Time dependence of electric field effects on cell membranes. 
A review for a critical selection of pulse duration for therapeutical applications

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Background. Electropulsation is one of the non-viral methods successfully used to transfer drugs and genes into living cells in vitro as in vivo. This approach shows promise in field of gene and cellular therapies. This presentation first describes the temporal factors controlling electroporation to small molecules (< 4kDa) and then the processes supporting DNA transfer in vitro. The description of in vitro events brings our attention on the processes occurring before (s), during (ms) and after electropulsation (ms to hours) of DNA and cells. They all appear to be multistep events with well defined kinetics. They cannot be described as just punching holes in a lipid matrix in a two states process.

Conclusions. The faster events (may be starting on the ns time scale) appear to be under the control of the external field while the slower ones are linked to the cell metabolism. Investigating the associated collective molecular reorganization by fast kinetics methods and molecular dynamics simulation will help in their safe developments for the in vivo processes and their present and potential clinical applications.

Key words: electropulsation; electroporation; electrotransfection; electroporation

Introduction
The application of electric field pulses to cells leads to the transient permeabilization of the membrane (electropermeabilization).1 This phenomenon brings new properties to the cell membrane: it becomes permeabilized, fusogenic and exogenous membrane proteins can be inserted. It has been used to introduce a large variety of molecules into many different cells in vitro.2,3 Clinical applications of the electropermeabilization are now under development as a results of the EU Cliniporator and Esope programs. A local antitumoral drug delivery to patients (a method called electrochemotherapy) is under clinical trial.4-8 Transdermal drug delivery is obtained in vivo.9 More recently, electropermeabilization has been also used to transfer DNA in vivo, into the skin, liver, melanoma and skeletal muscle cells.10-15 It
Electropermeabilization

Theory of membrane potential difference modulation.

An external electric field modulates the membrane potential difference.\(^1\) From the physical point of view, a cell can be described as a spherical capacitor which is charged by the external electrical field. The transmembrane potential difference induced by the electric field, \(\Delta \Psi_i\), is a complex function \(g(\lambda)\) of the specific conductivities of the membrane \((\lambda_{m})\), the pulsing buffer \((\lambda_{out})\) and the cytoplasm \((\lambda_{cyt})\), the membrane thickness and the cell size. Thus,\(^1\)

\[
\Delta \Psi_i = f. g (\lambda). r. E.\cos \theta \tag{1}
\]

in which \(\theta\) designates the angle between the direction of the normal to the membrane at the considered point on the cell surface and the field direction, \(E\) the field intensity, \(r\) the radius of the cell and \(f\), which is a shape factor (a cell being a spheroid). Therefore, \(\Delta \Psi_i\) is not uniform on the cell surface. It is maximum at the positions of the cell facing the electrodes. These physical predictions were checked experimentally by videomicroscopy by using the potential difference sensitive fluorescent probes.\(^{19-21}\)

The pulse duration plays a critical role when shorter than the capacitive loading time of the membrane. In the previous part of the paper, it was considered that the pulse was long enough to bring the potential steady state value. The loading time \(\tau_{load}\) brings a limit in this description.\(^1\)

\[
\Delta \Psi_i = f. g (\lambda). r. E.\cos \theta \left(1 - \exp \left(- \frac{t}{\tau_{load}} \right) \right) \tag{2}
\]

Assuming that the membrane is a true dielectric with no electric leak, the loading time. \(\tau_{load}\), is given by

\[
\tau_{load} = rC_m \left(1/2\lambda_{out} + 1/\lambda_{cyt}\right) \tag{3}
\]

\(C_m\) is the membrane capacitance, \(\lambda_{out}\) and \(\lambda_{cyt}\) respectively, the conductance of the external buffer and of the cytoplasm. \(\tau_{load}\) is longer for larger cells in a heterogeneous population. Longer pulses are needed to reach the asymptotic electrically induced transmembrane voltage value (Eq. 1). A key assumption in this physical description is that the electric pulse is a sharp square wave.\(^{22}\) This description is under the assumption that the cell is a sphere. A more complex description is needed for spheroidal cells and their orientation relative to the field has to be taken into account.\(^{23,24}\)

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$\tau = r \ C_m \ (\lambda_{\text{c}} + 2 \lambda_{\text{o}}) / (2 \ \lambda_{\text{c}} \lambda_{\text{o}} + r \lambda_{\text{m}} \ (\lambda_{\text{c}} + 2 \ \lambda_{\text{o}})/d)$ \[4\]

As $\lambda_{\text{m}}$ is dependent on the membrane leakiness, the loading time of the membrane will decrease with an increase in the membrane leakiness. The pulse duration plays a more critical role in such a case. But under physiological conditions, where $\lambda_{\text{o}}$ is larger than 10 mS/cm, as $\lambda_{\text{c}}$ is about 4 mS/cm, $\tau_{\text{load}}$ is always of the order of 1 $\mu$s for mammalian cells.

