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Impact of the number of electric pulses on cell electrochemotherapy in vitro: limits of linearity and saturation

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ABSTRACT

In this study the evolution in the efficiency of electrochemotherapy (reversible electroporation) with pulse number was assessed in vitro. Experiments were performed using 100 μ s pulses at different electric field intensities and the chemotherapeutic agent bleomycin. Additionally, electrical impedance spectroscopy measurements were used as a different method to study in real time the changes produced on cells with pulse number during trains of consecutive pulses. Our results show that the relation between pulse number and the observed outcome is complex and difficult to fully characterize. This relation can display a highly linear behaviour up to a certain number of pulses and/or field intensity applied. However, the relation between the number of pulses and the observed outcome always evolves to a saturation or at least a reduction in the electric field effects that is displayed when either electric field intensity or pulse number are increased. An exponential model was found to best describe this relation within the range of experimental conditions considered. Electrical impedance measurements confirmed the results and gave a more precise quantification of this dependence. The study highlights the importance that pulse number has in the electrochemotherapy protocols and establishes some limits in the use of this parameter.

1 Introduction

The application of high intensity pulsed electric fields to biological samples is widely used to provoke the transient permeabilization of the cells membrane in a phenomenon termed electroporation, also known as electropermeabilization. This is routinely used as a technique to incorporate membrane-impermeant molecules into the cells. A deep understanding of the technique and of the main applications of the phenomenon can be found in [1]. In particular, one of the most relevant medical applications of this technique, called Electrochemotherapy (ECT), consists in the combination of the electric field with a chemotherapeutic agent, usually bleomycin, or cisplatin, for the local treatment of cancer [2–6]. The classical electrical protocol used in the clinics consists in the application of 8 monopolar 100 μ s pulses.

Bleomycin is a non-permeant molecule that cannot cross the membrane by diffusion because of its hydrophilicity and molecular weight. In the absence of electric pulses, the bleomycin molecules showing cytotoxic effectiveness bind to a receptor [7] and it has been shown in vitro that they penetrate the cells by a receptor-mediated endocytosis [8]. In vivo, in the absence of electric pulses delivery, the same mechanism should apply, but it has only been demonstrated for the nucleic acids, another type of non-permeant molecules [9]. When electroporation pulses are applied, the cytotoxicity of bleomycin can be increased by more than 100-fold in vivo. The transient permeabilization of cell membrane allows the bleomycin to enter the cells and to exert its cytotoxic effect, which is significantly higher in the tumour cells because, at low concentrations, bleomycin induces a mitotic cell death [10]. Therefore, a very interesting specificity is achieved, causing tumour cell death while preserving the surrounding normal cells that do not divide like tumour cells. Unlike other antitumor treatments based on physical phenomena, ECT is the sole treatment able to exert a specific effect at the cellular level, as it only kills the dividing (thus the tumoral) cells in the treated volume.

Usually, the rule applied by medical doctors to increase the efficiency of an electroporation treatment is to increase either the amplitude, duration or the number of electric pulses applied to the target tissue.

In order to minimize certain undesired effects as Joule heating or just limited by the maximum power that the pulse generator can deliver, a regular practice is not to increase the amplitude or duration of the individual electric pulses, but to increase the number of the pulses applied. Especially, in the treatment of deep seated tumours, where the tissues can have high conductivities, there are important restrictions in the desired nominal parameters requiring large amperages that the pulse generators may not be able to deliver. For security reasons, the increase in the power of the pulse generators, while technically possible, is not sought over certain limits [11].

Many previous studies have investigated the role of the different pulse parameters on the efficiency of electroporation [1,12–18], mainly, the impact of the intensity of the applied electric field, its duration and possible equivalent combinations between these two parameters. In general, it is widely accepted that a decrease in the pulse amplitude can be compensated by an increase in the duration of the applied pulse down to a certain threshold at which the intensity of the electric field is not enough to produce electroporation. The same general idea applies when a reduction in the amplitude of the pulses is compensated by an increase in the number of pulses applied. However, this second relation has been poorly studied in the case of the 100 μ s pulses [19–21] and there is still some controversy about the limits that an increase in the number of pulses applied has on the treatment efficiency.

There is a general agreement in the fact that the increase in the effect produced by repetitive pulses is not linear and there exists a saturation phenomenon, where an increase in pulse number is not translated in a more intense effect. This was observed for example for CHO (Chinese Hamster Ovary) cells in vitro for the uptake [22] or the release [23] of different molecules. However, in both cases and under similar experimental conditions, the limits for the observation of this saturation were considerably different and thus dependent on the detection technique used. Theoretical modelling has also shown this saturation effect using trains of 20 conventional 100 μ s pulses at 1 Hz repetition frequency [24]. In this study, an exponential decrease in pore number formation despite the application of additional pulses was shown. However, the reasons and the mechanisms behind this loss of efficiency are not clear. Usually, it is believed that inducing a transmembrane potential in an already permeabilized membrane would be more difficult and could thus explain the observed gradual loss of

the electric field effect. However, this hypothesis has been only studied in a recent article using 200 ns pulses where the results regarding the membrane repolarization dynamics after the pulses were not conclusive due to limitations in the fluorescent dye used [25]. Another study on pulse trains termed this saturation phenomenon “electro-desensitization”, and related a decrease in pulse repetition rate with a greater membrane permeabilization over a wide range of pulse conditions [26]. A non-linear scaling law with pulse number was also proposed for nanosecond pulses [27]. In this case a square root relation was established. Finally, and contrary to the rest of studies, one single study showed a linear dependence between permeabilization and pulse number, at least up to 100 pulses, using 60 ns pulses and the classical propidium iodide and YoPro-1 dyes [28].

In the present study the dependence of electrochemotherapy efficiency *in vitro* on pulse number is studied for different electric field intensities using bleomycin. The limits of the detection technique and how they could impact in the observed saturation phenomenon are explored for different bleomycin concentrations. Additionally, electrical impedance spectroscopy measurements are used in a different scenario to measure in real time the changes produced on cells with pulse number during trains of pulses for different field intensities.

2 Materials and Methods

2.1 Cell culture

Chinese hamster lung cell line DC-3F [29] was grown in complete cell culture medium which consists of MEM - Minimum Essential Medium (Life Technologies, Cergy-Pontoise, France) with addition of 10% fetal bovine serum (Life Technologies) and supplemented by antibiotics (100 U/ml penicillin, and 100 µg/ml streptomycin (Life Technologies). The cells were maintained in a humidified atmosphere at 37°C and 5% CO₂ and routinely passed every two days.

