intermediate repetition rate can be found. To our knowledge, three *in vivo* studies address the question of repetition rate [7,16,34]. All three show a tendency for higher efficiency of the low repetition rates although in a less straight forward manner than *in vitro* or in plants. As mentioned above, a systemic reaction like the vascular lock might slightly blur the outcome of *in vivo* experiments. In fact, a study has shown that blood occlusion can be detected by impedance measurements approximately 10 s after pulse delivery [33].

5.3. Dependence on the repetition rate in permeabilization by nanosecond pulses

Our experiments carried out on potato samples but with nsPEF show a greater permeabilization when pulses are delivered at very low repetition rates. At the most, it turned out that 0.01 Hz could induce permeabilization on potato with only five pulses of 10 ns duration. We might note here that duration of excitation (*i.e.* duration of the pulse) is 10^{10} times shorter than the duration of relaxation (*i.e.* the time interval between two consecutive pulses). A similar study on the impact of repetition rate with pulses of different length has been recently performed *in vitro* on CHO cells [13]. For all the tested pulse lengths, from 0.3 μs to 9 μs, authors have shown a greater impact of the very low repetition rate. Similarities between microsecond and nanosecond pulses regarding the repetition rate are thus reported for the second time although the biological models were completely different (potato tissue *versus* cells in suspension).

5.4. Comparison between exposure to micropulses and nanopulses

It is interesting to note that the general trend of the effects of several pulses as a function of the pulse repetition frequency is similar when comparing the delivery of micropulses or nanopulses (at least in the potatoes, since we could not set adequate conditions to apply the nanopulses to the mice livers under completely controlled conditions). Actually, we have already shown that, using appropriate field amplitudes (adapted to the respective duration of the micro- and nanopulses), the response to a single pulse, whether of 100 μs or of 10 ns, may result in the reversible permeabilization of all the exposed cells and that the kinetics of the impermeability recovery are almost identical (A. Silve et al., 2012 and unpublished data). Our data adds new evidence to the fact that the consequences of nanopulses and micropulses are similar, even though we do not know if the very fundamental mechanisms of their effects are identical or not.

5.5. Hypothesis regarding the repetition rate dependency: the electro-desensitization concept

Several hypotheses have been suggested to explain the reasons why low repetition rates are more efficient in inducing permeabilization. One hypothesis was developed following observations of permeabilization of apple tissue. It stressed that the damages induced by the electric pulses are a phenomenon of correlated perfusion governed by two key processes: the resealing of the membrane and the water transfer inside [18]. This percolation model cannot account however for the fact that similar behaviors were observed on individual cells in suspension [1,13,14]. Pakhomova and colleagues proposed a hypothesis based on an 'electroporation-induced sensitization'. Possible reasons for this sensitization could be: 1) a depletion of the energy of the cells after they reseal the damage from the first pulse, 2) a modification of the cell physiology due for example to prolonged time intervals with high internal Ca^{2+} or 3) a swelling of the cells resulting in a diameter increase and thus a lower permeabilization threshold in terms of electric field magnitude [13]. In the case of potato, cell swelling is unlikely since the cell wall prevents it. Moreover, an impact on the metabolism (for example due to rushes of Ca^{2+} ions or to energy depletion) is important to consider when permeabilization is assessed by cell death measurements but it is quite unlikely to modify the results when electroporation is assessed by measuring the electrical properties of the tissues (which is the method that we have used on potato). Furthermore, potato tuber is a reserve tissue with very reduced metabolism and very low energy consumption.

Since the efficiency of low repetition rate has been demonstrated on so many different models, the reason for it should be model-unspecific. Our hypothesis is that the higher efficiency of low repetition rate is related to the resealing speed of the membranes. Indeed, to generate new conducting structures (pores or membrane defects) it is necessary to establish a transmembrane potential difference above a threshold value: applying a pulse on a permeabilized cell membrane is likely to be less efficient since the existing conducting structures might prevent the establishment of the transmembrane potential. Thus the electropermeabilization itself electro-desensitizes the cell membrane.

During the application of a first electric pulse, a high number of highly conductive pathways are created in the membrane inducing a high conductivity increase of the membrane (see cartoon 1 in Fig. 16). This is what has been predicted by all models, whether they are analytical models based on physics of membrane [53–55] or numerical models like those generated by molecular dynamics simulations [56–59]. Additionally, stable defects are created, which explain the long term effects of permeabilization (like molecule uptake). Such a dual phenomenon had already been proposed [55] and many scientists agree on the existence of such stable defects although their nature has not been yet unraveled. Several groups proposed the existence of hydrophilic pores associated with a reorganization of the lipids. Since the nature of those defects is not the core of this paper, we represent them with a question mark in our cartoons. If another pulse is applied immediately after the first one, the high conductance state of the membrane prevents the creation of additional defects. Conversely if a long enough time spans before the following pulse, the surviving stable defects, which do not contribute too much to membrane conductance, would not cause major electric short-circuiting of the membrane (cartoon 2 in Fig. 16) and thus would not impair the creation of new defects (and a fraction of the one new ones would survive after time). Thus, up to a point, time after pulses could be thought of as a "natural" selection mechanism for selecting the stable pores or defects. As the inter-pulse time increases (or the pulse repetition frequency decreases), more and more non-stable defects are removed between two consecutive pulses and, therefore, during a new pulse, the chances of creating new stable defects increases (less futile short-circuiting) which will be added to the long-lasting defects created before. Additionally, areas of the membrane highly permeabilized (already containing a high number of stable defects) are initially located at the poles of cells facing anode and cathode. With time, diffusion will redistribute defects more evenly in the whole membrane surface (cartoon 3 in Fig. 16) thus increasing the probability for new defects to be created at the anode and cathode where the field is more intense.

