November 15-21, 2009
Ljubljana, Slovenia

Proceedings of the

# Electroporation based Technologies and Treatments

International SCIENTIFIC WORKSHOP and POSTGRADUATE COURSE


**Edited by:**

Peter Kramar
Damijan Miklavčič
Lluis M. Mir


**Organised by:**

University of Ljubljana
Faculty of Electrical Engineering

Institute of Oncology, Ljubljana

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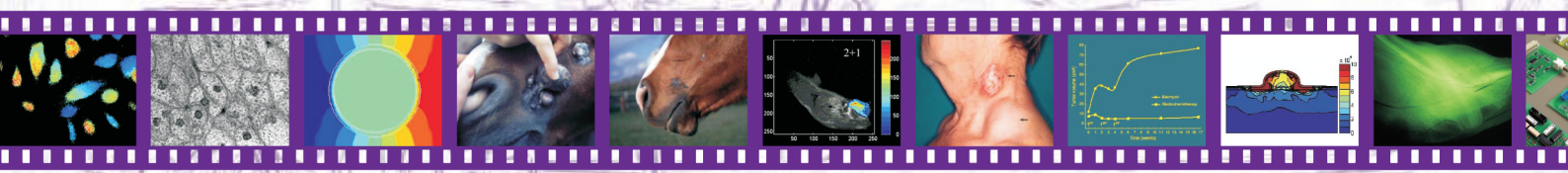

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# Welcome note

Dear Colleagues, Dear Students,

The idea of organizing the Workshop and Postgraduate Course on Electroporation Based Technologies and Treatments at the University of Ljubljana had been developing for several years. After preliminary discussions, the Workshop and Course was organised for the first time in 2003. It is now for the fourth time that we meet. Again it is with great pleasure that we can say: »with participation of many of the world leading experts in the field«. The intended audience are all those interested in applications of electroporation *in vitro*, *in vivo*, and in clinical environment and with first clinical reports. The most advanced among these applications are electrochemotherapy of tumors, which has already paved its way into clinical environment, and electroporative assisted drug and gene delivery, which is becoming more and more widely used in the experimental environment. The aim of the lectures at this Workshop and Course is to provide the participants with sufficient theoretical background and practical knowledge to allow them to use electroporation effectively in their working environments.

The consequences of exposing a cell to electric pulses are changes in membrane structure which facilitate the transmembrane flow of molecules that otherwise cross the membrane only in minute amounts, if at all. Electroporation can be used in all kinds of isolated cells as well as in tissues. The electric field to which one exposes the target cell has to be of sufficient strength, and the exposure of sufficient duration. The magnitude of electric field to be used depends on cell type, size, orientation and density, pulse duration and number of pulses. The selection of pulse parameters is influenced also by the size and type of molecule that we intend to internalize. Depending on the location and size of the targeted tissue, electric pulses will be delivered via appropriate electrodes chosen among a number of different types. Geometry and positioning of electrodes affect electric field distribution, which is important for effective *in vivo* electropermeabilization.

This year the program of lectures also includes lectures on recent safety issues and toxicity of in vivo reversible electroporation, as well as on the principles and applications (preclinical and clinical) of non-thermal irreversible electroporation. Recent developments on the delivery of ultra-short, high-intensity pulsed electrical will also constitute the core of two lectures: these pulses permeabilize the plasma membrane as well as the cell internal membranes, and they constitute a new promising tool for cell electromanipulation.

Finally, we would like to express our sincere thanks to the colleagues working in our and collaborating laboratories, to the agencies that have been sponsoring our research work for years, and to Slovenian Research Agency, Bioelectrochemical Society, Institute for technology transfer of the University of Bielefeld, and to the CliniGene Network of Excellence for their financial support. We also would like to thank Igea (Italy), Iskra Medical (Slovenia) and BIA separations (Slovenija) whose financial support allowed us to assist many students participating in this Workshop and Course by waiving their fee or providing them with accommodation.

Thank you for participating in our Workshop and Course. We sincerely hope that you will benefit from being with us both socially and professionally.

Sincerely Yours,

*Damijan Miklavčič and Lluis M. Mir*

# LECTURERS' ABSTRACTS

# Biological Cells in Electric Fields

Tadej Kotnik
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**Abstract:** This introductory lecture describes the basic interactions between biological cells and electric fields. Under physiological conditions, a resting voltage in the range of tens of millivolts is continually present on the cell membrane. An exposure of the cell to an external electric field induces an additional component of transmembrane voltage, proportional to the strength of the external field and superimposing onto the resting component for the duration of the exposure. Unlike the resting voltage, the induced voltage varies with position, and also depends on the shape of the cell and its orientation with respect to the electric field. In cell suspensions, it also depends on the volume fraction occupied by the cells. There is a delay between the external field and the voltage induced by it, typically somewhat below a microsecond, but larger when cells are suspended in a low-conductivity medium. As a consequence of this delay, for exposures to electric fields with frequencies above 1 MHz, or to electric pulses with durations below 1 μs, the amplitude of the induced voltage starts to decrease with further increase of the field frequency or further decrease of the pulse duration. With field frequencies approaching the gigahertz range, or with pulse durations in the nanosecond range, this attenuation becomes so pronounced that the voltages induced on organelle membranes in the cell interior become comparable, and can even exceed the voltage induced on the plasma membrane.

## THE CELL AND ITS PLASMA MEMBRANE

A biological cell can be considered from various aspects. We will skip the most usual description, that of a biologist, and focus on two more technical ones, electrical and geometrical.

From the electrical point of view, the cell can roughly be described as an electrolyte (the cytoplasm) surrounded by an electrically insulating shell (the plasma membrane). Physiologically, the surroundings of the cell also resemble an electrolyte quite closely. Under such conditions, when a cell is exposed to an external electric field, the electric field in its very vicinity concentrates within the membrane. This results in an electric potential difference across the membrane, termed the induced transmembrane voltage, which superimposes onto the resting transmembrane voltage typically present under physiological conditions. As the electric field vanishes, so does the induced component of transmembrane voltage. This voltage affects the functioning of voltage-gated membrane channels, initiate the action potentials, stimulate cardiac cells, and when sufficiently large, it can also lead to cell membrane electroporation

With rapidly time-varying electric fields, such as waves with frequencies in the megahertz range or higher, or electric pulses with durations in the submicrosecond range, both the membrane and its surroundings have to be treated as materials with both a non-zero electric conductivity and a non-zero dielectric permittivity.

From the geometrical point of view, the cell can be characterized as a geometric body (the cytoplasm) surrounded by a shell of uniform thickness (the membrane). For suspended cells, the simplest model of the cell is a sphere surrounded by a spherical shell. For augmented generality, the sphere can be replaced by a spheroid or an ellipsoid, but in this case, the requirement of uniform thickness complicates the description of the shell substantially. If its inner surface is a spheroid or an ellipsoid, its outer surface lacks a simple geometrical characterization, and vice versa.[1] Fortunately, this complication does not affect the steady-state voltage induced on the plasma membrane of such cells, which can still be determined analytically.

Spheres, spheroids, and ellipsoids may be reasonable models for suspended cells, but not for cells in tissues. No simple geometrical body can model a typical cell in a tissue, and furthermore every cell generally differs in its shape from the rest. With irregular geometries and/or with cells close to each other, the induced voltage cannot be determined analytically, and thus cannot be formulated as an explicit function. This deprives us of some of the insight available from explicit expressions, but using modern computers and numerical methods, the voltage induced on each particular irregular cell can still be determined quite accurately.

## RESTING TRANSMEMBRANE VOLTAGE

Under physiological conditions, a voltage in the range of –90 mV up to –40 mV is always present on the cell membrane [1,2]. This voltage is caused by a minute deficit of positive ions in the cytoplasm

[1] This can be visualized in two dimensions by drawing an ellipse, and then trying to draw a closed curve everywhere equidistant to the ellipse. This curve is not an ellipse, and if one is content with an approximation, the task is actually easier to accomplish by hand than with typical drawing programs on a computer.

relative to the negative ones, which is a consequence of the transport of specific ions across the membrane. The most important actors in this transport are: (i) the Na-K pumps, which export $Na^+$ ions out of the cell and simultaneously import $K^+$ ions into the cell; and (ii) the K leak channels, through which $K^+$ ions can flow across the membrane in both directions. The resting transmembrane voltage reflects the electrochemical equilibrium of the action of these two mechanisms, and perhaps the easiest way to explain the occurrence of this voltage is to describe how the equilibrium is reached.

The Na-K pump works in cycles. In a single cycle, it exports three $Na^+$ ions out of the cell and imports two $K^+$ ions into it. This generates a small deficit of positive ions in the cytoplasm and a gradient of electric potential, which draws positive ions into the cell, and negative ions out of the cell. But at the same time, the pump also generates concentration gradients of $Na^+$ and $K^+$, which draw the $Na^+$ ions into the cell, and the $K^+$ ions out of the cell. The $K^+$ ions are the only ones that possess a significant mechanism of passive transport through the membrane, namely the K leak channels, and through these the $K^+$ ions are driven towards the equilibration of the electrical and the concentration gradient. When this equilibrium is reached, the electrical gradient across the membrane determines the resting transmembrane voltage, which is continually present on the membrane.

The unbalanced ions responsible for the resting transmembrane voltage represent a very small fraction of all the ions in the cytoplasm, so that the osmotic pressure difference generated by this imbalance is negligible. Also, the membrane acts as a charged capacitor, with the unbalanced ions accumulating close to its surface, so that the cytoplasm can in general be viewed as electrically neutral.

## INDUCED TRANSMEMBRANE VOLTAGE

When a biological cell is placed into an electric field, this leads to a local distortion of the field in the cell and its vicinity. As outlined in the introductory section of this paper, due to the low membrane conductivity, in the vicinity of the cell the field is concentrated in the cell membrane, where it is several orders of magnitude larger than in the cytoplasm and outside the cell. This results in a so-called induced transmembrane voltage, which superimposes to the resting component. In the following subsections, we describe in more detail the transmembrane voltage induced on cells of various shapes and under various conditions. In each considered case, the principles of superposition allow to obtain the complete transmembrane voltage by adding the resting component to the induced one.

### Spherical cells

For an exposure to a DC homogeneous electric field, the voltage induced on the cell membrane is determined by solving Laplace's equation. Although biological cells are not perfect spheres, in theoretical treatments they are usually considered as such. For the first approximation, the plasma membrane can also be treated as nonconductive. Under these assumptions, the induced transmembrane voltage $\Delta\Phi_m$ is given by a formula often referred to as the (steady-state) Schwan's equation [3],

$$\Delta\Phi_m = \frac{3}{2} E R \cos\theta , \qquad (1)$$

where $E$ is the electric field in the region where the cell is situated, $R$ is the cell radius, and $\theta$ is the angle measured from the center of the cell with respect to the direction of the field. Thus, $\Delta\Phi_m$ is proportional to the applied electric field and to the cell radius. Furthermore, it has extremal values at the points where the field is perpendicular to the membrane, i.e. at $\theta = 0°$ and $\theta = 180°$ (the "poles" of the cell), and in-between these poles it varies proportionally to the cosine of $\theta$ (see Fig. 1, dashed).

$\Delta\Phi_m$ as given by (1) is typically established several microseconds after the onset of the electric field. With exposures to a DC field lasting hundreds of microseconds or more, this formula can safely be applied to yield the maximal, steady-state value of the induced transmembrane voltage. To describe the transient behavior during the initial microseconds, one uses the first-order Schwan's equation [4],

$$\Delta\Phi_m = \frac{3}{2} E R \cos\theta \left(1 - \exp(-t/\tau_m)\right), \qquad (2)$$

where $\tau_m$ is the time constant of membrane charging,

$$\tau_m = \frac{R\,\varepsilon_m}{2d\,\dfrac{\sigma_i \sigma_e}{\sigma_i + 2\sigma_e} + R\sigma_m} \qquad (3)$$

with $\sigma_i$, $\sigma_m$ and $\sigma_e$ the conductivities of the cytoplasm, cell membrane, and extracellular medium, respectively, $\varepsilon_m$ the dielectric permittivity of the membrane, $d$ the membrane thickness, and $R$ again the cell radius.

In certain experiments *in vitro*, where artificial extracellular media with conductivities substantially lower than physiological are used, the factor 3/2 in (1) and (2) decreases, as described in detail in [5]. But generally, Eqns. (2) and (3) are applicable to exposures to sine (AC) electric fields with frequencies below 1 MHz, and to rectangular electric pulses longer than 1 μs.