After trypsinization of exponentially growing cells and inactivation of trypsin (Life Technologies) by the serum factors of the complete medium, cells were centrifuged for 10 min at 150 g and resuspended at a density of $4,2 \times 10^6$ cells/ml in S-MEM, which is a Minimum Essential Medium modified for the culture of cells in suspension (Life Technologies).

2.2 Electric field exposure

Cells suspended in S-MEM were exposed in conventional electroporation 1 mm cuvettes from Cell Project (Harrietsham, United Kingdom). It must be noticed that precise measurements of the distance between the electrodes were performed in triplicates using an electronic calliper. These measurements indicated that the real distance was slightly higher, precisely 1.18 ± 0.02 mm for the 1 mm cuvettes. This measured distance was considered as the real inter-electrode distance to be used to evaluate the electric field inside the cuvette. The voltages to be applied were chosen in order to deliver electric pulses with a voltage to distance ratio (V/d) ranging from 760 V/cm to 1100 V/cm. This range was chosen based on our previous experience and corresponds to the limits of reversible electroporation when 8 pulses are applied under these particular experimental conditions.

Pulses of 100 µs duration were delivered at a repetition frequency of 1Hz by an electroporation power supply (CliniporatorTM, IGEA, Carpi, Italy) able to generate high-voltage square-wave pulses. Because this device cannot deliver more than 20 pulses in a row, two or more series of 20 pulses were consecutively applied when the treatment needed more than 20 pulses. In these cases, an interval of 20-30 seconds between each series was necessary in order to re-program the following series of

pulses. Moreover, this interval also ensured that no temperature increase occurred in the cuvette. It was also taken advantage of this period of time to gently shake the cuvettes for homogenization and to minimize bubbles formation at the metallic part of the cuvettes,

2.3 Assessment of electrochemotherapy efficiency

Electrochemotherapy efficiency was studied using bleomycin, a cell membrane impermeant chemotherapeutic agent that selectively kills only the permeabilized cells. This method indirectly measures cell survival as a reflect of the electroporation-mediated uptake of bleomycin inside cells. The cuvettes containing the cells and the desired concentration of bleomycin (final concentrations ranging from 1 nM to 30 nM) were subjected to the electric pulses. Tests were also performed without bleomycin in order to verify other collateral effects of the micropulses only (irreversible electroporation, thermal effects, etc.).

After the delivery of the electric pulses, cells were kept for 10 min at room temperature. They were then diluted in complete medium. After dilution, cells were seeded in triplicate in complete culture medium (250 cells per cell culture dish, 35 mm in diameter) to measure their viability through a quantitative cloning efficacy test. After 5 days maintained in a humidified atmosphere, colonies were fixed, stained (with a solution of 5% formaldehyde containing crystal violet) and the number of clones N for each condition was counted. Viability was normalized to the number of clones in the control (N_{control}) and reported as a percentage of survival: $N/N_{\text{control}} \times 100$. In experiments without bleomycin, the control condition refers to cells submitted to no treatment. In experiments with bleomycin, the control condition refers to cells, in contact with the bleomycin, that did not receive electric pulses. The cells in the controls were handled exactly the same way than the exposed cells including incubation times in cuvettes and dilutions. All the experiments were repeated at least three independent times in at least two different days.

2.4 Electrical impedance measurement system

The electrical impedance spectra of cell suspensions during the application of trains of 20 electroporation pulses were acquired in real time at different electric field intensities. These

measurements were done as a parallel study and the comparison between ECT and impedance results should be done carefully. Specifically, in order to increase the sensitivity to the impedance changes produced by the electroporation pulses, the same cell line (DC-3F) was prepared in suspension but at a higher density ($100 \cdot 10^6$ cells/ml) and the buffer used was a low conductivity electroporation buffer. The buffer consisted off of 250mM sucrose, 10mM Hepes and 1mM MgCl₂ (pH 7, osmolarity 287 mOsm/L, conductivity 0,1 S/m).

This method enabled us to have direct access to the temporal evolution of the effects of the electric field on cells pulse after pulse. Similar to the previous experiments, 100 μ s pulses at a repetition frequency of 1Hz were delivered by a CliniporatorTM and the cells were pulsed and measured in conventional 1 mm electroporation cuvettes. The measuring system was synchronized with the pulse generator in order to measure the impedance spectrum of the cells suspensions immediately after each individual electroporation pulse of a train of pulses. The custom made measuring system was based on a PXI platform (National Instruments, Austin, TX), comprising an arbitrary waveform generator (NI-PXI 5422), a two channels digitizer (NI-PXIe 5122) and a high density multi-configuration multiplexer matrix (NI-PXIe 2529). Additionally, a custom-made analog front-end was used to interface the measuring signals with the electrodes (measuring signal current level < 1 mA). More details about a very similar setup can be found in [30]. In this setup, the impedance measurements were performed using a two-electrode arrangement. In this configuration the two metallic plates of the cuvettes were used as the measuring electrodes both for current injection and voltage measurement.

A fast time-domain measuring technique based on multisine excitations was used to acquire impedance spectral information in the gap between consecutive pulses at a temporal resolution of 1 spectrum/ms. The multisine excitation burst contained 26 frequencies distributed from 1 kHz to 1 MHz. A custom software was developed in LabVIEW (National Instruments) to control the whole system. The measurements sequence consisted in a continuous acquisition of 100 spectra (100 ms) after a fixed delay of 500 ms after each pulse application until completing the 20 pulses protocol. Each 100 spectra segment was averaged to produce a single measurement point after each pulse. All the acquisitions were performed at least three independent times.

3 Results and Discussion

3.1 Effect of the electric field intensity and pulse number on cell electrochemotherapy

Cells were exposed to a varying number of micropulses (ranging from 1 to 20) and different fields amplitudes (ranging from 760 V/cm to 1100 V/cm) in the presence of 30 nM of bleomycin. For each condition, cell permeabilization to bleomycin was assessed as explained in Materials & Methods. In Fig.1a, the evolution in the percentage of cell survival with pulse number is shown for each of the applied electric field intensities. In this set of experiments, the number of pulses was gradually increased until a plateau in cell death was obtained. The maximum number of pulses assayed was 20 for 760 and 850 V/cm and 15, 10 and 5 for the field intensities of 930, 1020 and 1100 V/cm, respectively. It is interesting to notice how the plateau level, calculated as the mean value of the three last experimental points for each condition, varies between 10-20 % and does not reach zero (cell death of the entire cell population). Particularly, plateau values of 18 ± 3.8 %, 15 ± 2.9 % and 11 ± 3.5 % were respectively obtained for 930, 1020 and 1100 V/cm. This plateau value, which seems to decrease with the increase of the electric field amplitude, shows that there are restrictions in the uniform exposure of the cells either to the electric field, the bleomycin, or both that avoid a 100 % response. Bleomycin accessibility to cells in these experiments should be assured since 30 nM is considered already as a “saturating” concentration when cells are electropermeabilized. On the other hand, for the electric field exposure, there may be a lack of uniformity in the cuvettes maybe due to the meniscus in the liquid causing this remaining cell survival. Also, it is known that cells can shield the field to which the neighbouring cells are exposed [31–33]. These two last reasons are probably the major causes for the plateau values not reaching a 0 % of cell survival. In particular, considering that after the detachment of the cells from their culture substrate (whatever the method of detachment, using trypsin or not), some cell aggregates may subsist as cell suspensions are difficultly monodispersed.

