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review

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 electropermeabilization 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; electropermeabilization; 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

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has the main advantages of being easy to use, fast, reproducible and safe.

While during 30 years due to technological limits, pulse duration was always larger than 1 microsecond, the recent availability of high voltage (tens of kV) nanosecond long pulse generators opens the way to a new approach. Very fast perturbations under strong fields are induced in the membrane organization.[16,17] A new field of development is now present for electropermeabilization and promising results for clinical applications were reported.

One of the limiting problems remains that very few is known on the physicochemical mechanisms supporting the reorganisation of the cell membrane. The molecular target of the field effect remains unclear.

The present review focuses on the critical role played by the pulse duration in the electropermeabilization to small molecules (< 4kDa) and on its support to the processes associated to DNA transfer *in vitro*. Pulse durations are easy to adjust for an optimization of the clinical target: electrochemotherapy, irreversible electropermeabilization or gene therapy as suggested as a final conclusion.

Electropermeabilization

Theory of membrane potential difference modulation.

An external electric field modulates the membrane potential difference.[18] 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 (λ_m), the pulsing buffer (λ_{out}) and the cytoplasm (λ_{cyt}), the membrane thickness and the cell size. Thus,[1]

$$\Delta\Psi i = f.\ g(\lambda).\ r.\ E.\cos\theta \qquad [1]$$

in which θ 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 τ_{load} brings a limit in this description.[1]

$$\Delta\Psi i = f.\ g(\lambda).\ r.\ E.\cos\theta\ (1 - \exp(-t / \tau_{load})) \qquad [2]$$

Assumming that the membrane is a true dielectric with no electric leak, the loading time. τ_{load}, is given by

$$\tau_{load} = rC_m\ (1/2\lambda_{out} + 1/\lambda_{cyt}) \qquad [3]$$

C_m is the membrane capacitance, λ_{out} and λ_{cyt}, respectively, the conductance of the external buffer and of the cytoplasm. τ_{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]

The membrane leakiness affects the loading time of the membrane when the field is applied.[1] Its physical definition is given in [26] by:

$$\tau = r\,Cm\,(\lambda_{cyt} + 2\lambda_{out}) / (2\,\lambda_{cyt}\lambda_{out} + r\lambda_m\,(\lambda_{cyt} + 2\,\lambda_{out})/d) \qquad [4]$$

As λ_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 λ_{out} is larger than 10 mS/cm, as λ_{cyt} is about 4 mS/cm, τ_{load} is always of the order of 1 μs for mammalian cells

Critical parameters affecting electropermeabilization

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 $\Delta\Psi o$ 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 (Ep) must be applied to the cell suspension. From Eq. [1], permeabilization is first obtained for θ close to 0 or π. Ep is such that:

$$\Delta\Psi perm = f\,g\,(\lambda)\,r\,Ep \qquad [5]$$

Parts of the cell surface facing the electrodes are affected. The extent of the permeabilized surface of a spherical cell, Aperm, is given by:

$$Aperm = Atot\,(1 - Ep/E)/2 \qquad [6]$$

where Atot is the cell surface and E is the applied field intensity. Increasing the field strength (decreasing Ep/E) will increase the part of the cell surface, which is brought to the electropermeabilized 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 μs time scale. This will affect the mechanism of electropermeabilization 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 Ep 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 Ep 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. I*n 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:

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

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 ΔS 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.[35] 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.[36,37] 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) [38] while a twofold increase in volume was reported when using ms pulses.

There is a loss of the bilayer membrane asymmetry of the phospholipids.[39]

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).[40] 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)[5-7] or by irreversible permeabilization (IRE).[41.]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.[42,43]

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.[44.]