To determine the voltage induced by even higher field frequencies or even shorter pulses, the dielectric permittivities of the electrolytes also have to be

accounted for. This leads to a further generalization of Eqns. (2) and (3) to a second-order model [6-8], and the results it yields will be outlined in the last section of this paper.

**Spheroidal and ellipsoidal cells**

Another direction of generalization is to assume a cell shape more general than that of a sphere. The most straightforward generalization is to a spheroid (a geometrical body obtained by rotating an ellipse around one of its radii, so that one of its orthogonal projections is a sphere, and the other two are the same ellipse) and further to an ellipsoid (a geometrical body in which each of its three orthogonal projections is a different ellipse). To obtain the analogues of Schwan's equation for such cells, one solves Laplace's equation in spheroidal and ellipsoidal coordinates, respectively [9-11]. Besides the fact that this solution is by itself more intricate than the one in spherical coordinates, the generalization of the shape invokes two additional complications outlined in the next two paragraphs.

A description of a cell is geometrically realistic if the thickness of its membrane is uniform. This is the case if the membrane represents the space between two concentric spheres, but not with two confocal spheroids or ellipsoids. As a result, the thickness of the membrane modeled in spheroidal or ellipsoidal coordinates is necessarily nonuniform. By solving Laplace's equation in these coordinates, we thus obtain the spatial distribution of the electric potential in a nonrealistic setting. However, under the assumption that the membrane conductivity is zero, the induced transmembrane voltage obtained in this manner is still realistic. Namely, the shielding of the cytoplasm is then complete, and hence the electric potential everywhere inside the cytoplasm is constant. Therefore, the geometry of the inner surface of the membrane does not affect the potential distribution outside the cell, which is the same as if the cell would be a homogeneous non-conductive body of the same shape.[2] A more rigorous discussion of the validity of this approach can be found in [9]. Fig. 1 compares the transmembrane voltage induced on two spheroids with the axis of rotational symmetry aligned with the direction of the field, and that induced on a sphere.

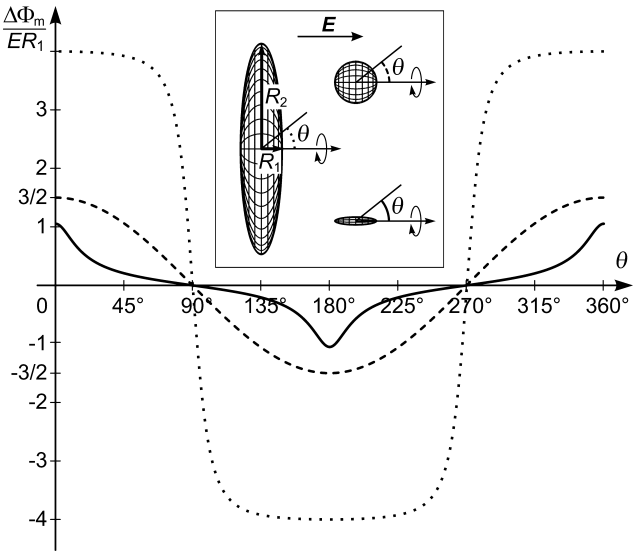


***Figure 1:*** Normalized steady-state $\Delta\Phi_m$ as a function of the polar angle θ for spheroidal cells with the axis of rotational symmetry aligned with the direction of the field. Solid: a prolate spheroidal cell with $R_2 = 0.2 \times R_1$. Dashed: a spherical cell, $R_2 = R_1 = R$. Dotted: an oblate spheroidal cell with $R_2 = 5 \times R_1$.

For nonspherical cells, it is generally more revealing to express $\Delta\Phi_m$ as a function of the arc length than as a function of the angle θ (for a sphere, the two quantities are directly proportional). For uniformity, the normalized version of the arc length is used, denoted by *p* and increasing from 0 to 1 equidistantly along the arc of the membrane. This is illustrated in Fig. 2 for the cells for which $\Delta\Phi_m(\theta)$ is shown in Fig. 1, and all the plots of $\Delta\Phi_m$ on nonspherical cells will henceforth be presented in this manner.

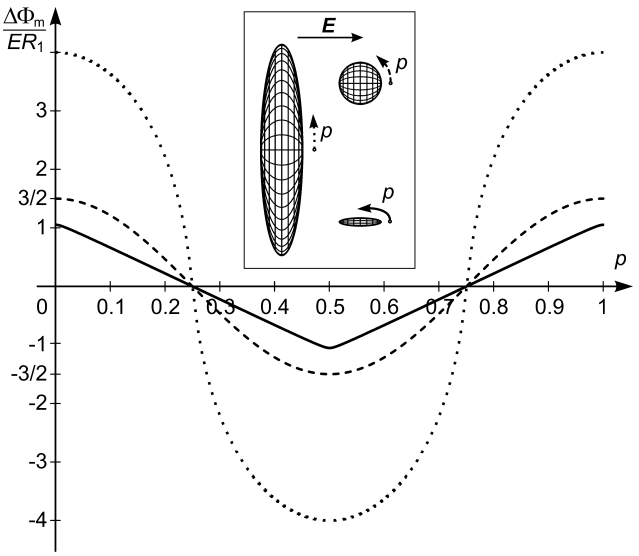


***Figure 2:*** Normalized steady-state $\Delta\Phi_m$ as a function of the normalized arc length *p* for spheroidal cells with the axis of rotational symmetry aligned with the direction of the field. Solid: a prolate spheroidal cell with $R_2 = 0.2 \times R_1$. Dashed: a spherical cell, $R_2 = R_1 = R$. Dotted: an oblate spheroidal cell with $R_2 = 5 \times R_1$.

[2] As a rough analogy, when a stone is placed into a water stream, the streamlines outside the stone are the same regardless of the stone's interior composition. Due to the fact that stone is impermeable to water, only its outer shape matters in this respect. Similarly, when the membrane is nonconductive, or "impermeable to electric current", only the outer shape of the cell affects the current density and the potential distribution outside the cell.

The second complication of generalizing the cell shape from a sphere to a spheroid or an ellipsoid is that the induced voltage now also becomes dependent on the orientation of the cell with respect to the electric field. To deal with this, one decomposes the field vector into the components parallel to the axes of the spheroid or the ellipsoid, and writes the induced voltage as a corresponding linear combination of the voltages induced for each of the three coaxial orientations [10,11]. Figs. 3 and 4 show the effect of rotation of two different spheroids with respect to the direction of the field.

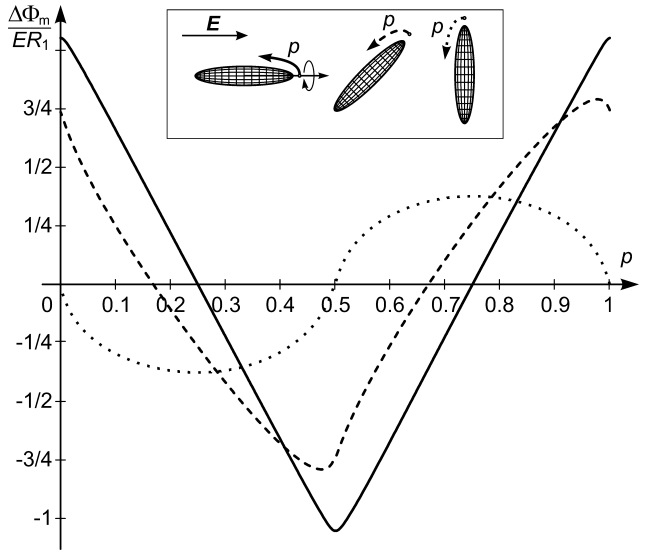


***Figure 3:*** Normalized steady-state $\Delta\Phi_m(p)$ for a prolate spheroidal cell with $R_2 = 0.2 \times R_1$. Solid: axis of rotational symmetry (ARS) aligned with the field. Dashed: ARS at 45° with respect to the field. Dotted: ARS perpendicular to the field.

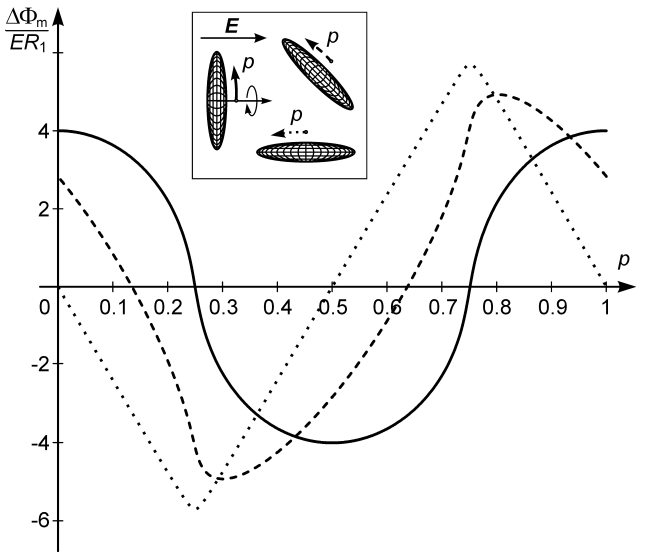


***Figure 4:*** Normalized steady-state $\Delta\Phi_m(p)$ for an oblate spheroidal cell with $R_2 = 5 \times R_1$. Solid: axis of rotational symmetry (ARS) aligned with the field. Dashed: ARS at 45° with respect to the field. Dotted: ARS perpendicular to the field.

**Irregularly shaped cells**

For a cell having an irregular shape, the induced transmembrane voltage cannot be determined exactly, as for such a geometry Laplace's equation is not solvable analytically. Using modern computers and finite-elements tools such as Maxwell or FEMLab, the voltage induced on a given irregular cell can still be determined numerically, as described in detail in [12]. While the results obtained in this manner are quite accurate, they are only applicable to the particular cell shape for which they were computed. Fig. 5 shows examples of two cells growing in a Petri dish and the voltages induced on their membranes.

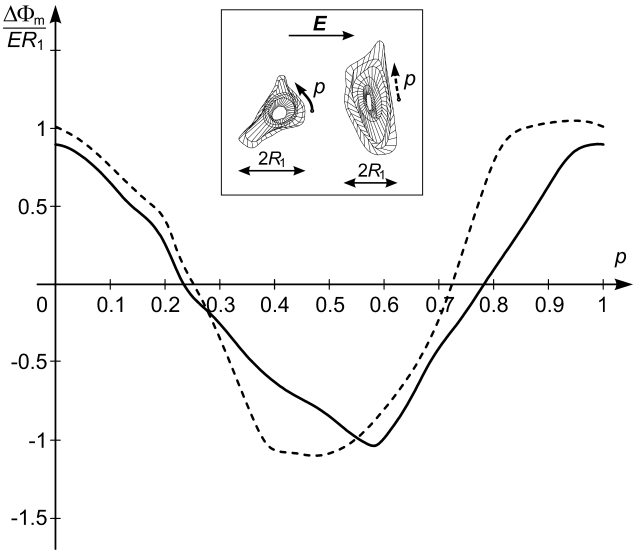


***Figure 5:*** Normalized steady-state $\Delta\Phi_m(p)$ for two irregularly shaped cells growing on the flat surface of a Petri dish.

**Cells in dense suspensions**

In dilute cell suspensions, the distance between the cells is much larger than the cells themselves, and the local field outside each cell is practically unaffected by the presence of other cells. Thus, for cells representing less than 1 % of the suspension volume (for a spherical cell with a radius of 10 μm, this means up to 2 million cells/ml), the deviation of the actual induced transmembrane voltage from one predicted by Schwan's equation is negligible. However, as the volume fraction occupied by the cells gets larger, the distortion of the local field around each cell by the presence of other cells in the vicinity becomes more pronounced, and the prediction yielded by Schwan's equation less realistic (Fig. 6). For volume fractions over ten percent, as well as for clusters and lattices of cells, one has to use appropriate numerical or approximate analytical solutions for a reliable analysis of the induced transmembrane voltage [13,14]. Regardless of the volume fraction they occupy, as long as the cells are suspended, they are floating

freely, and their arrangement is rather uniform. Asymptotically, this would correspond to a face-centered cubic lattice, and this lattice is also the most appropriate for the analysis of the transmembrane voltage induced on cells in suspension.