**Critical parameters affecting electroporation**

**Effects of the electric field parameters.** When the resulting transmembrane potential difference $\Delta \Psi (i.e. the sum between the resting value of cell membrane $\Delta \Psi_0$ and the electroinduced value $\Delta \Psi_i$) reaches threshold values close to 250 mV, membranes become permeable.$^{25-26}$

Permeabilization is controlled by the field strength. Field intensity larger than a critical value ($E_p$) must be applied to the cell suspension. From Eq. [1], permeabilization is first obtained for $\theta$ close to 0 or $\pi$. $E_p$ is such that:

$\Delta \Psi_{\text{perm}} = f \ g \ (\lambda) \ r \ E_p$ \[5\]

Parts of the cell surface facing the electrodes are affected. The extent of the permeabilized surface of a spherical cell, $A_{\text{perm}}$, is given by:

$A_{\text{perm}} = A_{\text{tot}} \ (1 - E_p/E)/2$ \[6\]

where $A_{\text{tot}}$ is the cell surface and $E$ is the applied field intensity. Increasing the field strength (decreasing $E_p/E$) will increase the part of the cell surface, which is brought to the electroporation state. This critical value of the transmembrane potential will be reached after a longer delay for the edges of the cap due to the loading time. But this delay remains always in the $\mu$s time scale. This will affect the mechanism of electroporation only for a very short pulse duration.

These theoretical predictions were assayed on cell suspension by measuring the leakage of metabolites (ATP)$^{27}$ or observed at the single cell level by digitised fluorescence microscopy.$^{28,29}$ The experimental results are in agreement with the predictions. The field strength must be larger than the threshold value $E_p$ to induce permeabilization. The permeabilized part of the cell surface is a linear function of the reciprocal of the field intensity. Permeabilization, due to structural alterations of the membrane, remained restricted to a cap on the cell surface when short lived pulses (microseconds) are applied. The area affected by the electric field depends also on the shape (spheroid) and on the orientation of the cell with the electric field lines.$^{24}$ If a train of 10 pulses is applied at a frequency of 1 Hz, it is observed that long pulses (more than 1 ms) slightly larger than $E_p$ bring a permeabilization on two caps on the cell surface, each facing one electrode.

Experimental results obtained either by monitoring conductance changes on cell suspension$^{34}$ or by fluorescence observation at the single cell level microscopy$^{28,29}$ shows that the local level of permeabilization is strongly controlled by the pulse duration.$^{27,28}$

As an electrical current is flowing, Joule heating is taking place. The temperature of the sample increases as a linear function of the pulse duration and of the square of the field intensity. In vitro, this deleterious by-effect is controlled by using a low ionic content pulsing buffer to deliver a limited amount of energy. This of course cannot be controlled by that means in vivo but the tissue can be considered as a heat sink.

Sieving of electropermeabilization

Electropermeabilization allows a post-pulse free-like diffusion of small molecules (up to 4 kDa) whatever their chemical nature. There is a size limit for permeabilization and the process for macromolecules is described in the second part of the text. Polar small compounds cross easily the electropermeabilized membrane. But the most important feature is that this membrane organisation is long-lived in cells. Diffusion is observed during the seconds and minutes following the ms pulse. Most of the exchange takes place after the pulse.\(^28,29\) Resealing of the membrane defects and of the induced permeabilization is a first order process, which appears to be controlled by protein reorganisation. For a given cell, the resealing time (reciprocal of k) is a function of the pulse duration but not of the field intensity as checked by digitised videomicroscopy.\(^27\) A precise analysis shows that several resealing processes are acting, two are very fast (ms, <s) while the last one remains present during several minutes at room temperature.\(^30\) Resealing of membrane defects is a metabolic process under control of the energy reserves of the cell.\(^31\) One can take advantage of this slow resealing to deliver the successive pulses at a 1kHz frequency to reduce the duration of the treatment in ECT.\(^32\)

These observations are in agreement with a model where the target of the field is under the control of the pulse duration. Due to their internal flexibility (mainly at the level of the polar heads), phospholipids are sensitive to short pulses. Their change in configuration brings an effect on the membrane proteins. The transmembrane ionic exchange induces a secondary effect on the cell organization. When long pulses are applied, they can induce long range reorganization (an electrophoretic drift of polar membrane components) and a direct effect on more rigid dipoles such as those associated with membrane proteins. Long pulses are not acting only on phospholipids but may affect directly the organization of membrane proteins.\(^33\) Of course, the transmembrane exchange will be larger as the density of membrane defects will be increased.