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.[45] But no free plasmid diffusion into the cytoplasm is detected while this was proposed in older works.[42] No plasmid membrane interaction occurs if the nucleic acids are added after electropermeabilizing cells as proposed in.[19,38] Negatively charged DNA molecules migrate when submitted to an electric field.[43,46] 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 Expr is shown to obey the following equation:

$$Expr = K\ N\ T^{2.3}\ (1 - Ep/E)\ f(DNA) \qquad [8]$$

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 electropermeabilized 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.[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.[51] The electric field induced transmembrane permeabilizing voltage is reached for the organelle as well for the plasma membrane.[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.[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.[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.[52,55-58] Preclinical studies of drug free tumor eradication were reported.[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.[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 [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-

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

Applications	Duration	Field strength	Number of pulses
Electrochemotherapy (ECT)	100 μs	1.3 kV/cm	8
Electrotransfection (EGT)	5 to 10 ms	0.5 to 0.8 kV/cm	8
Eradication (IRE)	300 μs	1.5 kV/cm	3 x 10
Nanopulse eradication (nsIRE)	60 ns	12 kV/cm	200

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 electropermeabilization (transfer of small molecules). Indeed, experimental results led to the conclusion that plasmids had to be present during electropulsation 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 Electrotransfection 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 electropermeabilized 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 electropulsation 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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References

1. Neumann E, Sowers AE, Jordan CA. Electroporation and electrofusion in cell biology. York: Plenum press; 1989.

2. Orlowski S, Mir LM. Cell electropermeabilization: a new tool for biochemical and pharmacological studies. *Biochim Biophys Acta* 1993; **1154:** 51-63.

3. Eynard N, Rols MP, Ganeva V, Galutzov B., Sabri N, Teissié J. Electrotransformation pathways of procaryotic and eucaryotic cells: recents developments. *Bioelectrochem Bioenerg* 1997; **44:** 103-10.

4. Mir LM, Roth C, Orlowski S, Quintin Colonna F, Fradelizi D, et al. Systemic antitumor effects of electrochemotherapy combined with histoincompatible cells secreting interleukin-2. Systemic antitumor effects of electrochemotherapy combined with histoincompatible cells secreting interleukin-2. J Immunother Emphasis Tumor Immunol 1995; **17:** 30-8 .

5. Cemazar M, Sersa G. Electrotransfer of therapeutic molecules into tissues.*Curr Opin Mol Ther* 2007; **9:** 554-62.

6. Sersa G, Miklavcic D, Cemazar M, Rudolf Z, Pucihar G, Snoj M. Electrochemotherapy in treatment of tumours. *Eur J Surg Oncol* 2008; **34:** 232-40.

7. Mir LM, Gehl J, Sersa G, Collins CG, Garbay JR, Billard V, et al. Standard operating procedures of the electrochemotherapy: Instructions for the use of bleomycin or cisplatin administered either systemically or locally and electric pulses delivered by the CliniporatorTM by means of invasive or non-invasive electrodes *Eur J Cancer 2006;* **42(Suppl 4):** 14-25.

8. Mir LM, Glass LF, Sersa G, Teissie J, Domenge C, Miklavcic D, et al. Effective treatment of cutaneous and subcutaneous malignant tumours by electrochemotherapy. *Br J Cancer* 1998; **77:** 2336-42.

9. Prausnitz MR, Bose VG, Langer R, Weaver JC. Electroporation of mammalian skin: a mechanism to enhance transdermal drug delivery. *Proc Natl Acad Sci USA* 1993; **90:** 10504-8.

10. Titomirov A, Sukarev S, Kistanova E. In vivo electroporation and stable transformation of skin cells of newborn mice by plasmid DNA. *Biochim Biophys Acta* 1991; **1088:** 131-4.

11. Heller R, Jaroszeski M, Atkin A, Moradpour D, Gilbert R, Wands J, et al. In vivo gene electroinjection and expression in rat liver. *FEBS Lett* 1996; **389:** 225-8.

12. Rols MP, Delteil C, Golzio M, Dumond P, Cros S, Teissie J. In vivo electrically mediated protein and gene transfer in murine melanoma. *Nat Biotechnol* 1998; **16:** 168-71.