In the case of the two lowest electric field intensities corresponding to 760 and 850 V/cm, although the beginning of a saturation effect was observed, we decided to perform an additional series of experiments increasing the number of pulses beyond 20 to verify if the saturation was maintained. In Fig. 1b, the results after the application of up to 80 pulses show that when cells were exposed to more

than 20 pulses, viability continued to decrease. It became possible to achieve about 80% of bleomycin-dependent cell death with more than 40 micropulses at 850 V/cm (plateau value 19 ± 2.3 %). However, for the field intensity of 760 V/cm it was not possible to reach a plateau even when 80 pulses were applied.

The relation between cell death and applied pulse number displays a nonlinear evolution. There is a first part of the curves that display a quite linear behaviour but when the number of pulses was further increased there was a reduction in the outcome caused by each supplementary pulse applied. This observation suggests the existence of a saturation phenomenon responsible for the loss of efficacy observed. Furthermore, the apparition of this phenomenon is dependent on the intensity of the electric field applied. For the highest electric field, this saturation is hard to observe as only 3 pulses were necessary to reach the maximum detectable effect. Thus, the saturation observed under the highest electric field conditions is likely due to a saturation in the bleomycin effect more than a saturation in the electric field effect. On the contrary, a saturation of the electric field effects is clearly observed for 850 V/cm up to 20 pulses. For an electric field of 760 V/cm the evolution with pulse number seems to be linear up to 20 pulses and it was necessary to increase the number of pulses above 20 to clearly observe this saturation.

Lastly, it has to be mentioned that there is a possible bias in the present analysis produced by the fact that for more than 20 pulses the exposure was not continuous. As explained in section 2.2, an interval of 20-30 seconds between each series was necessary in order to re-program the generator. As previously shown in several studies [34,35], the outcome obtained when the electric field dose is splitted in several delayed segments is sometimes higher than a single train exposure. This phenomenon called “electrosensitization” could have participated in the increase observed between 20 and 40 pulses for 850 V/cm in Fig. 1b. This means that, if all the pulses had been consecutively applied, even a more saturation effect would have been observed.

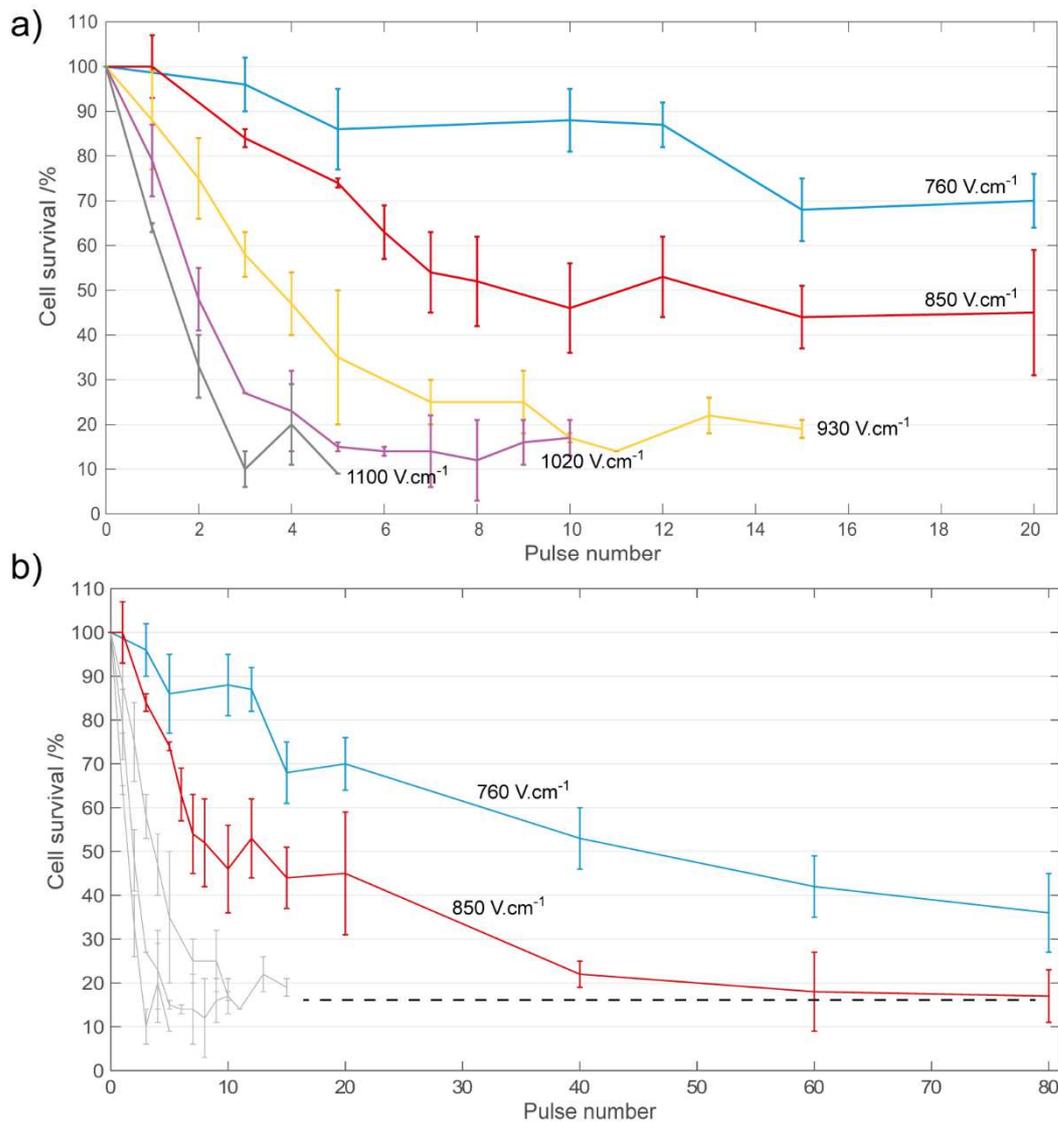


Fig. 1 Cell survival as a function of the number and intensity of 100µs micropulses in the presence of 30 nM bleomycin. Cloning efficiency tests were performed after exposure to micropulses (100 µs 1Hz) of a field amplitude of 760 V/cm (—), 850 V/cm (—), 930 V/cm (—), 1020 (—) and 1100 V/cm (—) in SMEM containing 30nM bleomycin. Results are represented as mean value ± SD (standard deviation) of three independent experiments. Cell survival is normalized to the control exposed to bleomycin alone. a) Results up to 20 pulses. b) Extended results up to 80 pulses for 850 and 760 V/cm only. Dashed line corresponds to the average of the plateau values for 1100, 1020 and 930 V/cm experiments.