Loading of macromolecules is a more complex mechanism, that will be described in details later.

Associated transmembrane exchange

Molecular transfer of small molecules (<4kDa) across the permeabilized area is mostly driven by the concentration difference across the membrane. Electrophoretic contribution to the transmembrane exchange during the pulse affects the loading of polar compounds.\(^29,30\) Free diffusion of low weight polar molecules after the pulse can be described by using the Fick equation on its electropermeabilized part.\(^27\) It plays the most important contribution to the loading. This gives the following expression for a given molecule S and a cell with a radius r:

\[
\Phi(S) = \frac{2\pi r^2 P_S \Delta S X(N, T) (1 - Ep/E) \exp(-k(N, T) t)}{\text{[7]}}
\]

where \(\Phi(S)\) is the flow at time t after the N pulses of duration T (the delay between the pulses being short compared to t), \(P_S\) is the permeability coefficient of S across the permeabilized membrane and \(\Delta S\) is the concentration gradient of S across the membrane. Ep depends on r (size). Permeabilization remains present for a longer time when cells are kept at low temperature. The cytoskeletal integrity plays also a major role.\(^33\)

Cellular responses

Reactive oxygen species (ROS) are generated at the permeabilized loci, depending
on the electric field parameters. These ROS can affect the viability. The amount of ROS is increased with an increase in pulse duration. Long pulses may therefore be toxic for cells.

When a cell is permeabilized, a transient osmotic swelling may result leading to an entrance of water into the cell. This increase of cell volume can lead to the rupture of the membrane. This swelling is under the strong control of the pulse duration. Using μs pulse does not trigger swelling (with the exception of red blood cells, the so called osmotic swelling) while a twofold increase in volume was reported when using ms pulses.

There is a loss of the bilayer membrane asymmetry of the phospholipids.

Carry-home messages on permeabilization

When cells are submitted to short lived electric field pulses, a free exchange of hydrophilic molecules takes place across the membrane. A leakage of cytosolic metabolites into the cytoplasm is obtained. Nevertheless, cell viability can be preserved under controlled electric field conditions. More drastic electric conditions affect strongly the cell viability. This effect is cell specific and some strains are weakly resistant to the electric trauma. Bringing them to a reversible permeabilized state needs a careful tuning of the electric parameters. The exchange of the hydrophilic compounds is strongly controlled by the pulse duration. Indeed the pulse duration plays a decisive role in the level of loading as it controls i) the density of permeabilized defects (X in Eq. 7) ii) the life time of these defects (k in Eq. 4). Short pulses will induce few short lived defects and a very limited loading (that cannot even be detected in many conditions). A larger loading (leakage) of the cytoplasmic content will occur with long pulses. The cell viability will be preserved more when using short pulses.

Clinical applications are supported either by the loading of therapeutic compounds (bleomycin) or by irreversible permeabilization (IRE). Electrochemotherapy is obtained by a low number of successive short lived pulses (100 μs) while IRE requires a much longer treatment. A delay between pulses as short as 1 ms can be observed to reduce the pain of the patient.

DNA electrotransfer

Gene expression is obtained after applying electric pulses to a cell DNA mixture. No transfected cells were detected in absence of electric field, in absence of DNA, or when DNA was added after the pulses.

Electrotransfection was only detected for electric field values leading to permeabilization. Transfection threshold values were the same as the ones for cell permeabilization when pulses lasting ms were applied.

Events during electropulsation:
Membrane –DNA interaction

Field strength is observed to have a critical role. Cell membrane must be permeabilized for plasmid-membrane interaction to occur. Plasmids interact only with the permeabilized cell surface. It is accumulated by the field associated electrophoretic drag as shown by fluorescence microscopy. But no free plasmid diffusion into the cytoplasm is detected while this was proposed in older works. No plasmid membrane interaction occurs if the nucleic acids are added after electropermeabilizing cells as proposed in. Negatively charged DNA molecules migrate when submitted to an electric field. But, electrophoretic DNA accumulation by itself is not enough to
bring transmembrane transfer and gene expression whatever the pulse duration.

Under permeabilizing field conditions, the pulse duration plays a critical role in the formation of the plasmid-cell complex. The complex between the plasmid and the cell surface is detected only when the pulse duration is at least 1 ms. This suggests that the density of defects is critical in the interaction between DNA and the permeabilized membrane.