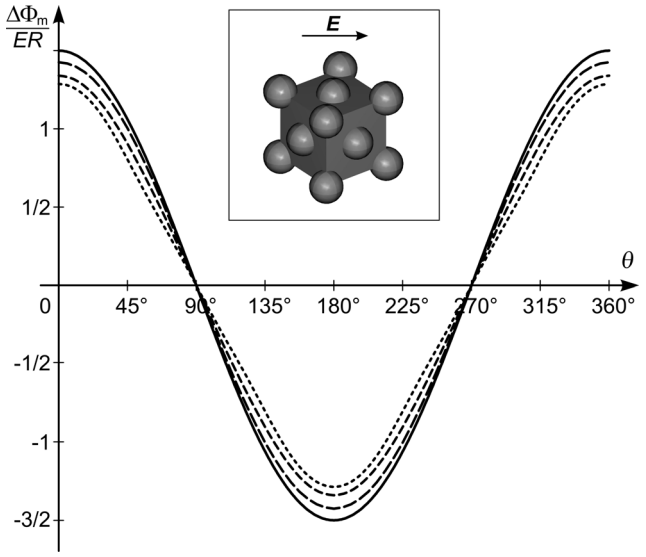


***Figure 6:*** Normalized steady-state $\Delta\Phi_m(\theta)$ for spherical cells in suspensions of various densities (intercellular distances). Solid: The analytical result for a single cell as given by (18). Dashed: numerical results for cells arranged in a face-centered cubic lattice and occupying (with decreasing dash size) 10%, 30%, and 50% of the total suspension volume.

For even larger volume fractions of the cells, the electrical properties of the suspension start to resemble that of a tissue, but only to a certain extent. The arrangement of cells in tissues does not necessarily resemble a face-centered lattice, since cells can form specific structures (e.g. layers). In addition, cells in tissues can be directly electrically coupled (e.g. through gap junctions). These and other specific features of the interactions between cells in tissues and electric fields will be considered in more detail in the paper that follows this one.

**High field frequencies and very short pulses**

The time constant of membrane charging ($\tau_m$) given by Eq. (3) implies that there is a delay between the time courses of the external field and the voltage induced by this field. As mentioned above, $\tau_m$ (and thus the delay) is somewhat below a microsecond under physiological conditions, but can be larger when cells are suspended in a low-conductivity medium. For alternating (AC) fields with the oscillation period much longer than $\tau_m$, as well as for rectangular pulses much longer than $\tau_m$, the amplitude of the induced voltage remains unaffected. However, for AC fields with the period comparable or shorter than $\tau_m$, as well as for pulses shorter than $\tau_m$, the amplitude of the induced voltage starts to decrease.

To illustrate how the amplitude of the induced transmembrane voltage gets attenuated as the frequency of the AC field increases, we plot the normalized amplitude of the induced voltage as a function of the field frequency. For a spherical cell, the plot obtained is shown in Fig. 6. The low-frequency plateau and the downward slope that follows are both described by the first-order Schwan's equation, but the high-frequency plateau is only described by the second-order model [6-8], in which all electric conductivities and dielectric permittivities are accounted for.

With field frequencies approaching the GHz range, or with pulse durations in the nanosecond range, the attenuation of the voltage induced on the cell plasma membrane becomes so pronounced that this voltage becomes comparable to the voltage induced on organelle membranes in the cell interior. In certain circumstances, particularly if the organelle interior is electrically more conductive than the cytosol, or if the organelle membrane has a lower dielectric permittivity than the cell membrane, the voltage induced on the membrane of this organelle can temporarily even exceed the voltage induced on the plasma membrane [15]. In principle, this could provide a theoretical explanation for a number of recent reports that very short and intense electric pulses (tens of ns, millions or tens of millions of V/m) can also induce electroporation of organelle membranes [16-18].

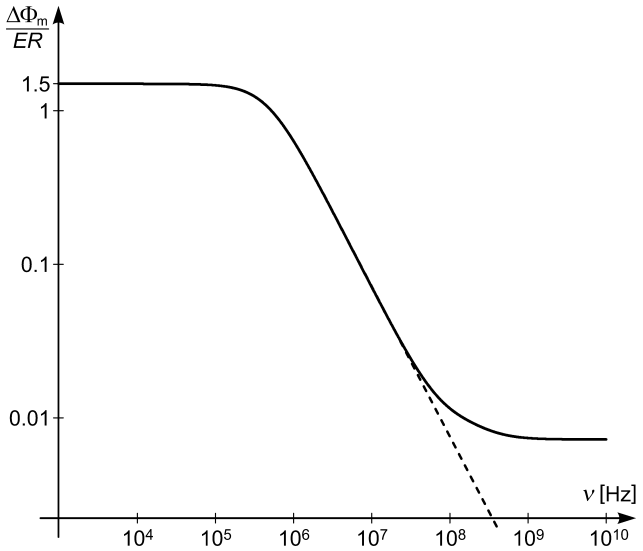


***Figure 7:*** The amplitude of normalized steady-state $\Delta\Phi_m$ as a function of the frequency of the AC field. The dashed curve shows the first-order, and the solid one the second-order Schwan's equation. Note that both axes are logarithmic.

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## NOTES

# Electric Properties of Tissues and their Changes During Electroporation

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**Abstract:** Passive electric properties of biological tissues such as permittivity and conductivity are important in applied problems of electroporation. The current densities and pathways resulting from an applied electrical pulse are dictated to a large extent by the relative permittivity and conductivity of biological tissues. We briefly present some theoretical basis for the current conduction in biologic materials and factors affecting the measurement of tissue dielectric properties that need to be taken into account when designing the measurement procedure. Large discrepancies between the data reported by different researchers are found in the literature. These are due to factors such as different measuring techniques used, the fact that macroscopic tissue properties show inhomogenity, dispersions, anisotropy, nonlinearity, as well as temperature dependence and changes over time. Furthermore, when biological tissue is exposed to a high enough electric field, changes in its bulk electric properties occur.

## INTRODUCTION

The electrical properties of biological tissues and cell suspensions have been of interest for over a century. They determine the pathways of current flow through the body and are thus very important in the analysis of a wide range of biomedical applications. On a more fundamental level, knowledge of these electrical properties can lead to the understanding of the underlying, basic biological processes. To analyze the response of a tissue to electric stimulus, data on the conductivities and relative permittivities of the tissues or organs are needed. A microscopic description of the response is complicated by the variety of cell shapes and their distribution inside the tissue as well as the different properties of the extracellular media. Therefore, a macroscopic approach is most often used to characterize field distributions in biological systems. Moreover, even on a macroscopic level the electrical properties are complicated. They can depend on the tissue orientation relative to the applied field (directional anisotropy), the frequency of the applied field (the tissue is neither a perfect dielectric nor a perfect conductor) or they can be time and space dependent (e.g., changes in tissue conductivity during electropermeabilization) [1-3].

## BIOLOGICAL MATERIALS IN THE ELECTRIC FIELD

The electrical properties of any material, including biological tissue can be broadly separated into two categories: conducting and insulating. In a conductor the electric charges move freely in response to the application of an electric field whereas in an insulator (dielectric) the charges are fixed and not free to move – the current does not flow [2].

If a conductor is placed in an electric field, charges will move within the conductor until the interior field is zero. In the case of an insulator, there are no free charges so net migration of charge does not occur. In polar materials, however, the positive and negative charge centers in the molecules do not coincide. An applied field, $E_0$, tends to orient the dipoles and produces a field inside the dielectric, $E_p$, which opposes the applied field. This process is called polarization [1, 4]. Most materials contain a combination of dipoles and free charges. Thus the electric field is reduced in any material relative to its free-space value. The net field inside the material, E, is then

$$E = E_0 - E_p$$

The net field is lowered by a significant amount relative to the applied field if the material is an insulator and is essentially zero for a good conductor. This reduction is characterized by a factor $\varepsilon_r$, which is called the relative permittivity or dielectric constant, according to

$$E = \frac{E_0}{\varepsilon_r}$$

In practice, most materials, including biological tissue, actually display some characteristics of both, insulators and conductors, because they contain dipoles as well as charges which can move, but in a restricted manner.

On a macroscopic level we describe the material as having a permittivity, $\varepsilon$, and a conductivity, $\sigma$. The permittivity characterizes the material's ability to trap or store charge or to rotate molecular dipoles whereas the conductivity describes its ability to transport charge. The permittivity also helps to determine the speed of light in a material so that free space has a permittivity $\varepsilon_0$ =8.85 x 10-12 F/m. For other media:

$$\varepsilon = \varepsilon_r \varepsilon_0$$

The energy stored per unit volume in a material, u, and the power dissipated per unit volume, p, are:

$$u = \frac{\varepsilon E^2}{2} \qquad p = \frac{\sigma E^2}{2}$$

Consider a sample of material which has a thickness, d, and cross-sectional area, A. If the material is an insulator, then we treat the sample as a capacitor with capacitance (C); if it is a conductor, then we treat it as a conductor with conductance (G):

$$C = \varepsilon \cdot A/d \qquad G = \sigma \cdot A/d$$

A simple model for a real material, such as tissue, would be a parallel combination of the capacitor and conductor. If a constant (DC) voltage V is applied across this parallel combination, then a conduction current $I_C$ = GV will flow and an amount of charge Q=CV will be stored. However, if an alternating (AC) voltage was applied to the combination:

$$V(t)=V_0\cos(\omega t)$$

The charge on the capacitor plates is now changing with frequency f. We characterize this flow as a displacement current:

$$I_d = dQ/dt = -\omega C V_0 \sin(\omega t)$$

The total current flowing through the material is the sum of the conduction and displacement currents, which are 90° apart in phase. The total current is $I = I_c + I_d$, hence

$$I=GV+C\cdot dV/dt = (\sigma + i\omega\varepsilon)A \cdot V/d$$

The actual material, then, can be characterized as having an admittance, Y*, given by:

$$Y^* = G + i\omega C = (A/d)(\sigma + i\omega\varepsilon)$$

where * indicates a complex-valued quantity. In terms of material properties we define a corresponding, complex-valued conductivity

$$\sigma^* = (\sigma + i\omega\varepsilon)$$

Describing a material in terms of its admittance emphasizes its ability to transport current. Alternatively, we could emphasize its ability to restrict the flow of current by considering its impedance Z*=1/Y*, or for a pure conductance, its resistance, R=1/G.

We can also denote total current as:

$$I = (\varepsilon_r - i\sigma/\omega\varepsilon_0)i\omega\varepsilon_0 A/d = C\frac{dV}{dt}$$

We can define a complex-valued, relative permittivity:

$$\varepsilon^* = \varepsilon_r - \frac{i\sigma}{\omega\varepsilon_0} = \varepsilon_r' - i\varepsilon_r''$$

with $\varepsilon_r'=\varepsilon_r$ and $\varepsilon_r''=\sigma/(\omega\varepsilon_0)$. The complex conductivity and complex permittivity are related by:

$$\sigma^* = i\omega\varepsilon^* = i\omega\varepsilon_0\varepsilon_r^*$$

We can consider the conductivity of a material as a measure of the ability of its charge to be transported throughout its volume by an applied electric field. Similarly, its permittivity is a measure of the ability of its dipoles to rotate or its charge to be stored by an applied external field. Note that if the permittivity and conductivity of the material are constant, the displacement current will increase with frequency whereas the conduction current does not change. At low frequencies the material will behave like a conductor, but capacitive effects will become more important at higher frequencies. For most materials, however, σ* and ε* are frequency-dependent. Such a variation is called dispersion. Biological tissues exhibit several different dispersions over a wide range of frequencies [1, 4].

Dispersions can be understood in terms of the orientation of the dipoles and the motion of the charge carriers. At relatively low frequencies it is relatively easy for the dipoles to orient in response to the change in applied field whereas the charge carriers travel larger distances over which there is a greater opportunity for trapping at a defect or interface. The permittivity is relatively high and the conductivity is relatively low. As the frequency increases, the dipoles are less able to follow the changes in the applied field and the corresponding polarization disappears. In contrast, the charge carriers sample shorter distances during each half-cycle and are less likely to be trapped. As frequency increases, the permittivity decreases and, because trapping becomes less important, the conductivity increases. In a heterogeneous material, such as biological tissue, several dispersions are observed as illustrated in Figure 1.

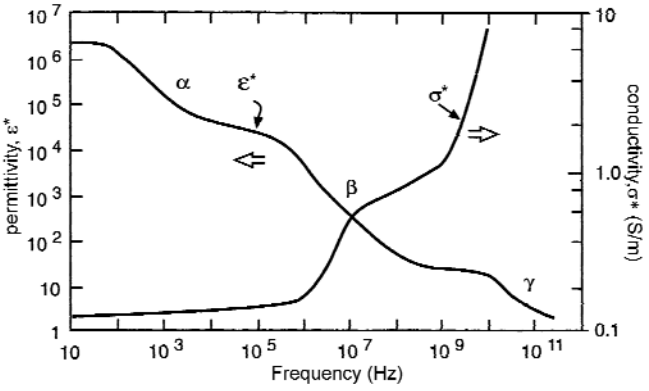


***Figure 1***: Typical frequency dependence of the complex permittivity and complex conductivity of a heterogeneous material such as biological tissue.