13. Aihara H, Miyazaki J. Gene transfer into muscle by electroporation in vivo. *Nat Biotechnology* 1998; **16:** 867-70.

14. Mir LM, Bureau MF, Rangara R, Schwartz B, Sherman D. Long-term, high level in vivo gene expression after electric pulse- mediated gene transfer into skeletal muscle. *C R Acad Sci III* 1998; **321:** 893-9.

15. Muramatsu T, Nakamura A, Park M. In vivo electroporation: A powerful and convenient means of nonviral gene transfer to tissues of living animals. [Review]. *Int J Mol Med* 1998; **1:** 55-62.

16. Pliquett U, Joshi RP, Sridhara V, Schoenbach KH. High electrical field effects on cell membranes. *Bioelectrochemistry* 2007; **70:** 275-82.

17. Pakhomov AG, Kolb JF, White JA, Joshi RP, Xiao S, Schoenbach KH. Long-lasting plasma membrane permeabilization in mammalian cells by nanosecond pulsed electric field (nsPEF). *Bioelectromagnetics* 2007; **28:** 655-63.

18. Neumann E, Schaefer-Ridder M, Wang Y, Hofschneider PH. Gene transfer into mouse lyoma cells by electroporation in high electric fields. *EMBO J* 1982; **1:** 841-5.

19. Gross D, Loew LM, Webb WW. Optical imaging of cell membrane potential changes induced by applied electric fields. *Biophys J* 1986; **50:** 339-48.

20. Lojewska Z, Farkas DL, Ehrenberg B, Loew LM. Analysis of the effect of medium and membrane conductance on the amplitude and kinetics of membrane potentials induced by externally applied electric fields. *Biophys J* 1989; **56:** 121-8.

21. Hibino M, Shigemori M, Itoh H, Nagayama K, Kinosita K jr. Membrane conductance of an electroporated cell analyzed by submicrosecond imaging of transmembrane potential. *Biophys J* 1991; **59:** 209-20.

22. Kotnik T, Pucihar G, Rebersek M, Miklavcic D, Mir LM. Role of pulse shape in cell membrane electropermeabilization. *Biochim Biophys Acta* 2003; **1614:** 193-200.

23. Pucihar G, Kotnik T, Valic B, Miklavcic D. Numerical determination of transmembrane voltage induced on irregularly shaped cells. *Ann Biomed Eng* 2006; **34:** 642-52.

24. Valic B, Golzio M, Pavlin M, Schatz A, Faurie C, Gabriel B, et al. Effect of electric field induced transmembrane potential on spheroidal cells: theory and experiment. *Eur Biophys J* 2003; **32:** 519-28.

25. Teissié J, Tsong TY. Electric field induced transient pores in phospholipid bilayer vesicles. *Biochemistry* 1981; **20:** 1548-54.

26. Teissié J, Rols MP. An experimental evaluation of the critical potential difference inducing cell membrane electropermeabilization. *Biophys J* 1993; **65:** 409-13.

27. Rols MP, Teissie J. Electropermeabilization of mammalian cells. Quantitative analysis of the phenomenon. *Biophys J* 1990; **58:** 1089-98.

28. Gabriel B, Teissie J. Direct observation in the millisecond time range of fluorescent molecule asymmetrical interaction with the electropermeabilized cell membrane. *Biophys J* 1997; **73:** 2630-7.

29. Gabriel B, Teissie J. Time courses of mammalian cell electropermeabilization observed by millisecond imaging of membrane property changes during the pulse. *Biophys J* 1999; **76:** 2158-65.

30. Pucihar G, Kotnik T, Miklavic D, Teissie J. Kinetics of transmembrane transport of small molecules into electropermeabilized cells. *Biophys J* 2008; **95:** 2837-48.