3.2 Collateral effects of the field application on cell viability (Limits for reversible electroporation)

Because of the very large number of pulses (up to 80 pulses) that were delivered at the lowest field amplitudes, it was necessary to verify the possible impact of these trains of pulses by themselves, i.e. in the absence of bleomycin, on cell viability. Fig. 2 shows the cell survival evolution with pulse number for up to 80 pulses for 760, 850 V/cm and, additionally, the effects for up to 20 pulses for an intensity of 1100 V/cm.

It can be seen how for the maximum electric field (1100 V/cm), pulses began to cause some cell death only when more than 8 pulses were applied. Cell death increased linearly with pulse number above 8 pulses and up to 20 pulses, when about 60% of the cell population was killed. This effect in cell viability, only detectable starting at pulse number 8, confirms that the cell death observed in Fig. 1 for this field intensity was only due to reversible electroporation.

In the case of 850 V/cm and 760 V/cm, the electric field (in the absence of bleomycin) started to be toxic for pulse numbers higher than 20 and 40, respectively. The accumulated cell death for these electric field intensities at pulse number 80 was around 55 % in the case of 850 V/cm and 20 % for 760 V/cm. Part of the cell death increase observed when a large number of pulses is applied in the presence of bleomycin at these field amplitudes (Fig. 1b) could then be due to irreversible permeabilization or other collateral effects. Among these other effects, we cannot exclude the toxicity due to an electrolytic contamination (partly revealed by the presence of bubbles in the cuvettes) or even thermal effects. This last was discarded after measuring the temperature rise using a temperature probe (LumaTest, Lumasense Technologies) whose optical fiber end was inserted inside a cuvette during pulse application. The change in temperature for the highest electric field intensity and 80 pulses was < 1 °C at the end of the treatment. Another reason that could explain the observed cell death is the pH changes produced at the electrodes interface, even though in a liquid suspension and at the pulse repetition rates used in our experiments, there is time to partly homogenise the solution and to compensate the pH changes at the opposite electrodes.

The results obtained in this section can be helpful for the interpretation of the results shown in Fig. 1b. Particularly, for 760 V/cm in the presence of bleomycin, the results in Fig. 1b suggests that no saturation is reached after 80 pulses. However, comparing the slopes of the cell survival decrease in Fig. 1b and Fig. 2a for this field intensity, it is clear that they are identical between pulse number 40 and 80. This observation can take us to consider that the decrease of cell survival from pulse number 40 to 80 in the presence of bleomycin (data in Fig. 1b) is in fact produced by the effects of the electric field alone. This way, in order to observe only the ECT effects, the data reported in Fig. 1b could be corrected with the data of Fig. 2a for 760 V/cm. This correction would produce a saturation of the electric field effects also for 760 V/cm (see Fig. 2b for the original (dashed) corrected data (solid)).

It is important to note that the same kind of correction (compensation) cannot be applied to the 850V/cm field amplitude because from pulse number 40 at this field amplitude, there is already a saturation in the maximum observable reversible effect (at 30 nM bleomycin) and the slopes are different.

In conclusion of this section, the results confirm that for the two lowest electric fields, the saturation observed in Fig. 1 is even more pronounced than the one displayed. If the cell death caused by the electric pulses alone is subtracted from the results obtained with bleomycin, a more significative saturation effect with pulse number is revealed. Also, the results of this section confirm the existence of limits for the increase of the applied number of pulses to reach a certain effect. The collateral effects of pulse application should be taken into account when a large number of pulses are applied.