Furthermore, this interpretation is supported by the observation that the DNA content in the complex, determined by the local fluorescence emission, is under the control of the field strength and the pulse duration.45

The reaction time of the DNA pushed against the part of the cell surface under the permeabilizing stress of the external field is increased by a longer pulse duration. This again is involved in the positive role of the pulse duration in gene electrotransfer.

This contribution of the pulse duration to the plasmid-membrane interaction has already been illustrated by a complex dependence of the gene expression.43 The associated gene expression \( \text{Expr} \) is shown to obey the following equation:

\[
\text{Expr} = K N T^{2.3} (1 - \frac{E_p}{E}) f(\text{DNA})
\]  

as long as the cell viability is not affected to a large extent by the pulse duration.45 All parameters are as described above, K being a constant. The dependence on the plasmid concentration (ADN) is rather complex as high levels of plasmids appear to be toxic.47

A very recent on line videomicroscopy study showed that plasmid DNA was trapped in the electropерmeabilized membrane where it forms aggregates.

The practical conclusion is that in vitro an effective transfer is obtained by using long pulses in order to drive the DNA towards the permeabilized area of the membrane by electropermeabilization but with a low field strength to preserve the cell viability.44,48 Nevertheless, the transfection was obtained with short strong pulses in the pioneering experiments 18 and with stem cells.49

Events after electropulsation

The main conclusion of the observations during the pulse is that plasmids do not cross the membrane during that step, even if the membrane is permeabilized (for small molecules). They form complexes at the permeabilized membrane level. More than 2 s appears to be needed to get a stable DNA membrane complex after a 5 ms pulse.66

Cell electroassociated DNA remains accessible to DNAaseI, a double-strand nucleic acid degrading enzyme, up to 60 s after the pulsation in the case of CHO cells.3 The DNA aggregates, which are anchored in the membrane after the electric field application, remain sensitive to the degrading action of the externally post pulse added nucleases, which are known not to cross the membrane.

High field nanosecond field pulses cellular effect

Under classical electropulsation conditions (micro-millisecond duration, a few kV/cm magnitude), the transmembrane voltage change is only present on the plasma membrane. The interior of the cell is shielded from the external field by the plasma membrane. The electrical behaviour of the cell is different under strong HV nanosecond lasting pulses. A fast charging capacitive effect is present.50 Two membrane capacitances, \( C_1 \) for the outer cell plasma membrane and \( C_2 \) for the inner organelle membrane, are being charged by currents from the exter-
nal voltage source. Charging of the external capacitance induces a transient voltage across the cytoplasm.\textsuperscript{52} It results a charging of the organelle capacitance. As the size of the organelle is very small and the conductivity of the cytoplasm is higher than that of the external buffer, the charging time of the organelle capacitor is much faster than that of the plasma membrane capacitor.\textsuperscript{51} The electric field induced transmembrane permeabilizing voltage is reached for the organelle as well for the plasma membrane.\textsuperscript{52} As soon as the plasma membrane is electropermeabilized, it is short circuited and only a fraction of the external field remains present on the organelles.\textsuperscript{50,51}

Another consequence of ultrashort pulses is the induction of an electrodeformation of the cell (electrostretching). The magnitude of this stress is high under low conductivities conditions. It brings an electrostretching of the membrane. This is supposed to contribute to the expansion step in the electropermeabilization. The induction of the force is very fast and is transiently present in the nanosecond range when a low conductivity buffer is present. The effect is not present when the buffer conductivity is close to the one of the cytoplasm.\textsuperscript{53,54} This stretching effect can be present only when the rise time of the electric pulse is very fast (ns). This is not the case with pulse generators routinely used nowadays. But this effect should be kept in mind with the new developments of ns HV pulse generators.

As a consequence, organelles can be electropermeabilized by nanosecond long pulses. This is indeed what is observed. Cytoplasmic stored Calcium pools are observed to be released by nanosecond pulses. Permeabilization of organelles can be used to trigger apoptosis and cell death without direct leak from the cytoplasm. This was proposed as an approach to destroy tumor cells.\textsuperscript{52,55-58} Preclinical studies of drug free tumor eradication were reported.\textsuperscript{59,60}

The nuclear enveloppe can be permeabilized. Therefore, a new strategy for plasmid transfer is present. In a first step, millisecond pulses are used to introduced plasmids in the cytoplasm as classically described. After a 30 min incubation, a nanosecond high strength field is applied to induce the destabilization of the nucleus membrane and to facilitate the nuclear transfer of the plasmid and its expression. A significant increase of expression can be apparently obtained.\textsuperscript{61}

This new methodology is a “hot” field of investigations where encouraging results have been published. But it is not yet a mature field as “classical” electropulsation.