## DIELECTRIC MEASUREMENTS OF TISSUES

There is a large discrepancy between various data on electrical properties of biological materials found

in the literature. The measurement of tissue dielectric properties can be complicated due to several factors, such as tissue inhomogeneity, anisotropy, the physiological state of the tissue, seasonal, age and disease-linked changes and electrode polarization [1].

**Inhomogeneity of tissues**

Tissue is a highly inhomogeneous material. The cell itself is comprised of an insulating membrane enclosing a conductive cytosol. A suspension of cells can be regarded at low frequencies simply as insulating inclusions in a conducting fluid. The insulation is provided by the cell membrane. At frequencies in the MHz range capacitive coupling across this membrane becomes more important. In tissue, the cells are surrounded by an extracellular matrix, which can be extensive, as in the case of bone, or minimal, as in the case of epithelial tissue. Tissue does not contain cells of a single size and function. The tissue is perfused with blood and linked to the central nervous system by neurons. It is thus difficult to extrapolate from the dielectric properties of a cell suspension to those of an intact tissue.

**Anisotropy of tissues**

Some biological materials, such as bone and skeletal muscle, are distinctly anisotropic. Therefore, when referring to measured conductivity and permittivity values, one needs to include data on the orientation of the electrodes relative to the major axis of the tissue; e.g., longitudinal, transversal or a combination of both. For example, muscles are composed of fibers, very large individual cells aligned in the direction of muscle contraction. Electrical conduction along the length of the fiber is thus significantly easier than conduction between the fibers in the extracellular matrix. Therefore, muscle tissue manifests typical anisotropic electric properties. The longitudinal conductivity is significantly higher than the transverse conductivity (can be up to 8 times higher).

Moreover, tissue anisotropy is frequency dependent. Namely, if the frequency of the current is high enough, the anisotropic properties disappear. Specifically for muscle tissue, that happens in the MHz frequency range.

**Physiological factors and changes of tissue**

Any changes in tissue physiology should produce changes in the tissue electrical properties. This principle has been used to identify and/or monitor the presence of various illnesses or conditions.

Tumors generally have higher water content than normal cells because of cellular necrosis but also irregular and fenestrated vascularization. Higher conductivity of tumors in the MHz frequency range could lead to their selective targeting by radio-frequency hyperthermia treatment. In addition, there may be differences in the membrane structure. Also, fat is a poorer conductor of electricity than water. Changes in the percentage of body fat or water are reflected in tissue impedance changes.

Further, tissue death or excision results in significant changes in electrical properties. Tissue metabolism decreases after the tissue has been excised and often the temperature falls. If the tissue is supported by temperature maintenance and perfusion systems, the tissue may be stabilized for a limited period of time in a living state in vitro (ex vivo). If the tissue is not supported, however, irreversible changes will occur, followed by cell and tissue death. For these reasons considerable caution must be taken in the interpretation of electrical measurements which were performed on excised tissues.

The electrical properties of tissue also depend on its temperature. The mobility of the ions which transport the current increases with the temperature as the viscosity of the extracellular fluid decreases. The rapid increase of conductivity with temperature was suggested to be used to monitor the progress of hyperthermia treatment. Also, possible other changes, such as cell swelling and edema, or blood flow occlusion, all have an effect on tissue properties.

**Electrode polarization**

The measurement of tissue electrical properties, in vivo, is complicated. Electrode polarization is a manifestation of molecular charge organization which occurs at the sample-electrode interface in the presence of water molecules and hydrated ions. The effect increases with increasing sample conductivity.

In a cell suspension a counterion layer can form at each electrode. The potential drop in this layer reduces the electric field available to drive charge transport in the bulk suspension, resulting in apparently lower suspension conductivity. As the frequency increases, the counterion layer is less able to follow the changes in the applied signal, the potential drop at the sample-electrode interface decreases, and the apparent conductivity of the suspension increases.

The process is more complicated in tissue. Insertion of electrodes can first cause the release of electrolytes from the surrounding tissue and later the development of a poorly-conductive wound region may occur. This region can shield part of the electrode from the ionic current and thus reduce the polarization effects compared to an ionic solution equivalent in conductivity to the intracellular fluid.

The material of the electrode plays an important part in determining its polarization impedance, the relative importance of which decreases with increasing frequency. It is good practice to measure tissue impedance in-vivo after waiting a sufficient

time for the electrode polarization processes to stabilize. A typical time might be on the order of thirty minutes.

Two different electrode set-ups are used to measure the electric properties of biological materials; the two-electrode and the four-electrode method.

*Two-electrode method*: Suitable for alternating current (AC) measurements. Cannot be used as such for direct current (DC) measurements because of the electrode polarization, which consequently gives incorrect results for the conductivity of the sample between the electrodes. For AC measurements the frequency range over which electrode polarization is important depends to some extent on the system being measured and the electrode material. For cell suspensions it is important up to nearly 100 kHz whereas for tissue measured in vivo it is significant only up to about 1 kHz. By varying the separation of the electrodes, the contribution of the electrode polarization can be determined and eliminated.

*Four-electrode method*: Can be used for both DC and AC measurements. Two pairs of electrodes are used: the outer, current electrodes and the inner, voltage electrodes. The current from the source passes through the sample. Voltage electrodes of known separation are placed in the sample between the current electrodes. By measuring the current as the voltage drop across a resistor in series with the sample and the voltage drop across the inner electrodes, one can determine the conductivity of the sample between the inner electrodes. The advantage of this method is that the polarization on the current electrodes has no influence on the voltage difference between the voltage electrodes. Polarization at the voltage electrodes is negligible for both DC and AC due to the high input impedance of the measurement system.

## ELECTRICAL RESPONSE OF TISSUE TO ELECTRIC FIELD

Changes in tissue conductivity have been observed in vivo if the tissue is subjected to a high enough electric field. Having said that, we can use the dielectric properties of liver and try to calculate the electrical response to a short rectangular voltage pulse having the duration of 100 μs and the rise time of 1 μs (typical pulse parameters used for electrochemotherapy). The complications arise from the facts that i) the pulse parameters (the pulse duration, the rise and the fall time) determine the span of its frequency spectrum and ii) the tissue conductivity and permittivity are frequency dependent. The obtained response for the first pulse is presented in Figure 2. At the onset of voltage pulse, capacitive transient displacement current is observed. As membranes charge, voltage across them rises and the measured current decreases. Soon steady state is reached and current stabilizes through the conductance of extracellular fluid. Since the model describing dielectric dispersions is linear, change of the applied voltage proportionally scales the amplitude of response current.

We can compare this calculated response with the measured response on rat liver *in vivo* for the same pulse train as above and different pulse amplitudes spanning up to electroporative field strengths (Fig. 3) [5]. For the lowest applied voltage we can see good agreement with calculated response. As the field intensity is increased, the electrical response of tissue is no longer linear and increase of conductivity during the pulse is observed. Measuring the passive electrical properties of electroporated tissues could provide real time feedback on the outcome of the treatment [5, 6].

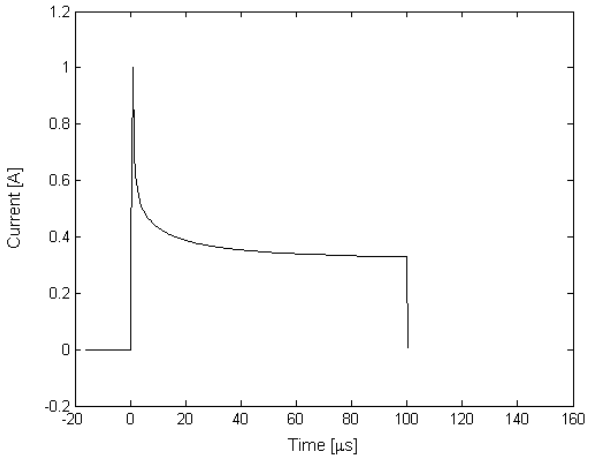


***Figure 2:*** Calculated tissue response during delivery of rectangular voltage pulse with the duration of 100 μs having the rise time of 1 μs and the amplitude of 120 V. Plate electrodes with 4.4 mm interelectrode distance were assumed.

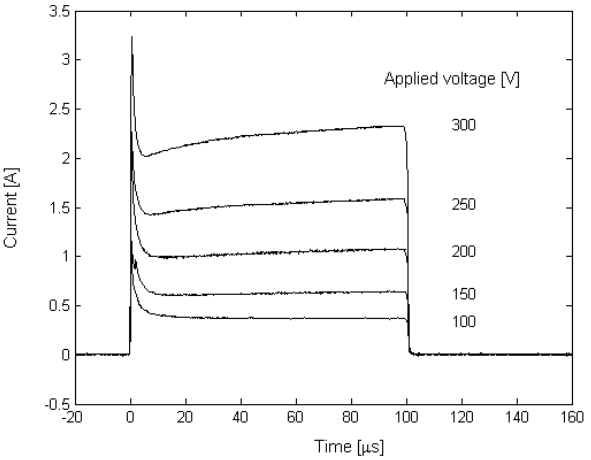


***Figure 3:*** Measured tissue response during delivery of 100 μs rectangular pulses of different amplitudes to rat liver *in vivo*. Adapted from Cukjati *et al*. [5]. Pulses were generated using Jouan GHT1287B; plate electrodes with 4.4 mm interelectrode distance were used.

The measured response is consistent with the hypothesis that the bulk tissue conductivity should also increase measurably since on a cellular level electroporation causes the increase of membrane conductance [7-10].

Further, in applications where electric pulses to skin or tissues underneath (such as subcutaneous tumor) are applied externally, through skin, one might expect high (too high) voltage amplitudes needed in order to breach the highly resistive skin tissue and permeabilize tissues underneath. Namely, tissues between the electrodes can be seen as serially connected resistors. Applying voltage on such a circuit (voltage divider) causes the voltage to be distributed between the resistors proportionally to their resistivities [11]. Upon applying electric pulses, almost the entire applied voltage thus rests across the most resistive (least conductive) tissue, in our case skin. That means a very high electric field in skin tissue, while the electric field in other tissues stays too low for a successful electropermeabilization. If our goal is the electrochemotherapy of the underlying tumor, one might wonder how a successful electrochemotherapy of subcutaneous tumors is possible when external plate electrodes are used. The answer lies in the increase in bulk conductivities of tissues during electroporation, a phenomenon that was also observed in vivo. This conductivity increase leads to a changed electric field distribution, which exposes the tumor to an electric field high enough for a successful permeabilization [12]. To support this hypothesis, we described this process with a numerical model, taking into account the changes of tissue bulk electrical properties during the electropermeabilization. In Figure 4 six steps of the electroporation process in the subcutaneous tumor model for the voltage of 1000 V between the electrodes are shown. The electric field distribution is shown in V/cm. Step 0 denotes the electric field distribution as it was just before the electroporation process started, thus when all the tissues still had their initial conductivities. When the voltage is applied to the electrodes, the electric field is distributed in the tissue according to conductivity ratios of the tissues in the model. The field strength is the highest in the tissues with the lowest conductivity, where the voltage drop is the largest, and the voltage gradient the highest. In our case, almost the entire voltage drop occurs in the skin layer which has a conductivity of about 10-100 times lower than the tissues lying underneath.

If we look at the last step of the sequential analysis, step 5, at 1000 V (Figure 4) the tumor is entirely permeabilized, in some areas the electric field is also above the irreversible threshold.

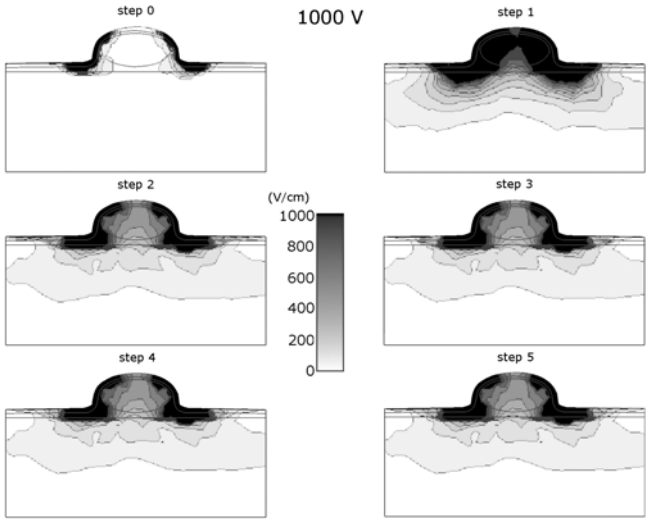


***Figure 4***: Six steps of the sequential analysis of the electroporation process in the subcutaneous tumor model at 1000 V between two plate electrodes with distance of 8 mm [12]. Time intervals between steps are in general not uniform. Different steps follow a chronological order but do not have an exact time value associated with them. The electric field distribution is shown in V/cm.