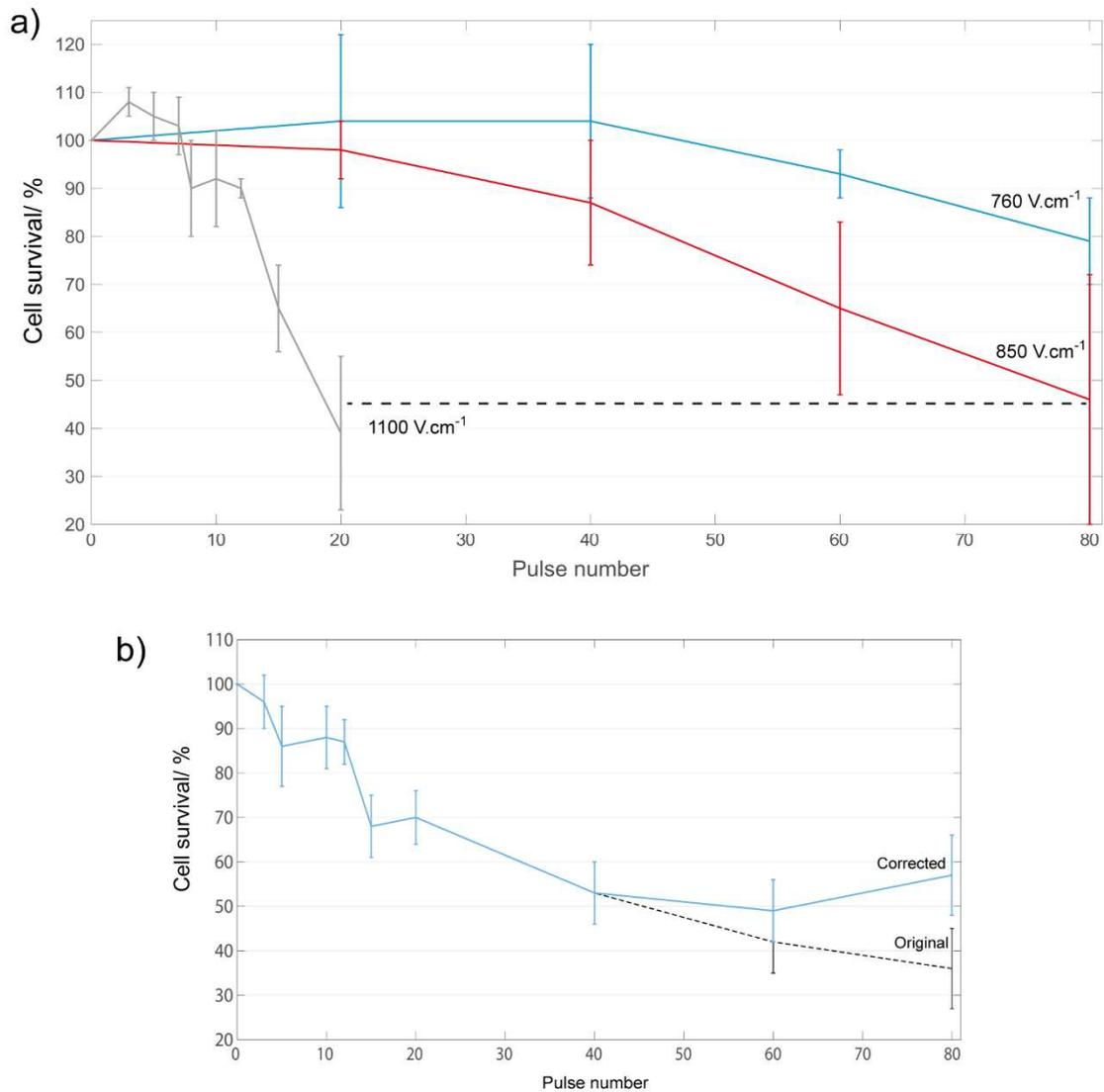


Fig. 2 a) Effects of electric field exposure alone. Cell survival is shown as a function of the number and intensity of 100 μ s pulses in the absence of bleomycin for 760 V/cm (—), 850 V/cm (—), and 1100 V/cm (—). Results are represented as mean value \pm SD (standard deviation) of three independent experiments. Cell survival is normalized to the control (non-exposed cells). b) Possible correction of irreversible electroporation. Cell survival for an intensity of 760 V/cm in the presence of bleomycin. Original data (---), shown in Fig. 1b, versus the corrected (—) after compensation for the effects of the electric field alone displayed in a) for the same electric field intensity.

3.3 Effect of the concentration of bleomycin (detection limits)

In order to assess whether the observed saturation as a function of the number of pulses is really under the dependence of the electric field amplitude or not, we analysed how a reduction in the concentration of bleomycin could impact the observed relation between electric field intensity and pulse number. With respect to the effects of the bleomycin alone, the extremely low bleomycin concentrations used (30 nM and lower concentrations) cause no effect or negligible effects if no pulse is delivered to the cell suspension (as we already reported in 1988 [36]). Fig. 3 shows the results of cells exposed to a fixed electric field intensity of 1100 V/cm at various bleomycin concentrations and various pulse numbers. It can be observed how, to achieve a similar cell survival rate, a reduction in the concentration of bleomycin needs to be translated in an increase in the number of pulses delivered. From 30 nM to 3 nM, a plateau (10-20 % of survival) is reached if the number of pulses increases up to 10. It is important to note that the results in Fig. 3 also confirm that, up to 8 pulses, there is no significant effect of the electric pulses alone (without bleomycin). However, at 1 nM the plateau is not reached even with 20 pulses, but, since pulses alone at 1100 V/cm were toxic, it did not seem profitable to explore the situation with more than 20 pulses.

The observed relation is similar to the one observed in section 3.1 for a variation in the electric field intensity applied. Specifically, at 1 nM, the relation between cell death and the number of pulses does not display a saturation phenomenon similarly to the behaviour observed previously for an electric field of 760 V/cm up to 20 pulses. However, it is noticeable that from pulse number 10 there is a reduction in the slope of the curve of survival rate versus pulse number, suggesting a decrease in the intensity of the effect of any supplementary pulse with increasing pulse number. Additionally, if for this low bleomycin concentration, the cell death produced by the pulses alone would be subtracted, the

reduction in the intensity of the effect of any supplementary pulse with increasing pulse number would be even more pronounced.

These results demonstrate that by increasing the detection sensitivity of the experimental technique used, in this case by reducing the bleomycin concentration, the apparent saturation observed nearly disappeared and the effects of the electric pulses became almost linear. This can at least partially agree with the study performed by Pakhomov et al. [28] where by using a highly sensitive technique and very short pulses they could not observe any saturation. The increase in the detection sensitivity would help in revealing the real additive effect of consecutive pulses. However, in the present study, even for the lowest concentration of bleomycin used, there is still a reduction in the effect when the number of pulses increases. This suggests that there would be always a saturation effect if the number of pulses applied is sufficiently high.

Interestingly, these results suggest that in the clinical application of ECT, a reduction in the dose of bleomycin administered to patients, for example, to those with high risk of lung fibrosis, could be compensated by increasing the number of pulses applied. This hypothesis, with relevant implications in the clinical practice, should be verified in animal models.

Finally, although all the previous results correspond to ECT efficacy *in vitro*, bleomycin has been used as a sort of permeabilization marker. A good correlation has been found between this method and other traditional assays based on the uptake of a fluorescent dye [37]. Consequently, using a similar approach, the main conclusions presented herein could be generalized for Reversible Electroporation by complementing the present study with other permeabilization assays (i.e. fluorescent dyes uptake). An optimization in the dye uptake conditions (e.g. by finely tuning the dye concentration and/or incubation time) should be performed to extend to detection limits of the technique.