**Conclusions**

“Classical” Electropermeabilization processes can be followed from microseconds up to days. Kinetic studies of electropermeabilization led to a description in 5 steps:

1- “**Induction step**”- The field induced the membrane potential difference increase which gave local defects (may be due to kinks in the lipid chains) when it reached a critical value (about 200 mV). A mechanical stress was present with a magnitude that depends on the buffer composition. This can be detected in less than 1 nanosecond but a limit is given by the charging time of the membrane. Molecular dynamic simulation suggests that it is much faster \textsuperscript{62} in agreement with the nanosecond experiments (μs to ns due to the pulse generator).

2- “**Expansion step**”- These defects expanded as long as the field was present and with a strength larger than a critical value. Again an electromechanical stress remained present. This may be due to a “coalescence” process (μs to ms)

3- “**Stabilisation step**”- As soon as the field intensity was lower than the threshold value, that is mentioned in step 1, stabilisa-
tion processes were taking place within a few milliseconds, which brought the membrane to the permeabilized state for small molecules (ms).

4- “Resealing step”- A slow resealing was then occurring on a scale of seconds and minutes. It was a first order process. It is driven by the cellular metabolism (s to min).

5- “Memory effect “- Some changes in the membrane properties remained present on a longer time scale (hours) but the cell behaviour was finally back to normal.

Experiments showed that the mechanism of DNA transfer was different from what was observed for electroporation (transfer of small molecules). Indeed, experimental results led to the conclusion that plasmids had to be present during electroporation but crossed the electropulsed membrane in the minutes following it. No gene transfer was detected with a post-pulse DNA addition. These results were obtained on bacteria, yeast and mammalian cells.37,63,64

We proposed a model in which electroporation appears as a multistep process 43 and brought its direct experimental evidence.45.

During the pulse,

i- electropermeabilization takes place (μs)

ii- plasmids are electrophoretically driven into contacts with the cell surface (ms)

iii- a metastable complex is formed between plasmids and the localised electropermeabilized part of the cell membrane (ms).

iv- a stable complex results (s)

v- long after the pulse, plasmids left the complex and diffused in the cytoplasm (min)

vi- a small fraction crossed the nuclear envelope to be expressed (h).

This interaction between plasmids and electroporateelizabizd membrane is strongly controlled by the pulse duration (always on the ms time scale). Additive effects of successive pulses are obtained. Only the localised part of the cell membrane brought to the permeabilized state by the external field is competent for the transfer.43

**Practical consequences**

Electropermeabilization is one of the non-viral methods successfully used to transfer genes into living cells *in vitro as in vivo*. It has the main advantages of being easy to perform, fast, reproducible and safe. This approach appears promising for gene therapy and is a clinical routine for drug delivery. A careful choice of the pulse duration and delays between the successive steps of electroporation is needed to get successful applications (Table 1).65 This remains linked to phenomenological conclusions. Its further developments need a better understanding of the basic effects induced at the membrane, cellular and tissue levels by electrical events and the plasmid entry in the cell.

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**Table 1. Operating parameters for the *in vitro* applications of electroporation on mammalian cells**

<table>
<thead>
<tr>
<th>Applications</th>
<th>Duration</th>
<th>Field strength</th>
<th>Number of pulses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electrochemotherapy (ECT)</td>
<td>100 μs</td>
<td>1.3 kV/cm</td>
<td>8</td>
</tr>
<tr>
<td>Electroporation (EGT)</td>
<td>5 to 10 ms</td>
<td>0.5 to 0.8 kV/cm</td>
<td>8</td>
</tr>
<tr>
<td>Eradication (IRE)</td>
<td>300 μs</td>
<td>1.5 kV/cm</td>
<td>3 x 10</td>
</tr>
<tr>
<td>Nanopulse eradication (nsIRE)</td>
<td>60 ns</td>
<td>12 kV/cm</td>
<td>200</td>
</tr>
</tbody>
</table>

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Acknowledgements

This work was supported by the CNRS, the AFM (Association française pour les myopathies), the région Midi Pyrénées and the Proteus Slovenia-CNRS exchange program.

The authors wish to thank their colleagues: L. Mir in Villejuif (France), D. Miklavcic, T. Kotnik, M. Cemazar, G. Sersa and G. Pucihar, all from Ljubljana (Slovenia) and E. Neumann in Bielefeld (Germany) for so many helpful discussions.

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