A similar situation can be encountered when applying electric pulses on a skin fold with external plate electrodes as a method to enhance in vivo gene transfection in skin [13]. Skin consists of three main layers: epidermis, dermis and subcutaneous tissue. Skin epidermis is made up of different layers, but the one that defines its electrical properties the most is the outermost layer, the stratum corneum. Although very thin (typically around 20 μm), it contributes a great deal to the electrical properties of skin. Its conductivity is three to four orders of magnitude lower than the conductivities of deeper skin layers. Again, when electric pulses are applied on skin fold through external plate electrodes, almost the entire applied voltage rests across the stratum corneum, which causes a very high electric field in that layer, while the electric field in deeper layers of skin – the layers targeted for gene transfection – stays too low. Similarly as in the case of subcutaneous tumors, the increase in bulk conductivities of skin layers during electroporation exposes the skin layers below stratum corneum to an electric field high enough for a successful permeabilization [14].

Theoretical explanation of the process of electoporation offers useful insight into the understanding of the underlying biological processes and allows for predicting the outcome of the treatment. Therefore, due effort needs to be invested into measurements of tissue electrical properties and their changes during electroporation.

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His research areas are biomedical engineering and study of the interaction of electromagnetic fields with biological systems. In the last years he has focused on the engineering aspects of electroporation as the basis of drug delivery into cells in tumor models *in vitro* and *in vivo*. His research includes biological experimentation, numerical modeling and hardware development for electrochemotherapy and gene electrotransfer.

Damijan Miklavčič received the MAPHRE Award at the 2nd European Congress of Physical Medicine and Rehabilitation in Madrid in 1989 and the National Industrial Award from Krka Pharmaceuticals in 1993. With Lojze Vodovnik and Gregor Serša he shared the Award of the Republic of Slovenia for Scientific and Research Achievements in 1995. In 2003 he received national award Ambassador in science of the Republic of Slovenia.

**Nataša Pavšelj** was born in Slovenia, in 1974. She received her B.Sc., M.Sc. and Ph.D. degrees from the University of Ljubljana in 1999, 2002 and 2006, respectively. Her main research interests lie in the field of electroporation, including finite element numerical modeling of electric field distribution in different biological tissue setups (subcutaneous tumors, skin fold) and comparison of the theoretical results with the experimental work.

## NOTES

# Theory of Membrane Electroporation and Transport Processes for Medical Electroporation Treatments

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**Abstract:** The digression on the physical chemical theory of membrane electroporation (MEP) focuses on the thermodynamics of field pulse effects as well as on the kinetics of the various electroporation processes, including the transport flows, caused by the field-induced structural flows. The primary membrane electroporation process in the applied electric field force is rapid (ns up to ms and seconds) as compared to the very much slower structural pore resealing processes (s, min, h). Most of MEP-induced crossmembrane exchange transport of uptake and release of components occurs in this after-field resealing phase. The concomitant electromechanical Maxwell stress leads to elongations of lipid vesicles and cells. Transiently adsorbed particles facilitate, by curvature effects both, electroporation and material transport, as well as global shape changes. Most importantly, the theory provides a versatile analytical formalism for quantitative analysis and characterization of electroporation data of cells and tissue. Together with results obtained from flow analysis, using the concept of time-dependent flow coefficients, the thermodynamic and kinetic information may be used not only as a general guideline for the analysis of data correlations, but also for the goal-directed design of experiments and apparatus for the various medical electroporation treatments.

## INTRODUCTION

The medical disciplines of Electrochemotherapy and Gene Electrotransfer are based on the principle of "*functional electroporation*". The concept dates back to 1982, where originally trains of electric pulses (with a time interval of 3s between the individual pulses) have been applied to directly reprogram, i.e., transform mouse lyoma cells with naked DNA. The DNA solution had been simply added to the dense cell suspension and, after a couple of minutes, to allow for adsorption of the DNA to the cell envelope, the electroporation pulse trains had been applied [1,2**].** Previously, complementary to functional electro-uptake, the electric pulse technique had been used to achieve electro-release of cellular components, such as catecholamines, ATP and chromogranine proteins from isolated chromaffin granules of bovine adrenal medullae [3].

These initial physical chemical data on non-destructive electroporative uptake and release of molecules have been recently valued, among others, in Nature Methods [4] as seminal for the various biotechnological and medical applications of what now may be called *„ functional electroporation"*, i.e., the clinical application of voltage pulses combined with bioactive agents [5].

The chemical elctrodynamic concept of functional membrane electroporation (MEP) also specifies a molecular mechanism for the primary effect of the electric field forces [1, 5], as directly affecting the hydrated polar head groups of lipids, leading to the formation of local (hydrophilic) pores. Field induced translational motions of the polar lipids in the curved pore wall also rationalize the huge acceleration of lipid flip-flop and (intrawall) motions of phosphoryl inositol from the internal membrane monolayer to the outer monolayer. Electric pulses of low field intensity, but longer pulse duration, apparently facilitate via electroporation both, endocytotic uptake of external particles and exocytotic release of intracellular cell components, respectively.

The physical chemical theory of MEP for closed membrane shells, besides addressing the primary field-induced lipid processes, also rationalizes the observed longevity of the porous structures, in terms of the locally defined cooperativity of the lipids in the highly curved pore walls. The theory-based technical developments have culminated in the new clinical disciplines of electrochemotherapy and gene electrotherapy (L.Mir, R.Heller, D.Miklavcic, J.Gehl, J. Teissie, G. Sersa). For the clinical electroporator see R.Cadossi (IGEA Cliniporator$^{TM}$).

The following detailed summary is restricted to a critical appreciation of the contemporary physical chemical theory of MEP.

## PART I.
## PHYSICAL CHEMICAL THEORY OF ELECTROPORATION

The physical chemical theory goes beyond the classical, purely physical theories of MEP which concentrate on the pure physical aspects such as the electric polarization term of water entrance in pore formation, pore line tension and membrane surface tension, pore radius and pore expansion, but do not address explicitly the chemical free energy changes of pore formation and pore resealing, which underlay the observed permeability changes, particularly apparent

in conductance relaxations of planar lipid bilayers and cells suspensions. Changes in membrane structure are also indicated by electrooptical relaxation data of unilamellar lipid vesicles. The closed membrane shells of vesicles are judged as a good model for the curved lipid parts of cell membranes. On the other hand, most planar lipid bilayers are under torus tension that enhances field-induced electromechanical stress finally leading to bilayer breakdown (rupture).

In summary, the observed *exponential* time courses of the conductometric and the electrooptical signals indicate entrance of water in the lipid phase and global shape changes. The single current events of patch clamp measurements clearly indicate local transport sites, structurally specified as porous patches of hydrophilic pores.

In both, the membranes of unilamellar lipid vesicles [6] and of cell membranes of densely packed CHO cells [7], respectively there are at least two types of "electropores": (a) short-lived, hydrophilic pores (of type $P_1$, after-field life time of 1-5 ms [8], average pore diameter of $1.6 \pm 0.2$ nm), and (b) long-lived, larger electropores (of type $P_2$, after-field life times of 20-40 min, average pore diameter $\geq 2$ nm [7].

These $P_2$-pores are the candidates to rationalize the structural longevity of the porous membrane states for the observed long-lived mass transport after the pulse. Usually for cellular systems, the pulse times should be short to prevent cell damage, thus mass transport within the field duration is very small as compared to after-field transport. This structural feature of pore longevity is also instrumental for rationalizing some of the voltage pulse data for pulse train combination modes of high voltage (HV) pulses and low voltage (LV) pulses and the effects of a time interval between the pulses.

Viewed afterwards, the originally applied "exponential field pulses" [1, 2], with the longer RC-circuit discharge times, combine the HV part of the initial time course with the LV part of the slower part.

## Electric field is force on charge.

In order to understand the field effects involved in the various medical electroporation treatments, it is essential to recall that the *electric field E* (of a voltage pulse) *acts as a force (simultaneously) on (all) polar (ionic and dipolar) groups of the membrane components.* The induced membrane field, originating from the mobile ions near the two surface sides of the dielectric membrane, acts also over the membrane-adsorbed molecules. The field forces directly cause the structural reorganisations of the membrane lipids, finally leading to higher field-stabilized order of the local permeation sites. The porous structures, once established, permit cross-membrane transport of small ions and of externally applied (and transiently adsorbed) substances into the inside, and of intra-cellular components to the outside.

**Transport of small ions and larger molecules**

Due to the small size of isolated fluctuating pores, the migration of substances, including small ions, through the porous parts is *always interactive*, involving transient electrostatic complexes between sites of the transported substance and sites of the structured pore wall. Patch clamp current data suggest that larger molecules like proteins and polyelectroctrolytes like DNA and RNA, are in multiple contacts with the lipids [10]. It is thus essential that theory and analysis of transport also incorporates the thermodynamic interactions between the porous membrane patches and the adsorbed molecules, also during the transport phases.

The amplified large membrane field may induce a kind of percolation of the small hydrophilic pores in the contact area of charged particles to form larger, but occluded pores. Lengthwise adsorbed DNA within the membrane looks like occluding a long macro-pore. Similarly, larger globular molecules like proteins, and dyes and drugs appear to transiently stick in a crater-like pore looking like a transienty occluded large pore.

It is recalled that DNA and RNA are polyanions electromigrating in the direction opposite to the direction of the applied field. So, on the cathodic cell hemisphere (facing the cathodic, negative electrode), a given external field draws the DNA towards, and once adsorbed into, the porous membrane and on the anodic hemisphere away from the membrane. Once within (i.e. occluding) a porous structure, the DNA is an interactive part of this local membrane patch. This important feature rationalizes that the small-ion leak currents, measurable in patch clamp configuration of lipid surface-adsorbed DNA, are linearly dependent on the length of the adsorbed parts of long DNA molecules [10].

Once a part of, or wthin, the membrane, after pulse termination, the "membrane-bound particles" can thermally redistribute. Membrane-adherent DNA can dissociate after the pulses from the membrane into the cell interior, or, alternatively, can return into the outside solution. The lipids of the pore wall of the formerly occluded large pore, freed from the macromolecule, now can reorganize back to the closed membrane configuration. In the absence of the ordering field forces, the lipids of the ordered pore walls will go through many mismatched lipid-water associations before the closed lipid structure is re-established. So, the random thermal nature of the pore resealing rationalizes the relatively slow return to the closed membrane structure. It is realized that the

coupling of transport to preceding adsorption covering only the small contact part of the membrane surface, and the relatively small pore surface density, rationalizes the relatively small efficiency of interactive uptake of adsorbed molecules like DNA or proteins at lower concentrations into single cells in suspension. The higher cell density of cell aggregates, as encountered in tissue, together with the higher local concentrations of externally applied molecules, all increase the uptake efficiency.

**Chemical schemes for pore formation**

The data basis suggests that the structural transitions of pore formation and pore resealing involve a whole cascade of field-sensitive closed membrane states (C) and a sequence of porous states (P). Fundamental principles of chemical description and thermodynamic analysis of field effects had been initially outlined in terms an overall two-state fluctuation scheme [1]:

$$(C) \rightleftharpoons (P) \qquad (1)$$

This simple scheme, however, has to be extended and adjusted for the particular cases encountered. In some more detail, Scheme (1) may be viewed as presenting pore formation in terms of field-induced cooperative rearrangements of n (dipolar head groups) lipids, to locally form hydrophilic pores $L_n$ and $L_n(W)$ by water (W) entrance, according to:

$$nL \rightleftharpoons L_n \rightleftharpoons L_n(W) \qquad (2)$$

The water in the configuration $L_n(W)$ will be more polarized in the strong transmembrane field as compared to bulk water. Therefore, water entrance contributes strongly to the thermodynamic stability of the aqueous hydrophilic pore [11, 13].