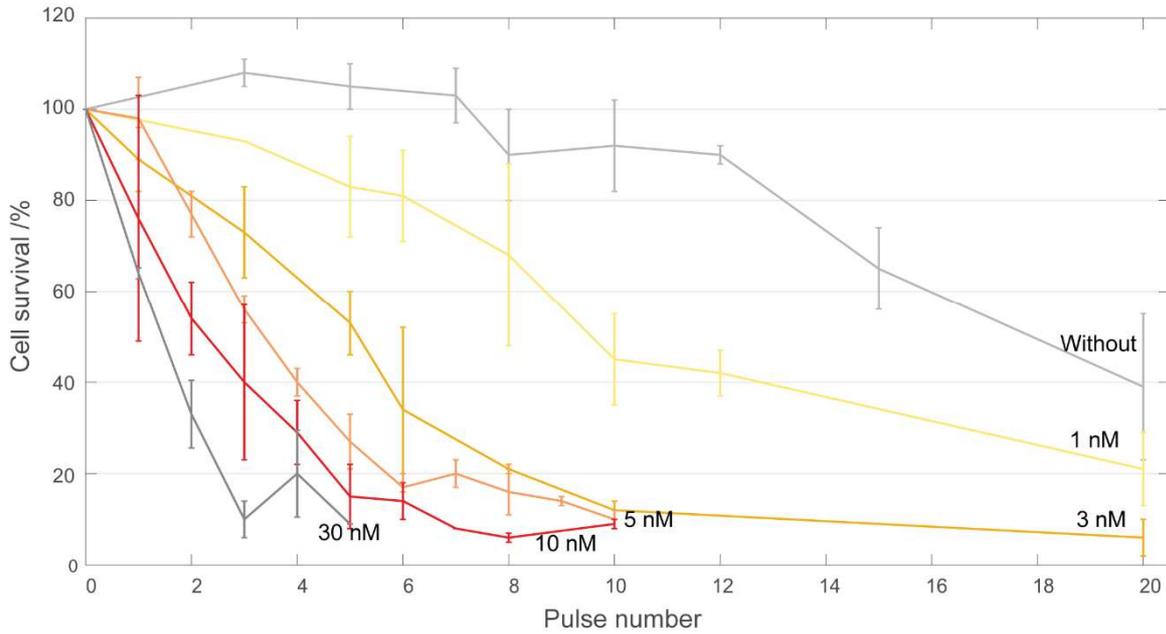


Fig. 3 Cell survival as a function of the number of pulses for different bleomycin concentrations: without (—), 1 nM (—), 3 nM (—), 5 nM (—), 10 nM (—) and 30 nM (—). A fixed electric field intensity of 1100 V/cm was used in all experiments. Results are represented as mean value \pm SD (standard deviation) of three independent experiments. Cell survival is normalized to unexposed cells for each bleomycin concentration.

3.4 Exponential model

In an attempt to mathematically model the ensemble of the data, and to describe in a more exhaustive way the relation between pulse number, the amplitude of the applied electric field and the observed outcome, we fitted the data to different mathematical functions and we found that the exponential function was the simplest and the most convenient approach. The analysis was performed in Matlab (The MathWorks, Inc.) using a nonlinear Least Squares approach and the Trust-Region algorithm. The data corresponding to a constant bleomycin concentration of 30 nM and different electric fields were first individually fitted to the general exponential function $f(N_{\text{pulse}}, E_{\text{field}})$ shown in Fig. 4a (left) and the values of the parameters a , b and c were extracted. It was observed that the values of the parameters a and c remained almost constant for the all the field intensities, meaning that this values are not dependent on the electric field applied. The final values for a (91) and c (12) were calculated from the mean values obtained for the different fields. The parameter c , which is the minimum level

reached by the model, corresponds to the plateau level in cell survival discussed before. The parameter a corresponds, thus, to the maximum effect attainable in the present experimental setup.

Contrarily, the values obtained for the parameter b were highly dependent on the field applied and, more interestingly, they followed a sigmoidal dependence upon the electric field intensity, at least inside the limits of reversible electroporation. To include this dependence on the model, the different values of b were fitted to a sigmoid function (Fig. 4a right panel) that was inserted in the final model equation. The sigmoidal behaviour found for the parameter b , which corresponds to the time constant of the exponential function, indicates that for a central range of electric field intensities, there is a strong influence of the field applied on the rate of decrease in cell survival with pulse number. Out of this range, a variation in the field applied will impact in a lesser extent the time constant of the exponential model and thus, a more similar evolution in the ECT outcome with pulse number will be found.

In Fig. 4b the resulting model computed for the different field intensities and the corresponding data are displayed. These results show the good agreement between the model and the experimental data for most cases. Observing the calculated residuals for the different conditions studied shown in Fig. 4c, an increase in the error with pulse number is obtained for the two lowest electric fields, but in average, the residuals values are acceptable.

This model therefore would allow for the calculation of the convenient number of pulses to compensate electric field amplitudes. Even though the numerical values of the function here shown are only strictly valid for the case of the DC-3F fibroblasts treated in vitro when cells are suspended in S-MEM medium at $4,2 \times 10^6$ cells/ml at the time of the exposure to the electric pulses, the exponential function should be of general use. For cells in vitro, the numerical values obtained could be a sufficient approximation for a number of cell lines. This result shows the interest of developing similar models that could be used as a guide in the clinical practice and that would help the physicians to take better decisions in the treatments planning.