31. Rols MP, Delteil C, Golzio M, Teissie J. Control by ATP and ADP of voltage-induced mammalian-cell-membrane permeabilization, gene transfer and resulting expression. *Eur J Biochem* 1998; **254:** 382-8.

32. Pucihar G, Mir LM, Miklavcic D. The effect of pulse repetition frequency on the uptake into electropermeabilized cells in vitro with possible applications in electrochemotherapy. *Bioelectrochemistry* 2002; **57:** 167-72.

33. Teissie J, Rols MP. Manipulation of cell cytoskeleton affects the lifetime of cell membrane electropermeabilization. *Ann N Y Acad Sci* 1994; **720:** 98-110.

34. Kinosita K Jr, Tsong TY. Voltage-induced conductance in human erythrocyte membranes. *Biochim Biophys Acta* 1979; **554:** 479-97.

35. Gabriel B, Teissie J. Generation of reactive-oxygen species induced by electropermeabilization of Chinese hamster ovary cells and their consequence on cell viability. *Eur J Biochem* 1994; **223:** 25-33.

36. Kinosita K Jr,Tsong TY. Hemolysis of human erythrocytes by transient electric field. *Proc Natl Acad Sci USA* 1977; **74:** 1923-7.

37. Golzio M., Mora MP, Raynaud C, Deltei C, Teissie J, Rols MP. Control by osmotic pressure of voltage-induced permeabilization and gene transfer in mammalian cells. *Biophys J* 1998; **74:** 3015-22.

38. Tsong TY. Electroporation of cell membranes. *Biophys J* 1991; **60:** 297–306.

39. Haest CW, Kamp D, Deuticke B. Transbilayer reorientation of phospholipid probes in the human erythrocyte membrane. Lessons from studies on electroporated and resealed cells. *Biochim Biophys Acta* 1997; **1325:** 17-33.

40. Rols MP, Teissie J. Electropermeabilization of mammalian cells to macromolecules: control by pulse duration. *Biophys J* 1998; **75:** 1415-23.

41. Al-Sakere B, Andre F, Bernat C, Connault E, Opolon P, Davalos RV, et al. Tumor ablation with irreversible electroporation. *PLoS ONE* 2007; **2(11):** e1135.

42. Klenchin VA, Sukharev SI, Serov SM, Chernomordik LV, Chizmadzev YuA Electrically induced DNA uptake by cells is a fast process involving DNA electrophoresis. *Biophys J* 1991; **60:** 804-11.

43. Wolf H, Rols MP, Boldt E, Neumann E, Teissie J. Control by pulse parameters of electric field-mediated gene transfer in mammalian cells. *Biophys J* 1994; **66:** 524-31.

44. Rols MP, Teissie J. Electropermeabilization of mammalian cells to macromolecules: control by pulse duration. *Biophys J* 1998; **75:** 1415-23.

45. Golzio M, Teissie J, Rols MP. Direct visualization at the single-cell level of electrically mediated gene delivery. *Proc Natl Acad Sci U S A* 2002; **99:** 1292-7.

46. Neumann E, Werner E, Sprafke A, Kruger K. Electroporation phenomena. Electrooptics of plasmid DNA and of lipid bilayer vesicles. In: Jennings BR, Stoylov SP, editors. Colloid and molecular electro-optics. Bristol: IOP publishing LTD; 1992. p. 197-206.

47. Rols MP, Coulet D, Teissie J. Highly efficient transfection of mammalian cells by electric field pulses. Application to large volumes of cell culture by using a flow system. *Eur J Biochem* 1992; **206:** 115-21.

48. Kubiniec RT, Liang H, Hui SW. Effects of pulse length and pulse strength on transfection by electroporation. *Biotechniques* 1990; **8:** 16-20.

49. Ferreira E, Potier E, Logeart-Avramoglou D, Salomskaite-Davalgiene S, Mir LM, Petite H. Optimization of a gene electrotransfer method for mesenchymal stem cell transfection. *Gene Ther* 2008; **15:** 537-44.