**Hysteresis**

Scheme (1) is also useful to feature another particular aspect of the electroporation-resealing cycle underlying the various transport phenomenona. The data suggest that the very structural transitions of pore formation as well as those of pore closure may be viewed as a hysteresis cycle. The chemical processes as such are reversible. In presence of the field, net pore formation is unidirectional. Net pore resealing, in the absence of the external field, also procedes unidirectional, but is purely thermal. So, poration and resealing are different, involving different structural intermediates. The thus different branches of the structural transitions constitute the hysteresis loop [9]. The directionality of the branches qualifies the field effect hysteresis as an irreversible phenomenon. If the resealed membrane state after pulse application is the same as that before pulsing, the hysteresis is called overall reversible.

**Equilibrium distribution constants**

The distribution equilibrium constant for the overall Scheme (1) is defined as state density ratio:

$$K = [(P)]/[(C)] = f/(1-f) \qquad (3)$$

The fraction f of pore states is defined as:

$$f = [(P)]/([(P)]+[(C)]] \qquad (4)$$

In line with the actual data both, K and f, increase with increasing field. The analysis of the kinetic normal modes of conductometric and electrooptic relaxation spectrometric data [6,7] require schemes like $(P^*) \rightleftharpoons (P_1)$ and $(P_1) \rightleftharpoons (P_2)$ to describe the, at least two, discernible kinetic phases. Here, the distribution constant $K_2=[P_2]/[P_1]= f_2/(1-f_2)$ of the $(P_1) \rightleftharpoons (P_2)$ transition to larger pores of type $P_2$ is related to the fraction $f_2=[(P_2)]/([(P_2)]+[(P_1)])$ in the range $0 \leq f_2 \leq 1$. Previously, the resticted assumption $f \ll 1$ was used [6,7]. Similar expressions for $f_1$ and $K_1$ are formulated for the transition $(P^*) = (P_1)$.

**General physical chemical energetics**

The overall Scheme (1) is suited to describe the general action of physical parameters on chemical processes in terms of a generalized van t' Hoff relationship [1], covering, as a total differential dlnK, change dT in the Kelvin temperature T, change dp in the pressure p and change dE in the strength E of the "locally active" electric field, relative to the molar energy unit RT:

$$RTd\ell nK = \Delta_r H^0_{p,E} dT - \Delta_r V^0_{T,E} dp + \Delta_r M^0_{p,T} dE \qquad (5)$$

Here, $R = k_B N_A$ is the gas constant, $k_B$ the Boltzmann constant and $N_A$ the Loschmidt-Avogadro constant. Eq. (5) refers to the different poration phenomena: electroporation is associated with the standard value $\Delta_r M° = M°(P) - M°(C)$ of the reaction dipole moment, sonoporation with the standard value of the reaction volume $\Delta_r V°$, "thermoporation" and thermal aspects of laser "optoporation" with the standard reaction enthalpy $\Delta_r H°$.

Note, for field effects, the total reaction energy at constant p,T and at given field strength E is the Legendre-transformed standard reaction enthalpy $\Delta_r \hat{H}^0 = \Delta_r \hat{G}^0 + T\Delta_r S^0$. The work potential is the Legendre-transformed Gibbs reaction energy in the

field E: $\Delta_r\hat{G} = \Delta_r G - EM$, where M is the projection of the total electric moment vector **M** onto the direction of **E**, $\Delta_r G$ the ordinary Gibbs reaction energy and $\Delta_r S$ the reaction entropy, all at constant pressure [11].

**Induced membrane potential (difference)**

The electric current density vector $\mathbf{j_m}$ for the cross-membrane ionic flows (of both cations and the anions) is given by:

$$j_m = \lambda_m(-\nabla\varphi_m) = \lambda_m E_m, \qquad (6)$$

where $\lambda_m$ is the specific conductance (or conductivity) of the membrane (all the conductive pores).An externally applied electric field ($E_{app}$), of field strength $E = E_{app}$, induces the cross membrane electric potential difference $\Delta\varphi_m$. For spherical membrane shells in conductive media (after rapid built-up of the ionic Maxwell –Wagner polarization), the *stationary value* $\Delta\varphi_m(\theta,E)_{ss}$ at the polar angle $\theta$ to the direction of the (homogeneous, plate condenser geometry) external field E, see Fig. 1, is given by [12]:

$$\Delta\varphi_m(\theta,E)_{ss} = -(3/2)\cdot a\cdot E\cdot f_\lambda\left|\cos\theta\right|. \qquad (7)$$

Here the term a is the outer radius (i.e., r = a) of the spherical membrane shell of a cell, ($a-d_m$) the inner radius and $d_m$ the membrane thickness. For the practical cases of larger cells of very low membrane conductivity, that is $d_m \ll a$ and $\lambda_m \ll \lambda_{ex}, \lambda_{in}$, with $\lambda_{ex}$ the external medium conductivity, $\lambda_{in}$ the conductivity of the cell interior, the conductivity factor for ionic media reduces to:

$$f_\lambda = 1 - \lambda_m\frac{2\lambda_{ex}+\lambda_{in}}{2\lambda_{ex}\lambda_{in}d_m/a} \qquad (8)$$

See the Appendix. Note, Eq. (7) is consistent with the Maxwell definition of E as the negative gradient $(E = -\nabla\varphi)$ of the electric potential $\varphi$. The induced membrane field at the polar angle $\theta$ is given by:

$$E_m(\theta) = \frac{U_m(\theta)}{d_m} = \frac{-\Delta\varphi_m(\theta)}{d_m} = E_m^{cap}\,|\cos\theta| \qquad (9)$$

Here $E_m^{cap}$ is the (maximum) membrane field at the pole caps, as required, same direction as $E_{app}$.

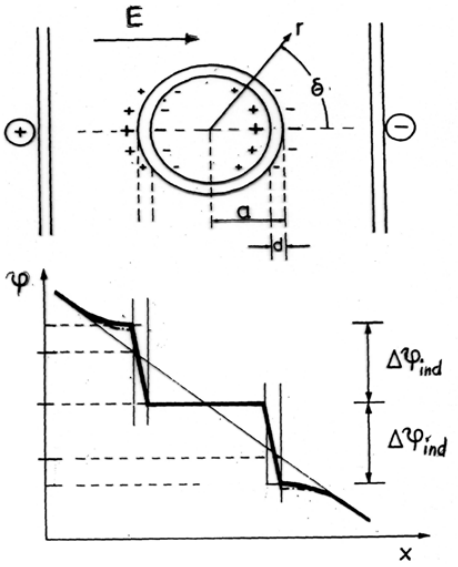
In the notation of the absolute $|\cos\theta|$, there is no change in sign when going from the cathodic pole cap, $1 \le \cos\theta \le 0$, to the anodic one, where $0 \ge \cos\theta \ge (-1)$. Hence Eq. (7) is unrestrictive, i.e., generally applicable for the description of current flows *in the direction* of the respective E vector, through the two electroporatable hemispheres of a spherical membrane shell.

The contribution of the natural membrane potential (difference) $\Delta\varphi_{nat}$ is readily incorporated as:

$$\Delta\varphi_m(\theta) = -\left((\frac{3}{2}\cdot a\cdot E\cdot f_\lambda + \Delta\varphi_{nat}/\cos\theta\right)\left|\cos\theta\right| \qquad (10)$$

**Potential and ionic current flow direction**

Fig. 1 graphically "visualizes" the direction of the **E** vector as the direction of current of the positive ions along and "down" the negative gradient of the potential from left to right, accounting for the minus sign in Eqs. (6-7).




$$\vec{E} = -\vec{\nabla}\varphi\,,\ \Delta\varphi_{ind} = -Ff_\lambda(E\cdot a)$$

$$\Delta\varphi_{ind}^{\Theta} = -\frac{3}{2}E\cdot a\cdot f_\lambda\cdot\left|\cos\Theta\right|$$

$$E_m^{\Theta} = -\frac{\Delta\varphi_{ind}^{\Theta}}{d} = \frac{3}{2}\cdot\frac{a}{d}\cdot E\cdot f_\lambda\cdot\left|\cos\Theta\right|$$

$$f_\lambda = 1 - \lambda_m\cdot\frac{a}{2d\lambda_{ex}} \qquad (\lambda_{in} >> \lambda_{ex})$$

***Figure 1:*** Field amplification by ionic membrane polarization (Maxwell-Wagner)

The absolute sign in $\left|\cos\theta\right|$ is also required for correct relations of other practical quantities. The $\cos\theta$-average within the boundaries $\theta = 0,\pi$ is $\langle\left|\cos\theta\right|\rangle = (1/2)\int\left|\cos\theta\right|\sin\theta d\theta = 1/2$. Hence, the $\cos\theta$ - average of the stationary membrane potential is given by:

$$\langle \Delta\varphi_m(\theta) \rangle = -(3/4)\, E \cdot a \qquad (11)$$

The average membrane field amplification is then:

$$\langle E_m(\theta) \rangle = (3/4)\, E\, a / d_m \qquad (12)$$

The geometrical amplification factor $a/d_m$ of a spherical shell with radius a = 5 μm and $d_m$ = 5 nm, for example, is as large as = $10^3$. It is this geometrical amplification which rationalizes that comparatively small external fields in the range of E =1 kV/cm are amplified to yield the large field strength $E_m$= $10^3$ kV/cm, which then has such high (electroporative) power on membrane structure. Eqs. (11-12) provide the correct tools for the respective estimates of membrane field $E_m$ and membrane voltage $U_m$,.

When an experimental (visibility) threshold field strength $E_{th}$ is used, the effectively porated area, $A_p$, may be expressed by: $A_p = A(1 - |\cos\theta|) = A(1 - E_{th}/E)$, where $A=(4/3)\pi a^3$ is area of the spherical shell.

**Molecular field effect analysis**

Usually the experimental signal (S), for instance, optical absorbance, current, or conductance, can be cast into a fractional "effect", for example, $f = S/S_{max}$, where $S_{max}$ is the maximum signal. It is the fractional extent f that connects experiment to thermodynamic distribution constant, K=f/(1-f), and thus to the field of strength E. For electroporation field effects, Eq.(5) is rewritten as:

$$\left(\frac{\partial \ell n K}{\partial E}\right)_{p,T} = \frac{\Delta_r M}{RT} = dX(E) \qquad (13)$$

Integration leads to:

$$K(E) = K_0 \exp X(E), \qquad (14)$$

where $K_0$ is to the state distribution constant at zero applied field, shown below to related to the half point field strength at f=0.5. The field effect term X(E) is specified in terms of the reaction moment $\Delta_r M$ as:

$$X(E_{loc}) = \int \Delta_r M\, dE_{loc}/RT \qquad (15)$$

**Reaction moment for permanent dipoles.**

If permanent dipoles are involved, the average molecular reaction moment is defined by: $\langle \Delta_r m \rangle = m(P) - m(C) = \Delta_r M / N_A$. Insertion into Eq. (15) leads to:

$$X(E_{loc}) = \frac{\int \langle \Delta_r m \rangle dE_{dir}}{k_B T} \qquad (16)$$

Note, in this context, E = $E_{loc}$, the local field at the (molecular) sites of field action. For permanent dipoles, $E_{loc} = E_{dir}$, the directing field, calculated from the induced membrane field $E_m$, see Eq. (9, 12).