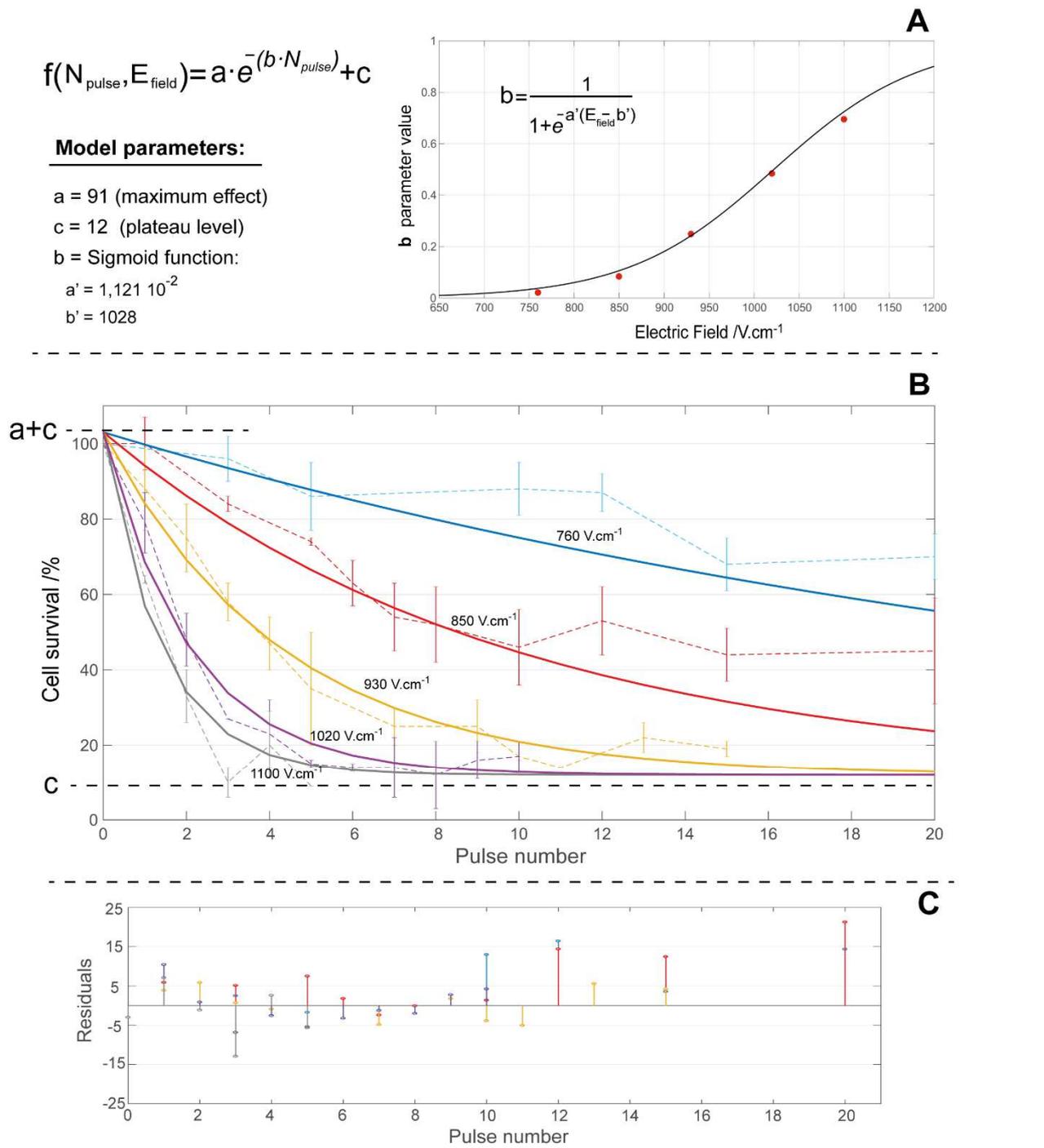


Fig. 4. Exponential model results. A) Left part shows the mathematical expression and the numerical values of the model. In the right part the sigmoidal dependence of b parameter (time constant of the exponential) upon the electric field is shown; red points correspond to the values obtained for each individual fitting and black line shows the resulting fitted function introduced in the final general equation. B) Representation of the model with the experimental data corresponding to 760 V/cm (—), 850 V/cm (—), 930 V/cm (—), 1020 (—) and 1100 V/cm (—) using 100 μs pulses at 1 Hz repetition frequency and a fixed bleomycin concentration of 30 nM. C) Residuals (data-model) of the model for the different conditions studied.

3.5 Real-time impedance measurements

Because all the data presented in the previous sections were somehow based in post-pulse evaluations of the effects of the entire trains of pulses (no data was collected on the effects of each additional pulse inside a given train of pulses), we considered that real-time data could give complementary information about the effects on cells with increasing pulse number. To this end we performed electrical impedance measurements of cell suspensions during the application of electroporation pulses. The measuring system allowed us to obtain measurements before, during (in the time gap between consecutive pulses) and just after a complete 20 pulses procedure. It is important to stress the fact that these measurements were performed in very different experimental conditions with respect to the previous experiments. Particularly, in order to increase the contribution of cells to the measured impedance, a dense cell suspension was used (volume fraction around 0,4). Also, the conductivity of the electroporation buffer was reduced to improve the acquired signal to noise ratio. Under these circumstances, a direct comparison between both sets of results should be avoided.

First, for each electric field intensity, control measurements were performed during the application of electric pulses to a cuvette containing the same buffer but without cells. These measurements were performed to ensure that other collateral effects such as corrosion of electrodes surface or small temperature variations did not produce changes in impedance that could lead to a misinterpretation of the results. As shown in supplementary information S1, no changes in impedance were detected when pulses were applied to the electroporation buffer alone.

In Fig. 5a an example of the obtained impedance spectra evolution during the application of an electric field of 1270 V/cm to a cell suspension is shown. The displayed data were calibrated for undesired system contributions (electrodes, cables, etc..) using different saline solutions measured in the exact same conditions and using a three-references calibration method [38]. Due to the fact that the measurements were performed using a two-electrodes arrangement, the impedance data at low frequencies were highly corrupted by the predominating electrode/electrolyte interface impedance and

could not be used. This is why the spectra displayed in Fig. 5a are truncated for the frequencies below 20 kHz. The arrows in the figure indicate the direction of the temporal evolution pulse after pulse.

A reduction in the measured impedance of the cell suspension is shown pulse after pulse, which is the expected behaviour during cell permeabilization [26,39–42]. However, according to theory and as shown in previous studies [42–44], the changes observed in the impedance magnitude, should be considerably different at low and high frequency. In the frequency band studied, at low frequency a non-permeabilized cell membrane behaves as an insulator, thus blocking the flow of current. When the frequency of the measuring signal increases, the membrane is gradually shunted allowing the flow of current at high frequencies. If the membrane is now permeabilized, the low frequency currents will be able to cross the membrane producing a bigger impedance change at low frequency than at high frequency impedance, where the current could already partially flow across the membrane. On the contrary, our results show a similar variation of the magnitude of the impedance for all the frequency range analysed. This can be explained by the fact that a massive change in conductivity is produced concomitant to cell permeabilization due to the leakage of intracellular components to the extracellular compartment in the cuvettes-based set-up and this can predominate over the changes produced on cells. Observing now the evolution of the phase of the impedance, it is clear that in this case, the changes produced are not affecting all the frequencies in a similar way. In particular, in the frequency range between 100 – 200 kHz these changes are considerably higher. This observation suggests that the phase information is less affected by the changes produced in the conductivity of the extracellular buffer and that should bring more precise information about the changes produced in the cell suspension, as previously shown in a recent study [43].

In Fig. 5b the normalized phase evolution at the frequency of 173 kHz, is shown for the different electric field intensities studied. It can be seen how for 760 V/cm the impedance measurements display no change, meaning that either this electric field is not causing cell permeabilization under these particular experimental conditions or that the measuring system is not able to detect the small changes produced by this low electric field intensity. The high density of the cells suspensions used in these experiments could at least partially cause a reduction in the effect of the same electric field intensity

compared to the previous situation. This is explained based on the shielding effect discussed in section 3.1, which could have a large effect under these experimental conditions. Also, the reduction in the conductivity of the pulsing buffer used can be proposed as a possibility for a change in the effectiveness of the applied electric field. However, a previous study using very similar experimental conditions, demonstrated that the dependence on the conductivity of the extracellular buffer was only important when a single 100 μs pulse was applied and negligible when a train of several pulses was used [45]. In any case, there is still the possibility that the measuring system is unable to detect the very small changes in impedance that this low electric field produces on the cell membrane.

The impedance changes for the next electric field tested (1016 V/cm) display a linear evolution with pulse number up to 20 pulses. On the contrary, for the two highest electric fields it is possible to observe a change in this linear trend when more than 14 and 10 pulses were applied at 1270 and 1525 V/cm, respectively. Above these limits, there is a clear reduction in the rate of impedance drop with pulse number suggesting a saturation in the effects of the electric field on the cell suspension.

It is important to stress the fact that, contrary to the experiments on cell viability, the information provided by the impedance measurements reflects in real time the immediate effects of the electric pulses on cells, avoiding the “intrinsic” saturation effects of the other methods. Because the absolute changes in the phase (Fig. 5a) are very small, we can thus conclude that the loss of linearity detectable in Fig. 5b when more than 14 and 10 pulses were applied for 1270 and 1525 V/cm reflects a real saturation, even if this saturation is not fully reached with only 20 pulses at these field amplitudes. Thus, our results confirm at the least that repetitive pulses are less and less efficient. The loss in efficiency hereby demonstrated supports the idea that saturation should exist independently of the experimental technique used.