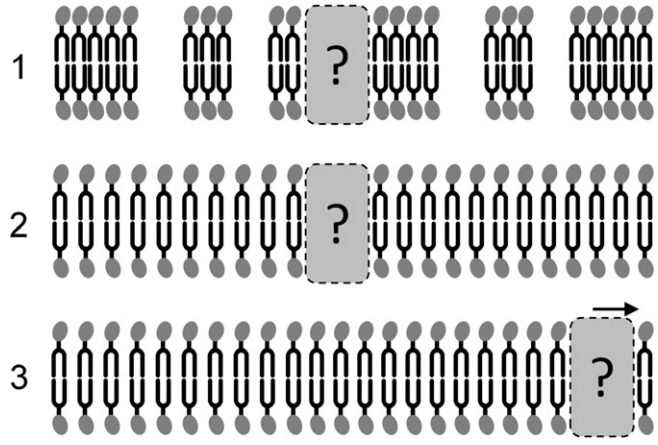

Fig. 16. Schematics of the electro-desensitization concept. 1) During and just after the pulse, the membrane contains a high density of conducting structures (hydrophilic pores) and some stable defects are created (hydrophobic pores?). The membrane is highly conductive. 2) Most conductive defects close rapidly but the stable defects remain. 3) Diffusion within the membrane plan drives stable defects away from their initial position (at the cell pole facing anode and cathode in a standard experiment with a homogeneous electric field).

According to the literature, the resealing of the membrane probably happens through many different pathways. Part of the resealing might be completely passive as suggested by molecular dynamics [58] or indicated by experiments on lipid bilayers [60–62]. The time scales associated with these processes range from a few nanoseconds to a few milliseconds. Additionally, re-organization of the stable defects driven by diffusion along the membrane plane might also be considered as a form of resealing. Indeed, this rearrangement brings unaffected membrane in the region where electroporation is most likely to happen (poles facing anode and cathode). Other pathways might involve active biological processes since the quantity of ATP and ADP available highly impacts on resealing time [63,64]. In general, membrane resealing through the different pathways consists of a continuous process starting immediately after the end of the pulse. Such process could explain why there is a continuous increase of the efficiency with the decrease of the repetition rate.

The experiments presented here highlight that low repetition rates are much more efficient in permeabilizing with both 100 μs and 10 ns pulses. Such similarities do not allow us to draw any conclusion on whether the mechanisms of permeabilization are the same or not. However, they support the possibility of having similar underlying mechanisms. In particular, if we assume that efficiency of low repetition rates is indeed directly related to repair mechanism, we might speculate on the fact that the type of damage induced by both long and short pulses is similar and that the resealing of such damage, a cellular process, happens through identical pathways.

In any case, in reversible conditions, there should be an extremely low cut-off frequency below which permeabilization should become less efficient, since the effects of the first pulse would be completely reversed. This remains to be explored.

5.6. Conclusion

Assessing the impact of repetition rate on the efficiency of electropermeabilization is not a trivial task. One of the most difficult aspects of this work is to figure out to what extent the dependency observed in an experiment really translates the dependency of

permeabilization to repetition rate. Indeed, several biases can complicate the interpretation of data. These biases include (but the list is probably not exhaustive): thermal effects, cell rotation, cell swelling between pulses, sedimentation of cells, *in vivo* systemic effects such as the vascular lock, as well as enhanced electrophoretic transport of permeabilization markers. From that point of view, the bio-impedance technique on potato tissue is a relatively safe approach since it intrinsically cannot include most of those biases. Only thermal effects could eventually bias the results in that case, but not under the pulse conditions tested in the present study, in which low repetition rates stood out as more efficient than high ones. Many other results indicate the same tendency on a large repetition range although there are some diverging results. The mechanism of 'electro-desensitization' proposed in the discussion enables us to understand all the results. It remains to be proven by specific experiments. For future directions, it could be convenient to test on single cells, how fast the effects of a first pulse disappear as far as the membrane charging is concerned. This can be done through the post-pulse measurement of the transmembrane voltage, for example with fluorescent dyes [65,66] and also using fast impedance techniques on single cells.

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