**Reaction moments for induced polarizations**.

For continuum polarization processes, the reaction dipole moment is expressed as $\Delta_r M = V_p \Delta_r P$, where $V_p = N_A \langle v_p \rangle$ is the polarization volume. Here $\langle v_p \rangle = d_m \pi \langle r_p^2 \rangle$ is the mean pore volume and $\langle r_p \rangle$ the mean pore radius (of the cylindrical pore model). The reaction polarisation for the entrance of water in the lipid phase forming an aqueous pore is specified by: $\Delta_r P = \varepsilon_0(\varepsilon_W - \varepsilon_L) E_m$, $\varepsilon_o$ the dielectric permittivity of the vacuum, $\varepsilon_W$ and $\varepsilon_L$ the dielectric constants of water and the lipid phase, respectively [13].

$$X(E_{loc}) = \frac{\langle v_p \rangle \varepsilon_0 (\varepsilon_W - \varepsilon_L) E_m^2}{2 k_B T} \qquad (17)$$

**The field dependence of the fractional extent f**

Substitution of Eq. (14) into Eq. (3),K=f/(1-f), yields:

$$f(E) = \frac{K_0 \exp X(E)}{1 + K_0 \exp X(E)} \qquad (18)$$

For each special case of application the respective field has to be used. For instance, if E = $E_m$, the polarisation field factor X(E) is specified as:

$$X(E_m) = \frac{\langle v_p \rangle \varepsilon_0 (\varepsilon_W - \varepsilon_L) E_m^2}{2 k_B T} = b E_m^2 \qquad (19)$$

The b-term is defined by:

$$b = \frac{\langle v_p \rangle \varepsilon_0 (\varepsilon_W - \varepsilon_L)}{2 k_B T} \qquad (20)$$

**Dependence on external field $E_{app}$**

Primary data correlations are usually in terms of the applied field strength, i.e., E = $E_{app}$. The membrane field, Eq. (12), is then: $\langle E_m \rangle = (3/4)\, E_{app}\, a / d_m$ and insertion into Eq. (19) leads to:

$$X(E_{app}) = b^* E_{app}^2 \qquad (21)$$

Since $b^* = b (3a/4 d_m)^2$, the b*-factor is given by:

$$b^* = \frac{9\langle v_p \rangle \varepsilon_0 (\varepsilon_W - \varepsilon_L) a^2}{32 k_B T d_m^2} \qquad (22)$$

**Halfpoint field strength**

Many experimental data correlations with respect to the applied field strength $E_{app}$ permit to estimate a half-point field strength $E_{app,0.5}$, defined for the fractional extent f = 0.5, $E_{app}(f=0.5) = E_{app,0.5}$. So, the half signal is defined by $f = S(E=E_{0.5})/S_{max} = 0.5$. Insertion of f =0.5 into Eq. (18) and specifying for $E=E_{app}$ leads to:

$$K_0 = \exp[-X(E_{app,0.5})] \qquad (23)$$

Insertion of Eq.(23) leads to those practical expressions, which can be applied to the signal fraction $f(S) = S/S_{max}$ as $f(S) = f(E_{app})$:

$$f(E_{app}) = \frac{\exp[b^*(E_{app}^2 - E_{app,0.5}^2)]}{1 + \exp[b^*(E_{app}^2 - E_{app,0.5}^2)]} \; . \qquad (24)$$

Or more compact:

$$f(E_{app}) = \frac{1}{1 + \exp[-b^*(E_{app}^2 - E_{app,0.5}^2)]}$$

Previously, current relaxations of densely packed cell suspensions have been analyzed, directly using only the nonlinear onset part of the amplitude values as a function of E, in terms of the simple first order approximation $f(E) = K_0 \exp(X)$, [7], readily derived from Eq.(18), for arbitrarily $K_0 \ll 1$.

For practical purposes, the fraction f(D) of uptake of dye or DNA, properly defined by experimentally accessable quantities, can be rationalized to reflect effective poration and coupled transport. So, one may set $f(D)=f(E_{app})$ and use Eq.(24) for quantitative analysis to yield physical parameters for judging the credibility of data and analysis. In particular the transport quantities are specific for the transported species. Permeabilization therefore has to specified with respect to the permeating species, is not a general term like structural poration.

**Threshold of detection**

In membrane electroporation, like any other non-linear dependence of experimental signals on the applied force parameter, data indicate an experimental "threshold field strength $E_{th}$ [14]". In particular at short pulse times, the threshold signal $S_{thr}$ at $E_{th}$ is dependent on pulse length, quantified in the known experimental strength-duration correlation.

Experimental experience approximates $E_{th}$ as that field strength estimate where effects become visible. Thus $E_{th}$ may be qualified as "visibility" threshold. In physical chemical theory, it is not customary to incorporate such an experimental detection threshold.

**Field effects are always finite**

In a field, there is *always some finite field effect*, provided the reaction dipole moment or reaction susceptibility is finite, no matter how small the acting field force is. The relevant question is how large is the effect, as compared to the randomizing thermal motion. In electroporation theory of lipid systems, the experimental threshold field strength, dependent on pulse length or not, had been used to formulate the energetic balance, of electric polarization and surface tension on the hand and of the (counter-acting) line tension energy on the other hand.

## PART II. ELECTROPORATION KINETICS AND TIME - DEPENDENT FLOW COEFFICIENTS

**Pore type and size from relaxation kinetics**

Experimental current relaxations of single cells as well as those of densely packed cell suspensions had been analyzed in terms of amplitudes and relaxation time constants of the single normal modes. At least two types of pores are apparent. The first type is like permselective "Nernst-Planck" pores, permitting flow of either of cations or of anions going separately through different pores of electroporated parts of the membrane shell. So, on average, half of the pores transport cations and, parallel to it, the other half of the pores transport anions. A transport of this type is a kind of overall ion exchange, e.g., cations go into the cell interior on one hemisphere and go out of the cell on the other hemisphere. Hence there is no net transport of ions, for instance, out of the cell. The data further suggest that these ion exchange pores (of radius $r_p$/nm =0.8 $\pm$ 0.2) can, at a higher pore density, occasionally develop to larger pores at the expense of the smaller ones. The larger pores ($r_p \geq 1$ nm) permit then net transport, for instance, net outflow of both cations and anions, for instance caused by Maxwell stress (on equatorial regions of a cell, causing cell elongation).

**Pore expansion re-defined**

The kinetic feature of exponential relaxation modes indicates that it is the *number of pores* of defined size ($r_p$) which increases with time and field strength [6, 7]. Therefore the transport quantities of ionic current, conductance, resistance indicate that the transport cross section for flow is structurally

controlled. These kinetic features indicating single pores (of given average size) require a re-evaluation of the concept of pore expansion. As a diffusive process, expansion *sensu strictu* is associated with a square root dependence on time. The time dependence of electroporation events is, however, exponential, consistent with defined types of pores.

**Lipid rearrangements**

The previously presumed lipid rearrangements during pore formation have been specified as directed rotations of the dipolar lipid head groups to form a specific pore wall like that in hydrophilic or inverted pore. In 1982, the dipolar head groups were drawn as aligned parallel to the external field direction [1]. This presumption is now supported by both, relaxation kinetic data obtained with small lipid bilayer vesicles, and by molecular dynamics simulations of the molecular rearrangements of lipid and water molecules involved in electric pore formation.

The technique of cell electroporation has been recently extended to ultra-short pulses with nominally very high external electric field strengths. The applied field rapidly increases and acts internally primarily on the intracellular organelles. Due to the short pulse duration, the field only marginally affects the slowly responding plasma membrane. Ultra-electroporation is expected to have powerful clinical potentials, for instance, for inducing cell apoptosis in malignant tissue J.C. Weaver).

Analytically, if the experimental electroporation times are expressed as a function of the respective directing field, (calculated from the external field using the dielectric constant of the polar environment), the entire field strength range, from moderate field strengths and short pulse times up to the very high external fields of the ultra-short –pulses, can be consistently described with one and the same permanent dipole moment. The thermodynamic analysis along the physical chemical theory yields the mean dipole moment associated with the elementary unit, involved in a defined dipolar rotation process. If we compare this value with the dipole moment of the zwitterionic phosphatidylcholine head group of $(70 \pm 5)\ 10^{-30}$ Cm ($21 \pm 2$ D), we may conclude:

The hydrated ionic and dipolar lipid head groups, where the water molecules in the asymmetric hydration shells of the ionic groups contribute to higher dipole moments, are the molecular receptors for the interaction of the local field with the membranes.

The data and calculations quantify alignment processes of these dipolar field receptors into field-parallel positions in the walls of the hydrophilic (inverted) electropores, as one type of the dominant elementary processes in membrane electroporation.

**Unidirectionality of hysteresis processes**

For the analysis reaction flows it is recalled that MEP is recognized as a hysteresis cycle of the (rapid) electroporation processes to produce long-lived porous structures and the (slow) resealing processes, which couple in, and are reflected in, the observed material transport through the electroporatively permeabilized structures. It is recalled that the longevity of the porous structures is the structural reason for the large after-field material transport, such as release of small ions, net uptake (or release) of DNA, RNA or proteins, larger dye drug molecules, through the slowly annealing MEP-structures.

**Conventional flows**

The concept of the overall hysteresis cycle intrinsically implies that the structural (equilibrium) transitions, along each branch of the hysteresis loop, occur net unidirectional. This important feature justifies the introduction of (unidirectional) structural reaction flows modifying (controlling) ordinary particle flow, by nature qualified as net unidirectional (irreversible). Thus, physical chemical electroporation theory also comprises, besides conventional kinetics, flow analysis aiming at numerical values of the flow coefficients. The flow coefficient includes the permeability coefficient (diffusion coefficient, thickness of transport area, Nernst-Planck distribution coefficient for the transport compartment and the outer compartments), the surface-volume ratio and the fraction of actually transporting area or pore fraction, both for the rapid in-field processes as well as for the slower after-field processes.

**Non-linear flow analysis**

Recently, the newly introduced concept of time-dependent flow coefficients [12] has turned out to be instrumental for proper flow analysis. It is recalled that in particular the after-field conductance relaxations (resealing curves) reflect transport through decreasing transport cross sections, i.e., current modulated by the decreasing number of pores.

The actual time course of the fraction $Y(t) = (g(t) - g(0))/g(0) = (I(t) - I(0))/I(0)$ of the after-field currents, I, or conductances, g, relative to the zero time current I(0), g(0) conductance, i.e., before pulse application, in the simplest case is given by [7]:

$$Y(t) = Y_{max}\,(1 - e^{(k_0 \tau_R)(1-\exp[-t/\tau_R])}) \qquad (25)$$

where $Y_{max}$ refers to the maximum value for the case of complete equilibration between the intracellular ion

concentration and that of the external medium. The experimental stationary value $Y_\infty = Y(t \to \infty) \le Y_{ss}$, refers to complete resealing before equilibration and is given by:

$$Y_\infty = \frac{1}{1 - e^{k_0 \tau_R}} \qquad (26)$$

In Eq.(14), $k_0$ is the flow coefficient at a given field intensisity at the time point t = 0 of switching off the single pulse or the pulse train, and $\tau_R$ the (field-independent zero-field) time constant of resealing of the fractional transporting area f (=$A_{trp}/A_0$) or the fraction of conductive pores. The time course of Y(t) indicates that the pore resealing starts with the value f(E) at the given field at the pulse end, denoted here as after-field $t_0 = 0$.. In the simplest case, the zero-field porous area resealing is exponential according to:

$$f(t) = f(E) \cdot e^{-t/\tau_R} \qquad (27)$$

This procedure of analysis has, for instance, been successfully applied for the resealing phase of densely-packed CHO cells. As rationalized with Eq. (25), the measured transport curves are therefore exponentials of exponentials. (The actually measured curve types can deceive smeared exponentials of the Kohlrausch-type). As seen in the case of exponential decrease in the pore fraction, the after-field kinetics is indicative for mechanistic details of the long-lived electroporative membrane states. This analytical framwork has been used to obtain the values of $k_0$ (E, $t_E$ ), dependent on E and on the pulse duration $t_E$ , and the time constant $\tau_R$ of the resealing process [7].

**Mole flow and mole flux**

Any analysis in terms of flow coefficients is suggested to start with the Nernst-Planck *mole flow* equation. For the unidirectional case (1-D), the mole flow is defined as $\partial n_i / \partial t$. where $n_i$ is the amount of substance of species or ion of type i. If the transport is *orthogonal* across a slap of thickness $d_m$, the *actually transporting area* is $A_{trp}$ within the membrane range $0 \le x \le d_m$ of the flow pathway. Explicitly:

$$\left(\frac{\partial n_i}{\partial t}\right)_x = A_{trp}\left(D_i(-|\nabla c_i|) + u_i c_i(-|\nabla \varphi|)\right) \qquad (28)$$

where $c_i = n_i / V$ is the molar concentration, volume V=1 $dm^3$, $u_i = z_i e_0 D_i / kT$ the electric mobility, with sign because ot the sign of the ion charge number $z_i$; $\nabla$ the nabla vector (of concentration and electric potential, respectively), $D_i$ the diffusion coefficient. The absolute signs refer to the scalar values of the gradients. For membranes, the actual transport area is $A_{trp} = N_p \pi r_p^2 = f_p A_0$, $f_p = A_{trp}/A_0$ the fraction of pores, $A_0$ the total area, $N_p$ the number of pores and $r_p$ the mean pore radius of the assumed cylindrical pore..