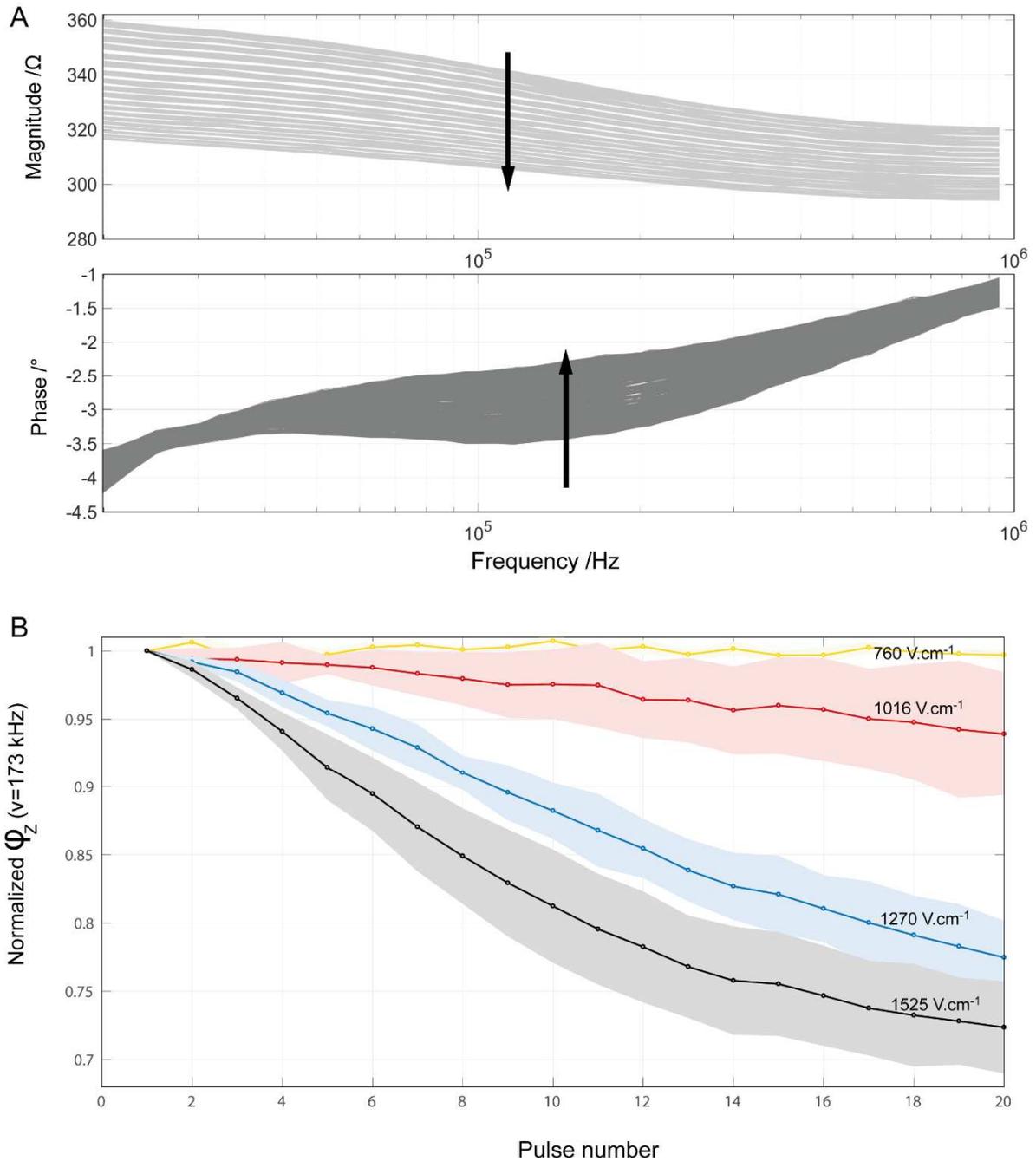


Fig. 5. Impedance measurements. A) Example of the evolution in the measured impedance spectra (magnitude and phase) for a 20 pulses sequence applying an electric field of 1270 V/cm. Arrows indicate the direction of the temporal evolution. B) Variation in the phase angle of the impedance at a frequency of 173 kHz normalized with respect to the initial pre-electroporation values for different electric field intensities. Data points correspond to the mean values of 100 spectra acquired at a fixed delay of 500 ms after each pulse. Shadow areas represent the \pm standard deviation.

4 Conclusions

This study demonstrates that the relation between the number of electroporation pulses applied, their intensity and the effects of these pulses on the cell membrane is not straightforward. Up to some extent, particularly when the pulses are applied for the treatment of cells with bleomycin in the scope of the antitumor electrochemotherapy, a reduction in the field amplitude (imposed, for example, by a limitation in the current intensity that the pulse generator is able to deliver) can be compensated by an increase in the number of pulses applied. According to this study, this compensation would follow an exponential law with a highly linear behaviour for a low number of pulses. The parameters of this exponential function have been determined for a fibroblastic cell line but they could approximate those of a number of cell lines in vitro.

However, there are limits to compensate a reduction in the field intensity by an increase in the number of pulses applied to obtain a similar accumulated effect. The loss of linearity, which results in a saturation (reached or not in our experiments, as a function of the experimental conditions), appears when a too high number of pulses and/or a too intense field amplitude are applied. Our study also shows that the observed saturation would not be only the result of the saturation in the transport of the bleomycin across the electropermeabilized membrane: in a different scenario the impedance measurements reveal an intrinsic saturation of the effects of the electric pulses in the membrane structure, i.e. in the structural changes of the membrane that are responsible for its permeability to otherwise non-permeant molecules. Further research is necessary to deeply understand and establish the causes and limits of this gradual loss in the efficiency of repetitive pulse application. These results should be complemented with other permeabilization assays to extend the present results to a more general conclusion in the electroporation field

Finally, this study reinforces the idea that other limitations in the increase of the number of pulses to increase the electroporation effect are imposed by the apparition of undesired collateral effects that impact the viability of the cells, like the cell death directly caused by the electric pulses which was detected in the absence of bleomycin.

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Author Contributions

T.G.S. conceived the paper, performed the impedance measurements, developed the mathematical model, analysed the data and contributed to the writing of the paper. I.L. performed the permeabilization experiments, analysed the data and contributed in the writing of the paper. M.R. and R.C initiated the study, provided financial support, supervised the study and reviewed the paper. L.M. conceived the study, contributed to the analysis of the data and to the writing of the paper.

Additional Information

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.