50. Frey W, White JA, Price RO, Blackmore PF, Joshi RP, Nuccitelli R, et al. Plasma membrane voltage changes during nanosecond pulsed electric field exposure. *Biophys J* 2006; **90:** 3608-15.

51. Kotnik T, Miklavcic D. Theoretical evaluation of voltage inducement on internal membranes of biological cells exposed to electric fields. *Biophys J* 2006; **90:** 480-91.

52. Tekle E, Oubrahim H, Dzekunov SM, Kolb JF, Schoenbach KH, Chock PB. Selective field effects on intracellular vacuoles and vesicle membranes with nanosecond electric pulses. *Biophys J* 2005; **89:** 274-84.

53. Muller KJ, Sukhorukov VL, Zimmermann U. Reversible electropermeabilization of mammalian cells by high-intensity, ultra-short pulses of submicrosecond duration. *J Membr Biol* 2001; **184**: 161-70.

54. Sukhorukov VL, Mussauer H, Zimmermann U. The effect of electrical deformation forces on the electropermeabilization of erythrocyte membranes in low- and high-conductivity media. *J Membr Biol* 1998; **163:** 235-45.

55. Beebe SJ, Fox PM, Rec LJ, Willis LK, Schoenbach KH. Nanosecond high intensity pulsed electric fields induce apoptosis in human cells. *FASEB J* 2003; **30:** 286–292.

56. Vernier TP, Sun YH, Marcu L, Craft CM, Gundersen MA. Nanoelectropore-induced phosphatidylserine translocation. *Biophys J* 2004; **86:** 4040–8.

57. Brown FD, Rozelle AL, Yin HL, Balla T, Donaldson JG. Phosphatidylinositol 4,5-bisphosphate and Arf-regulated membrane traffic. *J Biol Chem* 2001; **154:** 1007–17.

58. White JA, Blackmore PF, Schoenbach KH, Beebe SJ. Stimulation of capacitative calcium entry in HL-60 cells by nanosecond pulsed electric fields. *J Biol Chem* 2004; **279:** 22964–72.

59. Nuccitelli R, Pliquet U, Chen X, Ford W, James Swanson R, Beebe SJ, et al. Nanosecond pulsed electric fields cause melanomas to self-destruct. *Biochem Biophys Res Commun* 2006; **343:** 351-60.

60. Garon EB, Sawcer D, Vernier PT, Tang T, Sun Y, Marcu L, et al. .In vitro and in vivo evaluation and a case report of intense nanosecond pulsed electric field as a local therapy for human malignancies. *Int J Cancer* 2007; **121:** 675-82.

61. Beebe SJ, White J, Blackmore PF, Deng Y, Somers K, Schoenbach KH. Diverse effects of nanosecond pulsed electric fields on cells and tissues. *DNA Cell Biol* 2003; **22:** 785-96.

62. Tarek M. Membrane electroporation: a molecular dynamics simulation. *Biophys J* 2005; **88:** 4045-53.

63. Eynard N, Sixou S, Duran N, Teissie J. Fast kinetics studies of Escherichia coli electrotransformation. *Eur J Biochem* 1992; **209:** 431-6.

64. Ganeva V, Galutzov B, Teissie J. Fast kinetic studies of plasmid DNA transfer in intact yeast cells mediated by electropulsation. *Biochem Biophys Res Commun* 1995; **214:** 825-32.

65. Lebar AM, Sersa G, Kranjc S, Groselj A, Miklavcic D. Optimisation of pulse parameters in vitro for in vivo electrochemotherapy. *Anticancer Res* 2002; **22:** 1731-6.

66. Phez E, Faurie C, Golzio M, Teissié J, Rols MP. New insights in the visualization of membrane permeabilization and DNA/membrane interaction of cells submitted to electric pulses. *Biochim Biophys Acta* 2005; **1724:** 248-54.