The *mole flux* (or flow density) vector $\mathbf{J_i}$ here refers to, and is expressed as, the flow *perpendicular* across $A_0$, The scalar value of the mole flux is given by the (modified) Nernst-Planck equation:

$$J_i = \left(\frac{\partial n_i}{\partial t}\right)_x \frac{1}{A_0} = f_p \cdot \left(D_i(-|\nabla c_i|) + u_i c_i(-|\nabla \varphi|)\right) \qquad (29)$$

Note that for electro-diffusion in free solution corresponds to $f = A_{trp}/A_0 = 1$. The flux vector, involved in the expression for the electric current, besides the diffusion potentials of ions with different mobilities, reducing the actual field in the solution is solely given by the potential term, and is expressed in vector notation by:

$$\vec{J}_i = \vec{v}_i c_i = u_i c_i \vec{E}, \qquad (30)$$

where $\vec{v}_i = u_i \vec{E}$ is the drift velocity. The signed mobility $u_i$ is given by:

$$u_i = z_i D_i F/RT = z_i D_i e_0 / k_B T, \qquad (31)$$

In this context, note that the (ionic) Poisson equation for the potential $\varphi$ is given by:

$$\nabla^2 \varphi = -\nabla \cdot (-\nabla \varphi) = -\rho / \varepsilon_0 \varepsilon = (F / \varepsilon_0 \varepsilon) \Sigma z_i c_i \qquad (32)$$

where $\rho = F \sum_i z_i c_i$ is the ionic charge density and F the Faraday. The current density vector $j$ and the ionic solution conductivity are "ion-specified" as:

$$\vec{j} = \vec{E}\,\sigma = F\,(\sum_i z_i c_i\, u_i)\,\vec{E} \qquad (33)$$

and:

$$\sigma = F\,(\sum_i z_i c_i\, u_i)\,\vec{E} \qquad (34)$$

The electric current due to ion flows, cations in the direction of E, anions opposite to cations, where $z_i u_i$ is always positive, is then given by:

$$I = A\,F \sum_i z_i \left|\vec{J}_i\right| = A\,F\,(\sum_i z_i c_i\, u_i)\,E, \qquad (36)$$

Divergence (nabla scalar) operation on the current density yields:

$$\nabla \cdot \mathbf{j} = - F \sum_i z_i (\partial c_i / \partial t) \qquad (37)$$

The "conservative continuity equation", meaning inflow = outflow at the site of the concentration change with time, is expressed as:

$$\left( \frac{\partial c_i}{\partial t} \right)_{x,y,z} = -\nabla \vec{J}_i \qquad (38)$$

With Eq. (36), it is readily derived that the drift-diffusion contribution, with cations and anions flowing in the same direction, does not contribute as such to electric current I.

This framework of "ion equations" is necessary in a molecular interpretation of electro-poration currents and of transport flows of charged and uncharged particles.

**General integral flow equations**.

Inspecting Eq. (25), the time courses reflect, in a folded form, the change in the fraction of resealing pores, because the underlying mole flow is proportional to the flow area, i.e., decreasing number of pores. Therefore, proper analysis preferentially starts with the mole flow, and not with the mole flow density, in order to rationalize time-dependent flow coefficients. In addition, the flow coefficient at the time point of the end of the applied pulse yields the kinetic parameters for the rate limiting structural transitions, preceding the actual transport processes. The flow coefficient k comprises the (free) diffusion coefficient D, the Nernst distribution constant $\gamma$ for the distribution of the particle in the pore, relative to a bulk compartment, the thickness of the passage $d_m$. For instance, $k = (D\gamma / d_m) A_{trp}/V_0) = P f_p A_0 /V_0$, where $P=D\gamma/d_m$ is the permeability coefficient. It is thearea $A_{trp}$ or the pore fraction $f_p$ for electroporation processes are time dependent, e.g., $f(t)=f_0 \exp[-t/\tau_R]$, causing $k(t)=P f(t) A_0 /V_0$ dependent on time [12].Obvious for transport of larger molecules, the coeeficients for permeation are species dependent.

**Fractional signal change**

For proper flow analysis, for instance, the measured signals S(t) are expressed as the fraction $y(t)=S(t)/S_{ss}$, where the stationary signal $S_{ss}$ is the amplitude, $S_{ss} = S(t\rightarrow\infty)$. Next, the proper differential equation is selected, formally analogous to the linear form of dy(t)/d(t):

$$\frac{dy(t)}{dt} = -k(t)\left(y(t) - y_{ss}\right) \qquad (39)$$

Here $y_{ss}=S(t\rightarrow\infty)/S_{ss} = 1$. The integrated form is the integral flow equation according to [12]:

$$y(t) = y_{ss} \exp[-\int k(t) dt] \qquad (40)$$

If we use explicit flow coefficients:$k(t)= k_0 \exp[-t/\tau_R]$ or $k(t) = k_0 (1- \exp[-t/\tau_R])$ expressions of the type of Eq.(25) are obtained. It is stressed, that in each case, it must be carefully checked, which equation can be applied and whether existing equations have to be modified or expanded, as dictated by proper physical chemical reasoning along the fundamental laws of thermodynamics, in particular those of nonequilibrium (or flow) thermodynamics. This applies, too, to the claims of small electromagnetic field (EMF) effects where at a first glance the data appear "unbelievable for physical chemical reasons" [14, 16].

**ACKNOWLEDGEMENT**

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## NOTES

# Molecular Dynamics Simulations of Lipid Membrane Electroporation

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**Abstract:** The permeability of cell membranes can be transiently increased following the application of external electric fields. Here we show that theoretical approaches such as molecular modelling may provide a significant insight into the processes affecting, at the molecular level, the integrity of lipid cell membranes when these are subject to voltage gradients of a magnitude equivalent to those generated by patch clamp or electropulsation techniques.

## INTRODUCTION

Membranes constitute a ubiquitous component of tissues as they divide them into compartments and hold cells together [1]. Each membrane has a unique composition but in general, their main components are lipids, amphiphilic molecules characterized by a polar head group and two hydrophobic tails. In aqueous solutions, these lipids self assemble into a two-layered sheet (a bilayer) with all their tails pointing toward the center of the sheet. Even though they are only a few nanometers thick, lipid bilayers are impermeable to most water-soluble (hydrophilic) molecules, rationalizing their primary role in biological systems, which is to separate intracellular aqueous compartments from their surroundings (extracellular domain).

In order to overcome the high free energy associated to the transfer of such species (*e.g.* ions or water molecules) between the extracellular and intracellular aqueous medium, cells make use of transmembrane channels, pumps or carriers. Membranes may also become permeable to these compounds when subject to electrical stress. Indeed, application of high electric fields to cells or tissues produces aqueous-filled pores in the lipid bilayer [2-7] This process, first observed for planar bilayer lipid membranes [8, 9] is referred to as membrane breakdown, electropermeabilization, or electroporation [10, 11]. It finds today many applications [12-16] since, under certain conditions, it is reversible, and hence permits efficient transmembrane transfer of small molecules drugs, oligonucleotides, antibodies and plasmids.

The membrane electroporation process takes place in the following steps: (i) the application of millisecond to nanosecond electrical pulses produces transient, elevated, transmembrane voltage $\Delta V$ due to the dynamics of ions, that charge the membrane and create a local electric field, (ii) this field induces a rearrangement of the membrane components (water and lipid), (iii) followed by the formation of pores (the so-called aqueous or hydrophilic pores), whose presence increase by orders of magnitude the ionic and molecular transport through the cell membrane and (iv) under appropriate circumstances, the membrane recovers its integrity when the external field is switched off.

Despite its widespread use, the molecular level description of the electroporation phenomenon remains scarce. Indeed, due to the complexity and heterogeneity of membrane systems, it is often difficult to interpret experimental data in terms of atomically resolved structural and dynamical properties.

Moreover, in the context of electroporation, the electrostatic potential (EP) across a membrane is probably the most important property of the lipid bilayer. Its accurate evaluation requires a system description on the level of elementary charges. Full atomistic computer simulations, and in particular molecular dynamics (MD) simulations are the most suitable techniques capable of providing a detailed description of both the structural and dynamical properties of membranes, as well as a description of their electrostatic properties either at rest or when subject to an electric stress.

This paper reports on progress made so far using such simulations to model membrane -in particular lipid bilayer- electroporation. After a brief description of commonly used methodologies and protocols, we present an account of early results of "*in silico*" electroporation experiments, resulting from the direct application of an electric field. We will then describe MD simulations results obtained using a recently proposed method, which consists in generating $\Delta V$ by explicit ions dynamics.

## MD SIMULATIONS OF MEMBRANES

Molecular dynamics (MD) refers to a family of computational methods aimed at simulating macroscopic behaviour through the numerical integration of the classical equations of motion of a microscopic many-body system. Macroscopic properties are expressed as functions of particle coordinates and/or momenta, which are computed along a phase space trajectory generated by classical dynamics [17, 18] When performed under conditions

corresponding to laboratory scenarios, MD simulations can provide a detailed view of the structure and dynamics of a macromolecular system. They can also be used to perform "computer experiments" that could not be carried out in the laboratory, either because they do not represent a physical behaviour, or because the necessary controls cannot be achieved.

MD simulations require the choice of a potential energy function, *i.e.* terms by which the particles interact, usually referred to as a force field. Those most commonly used in chemistry and biophysics, *e.g.* GROMOS [19] CHARMM [20] and AMBER [21], are based on molecular mechanics and a classical treatment of particle-particle interactions that precludes bond dissociation and therefore the simulation of chemical reactions. Classical MD force fields consist of a summation of bonded forces associated with chemical bonds, bond angles, and bond dihedrals, and non-bonded forces associated with van der Waals forces and electrostatic interactions. The parameters associated with these terms are optimized to reproduce structural and conformational changes of macromolecular systems.

Conventional force fields only include point charges and pair-additive Coulomb potentials, which prevent them from describing realistic collective electrostatic effects, such as charge transfer, electronic excitations or electronic polarization, which is often considered as a major limitation of the classical force fields. Note that constant efforts are undertaken on the development of potential functions that explicitly treat electronic polarizability in empirical force fields [22-24] but none of these "polarizable" force fields is widely used in large-scale simulations for now, the main reasons for that being the dramatic increase of the computational time of simulation and additional complications with their parameterization. In this perspective, classical force fields provide an adequate description of the properties of membrane systems and allow semi-quantitative investigations of membrane electrostatics.

MD simulations use information (positions, velocities or momenta, and forces) at a given instant in time, t, to predict the positions and momenta at a later time, t + Δt, where Δt is the time step, of the order of a femtosecond, taken to be constant throughout the simulation. Numerical solutions to the equations of motion are thus obtained by iteration of this elementary step. Computer simulations are usually performed on a small number of molecules (few tens to few hundred thousand atoms), the system size being limited of course by the speed of execution of the programs, and the availability of computer power. In order to eliminate edge effects and to mimic a macroscopic system, simulations of condensed phase systems consider a small patch of molecules confined in a central simulation cell, and replicate the latter using periodic boundary conditions (PBCs) in the three directions of Cartesian space. For membranes for instance the simulated system would correspond to a small fragment of either a black film, a liposome or multilamellar oriented lipid stacks deposited on a substrate [25, 26].

Up to recently, most of membrane models consisted of simulating fully hydrated pure phospholipid bilayers, without taking into account the effect of salt concentration (see sections below). For such systems, the average structure of the lipid water interface at the atomic-scale may be provided by the density distributions of different atom types along the bilayer normal (Fig. 1), which can be measured experimentally on multilamellar stacks by neutron and X-ray diffraction techniques [27], as well as calculated from MD simulations. These distributions highlight the composition and properties of the membrane that appears as a broad hydrophilic interface, with only a thin slab of pure hydrocarbon fluid in the middle (Fig. 1). They indicate clearly the roughness of the lipid headgroup area and how water density decays smoothly from the bulk value and penetrates deeply into the bilayer at a region delimiting the membrane/water interface.

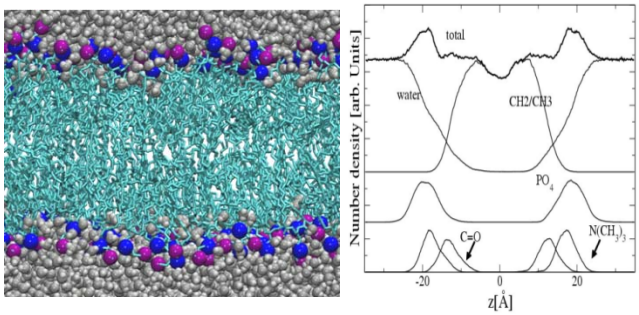


***Figure 1:*** *Left:* configuration of a Palmitoyl-Oleyl-Phosphatidyl-Choline (POPC) hydrated bilayer system from a well equilibrated constant pressure MD simulation performed at 300K. Only the molecules in the simulation cell are shown. Water molecules (O gray; H white) and the Phosphate (blue) and Nitrogen (purple) atoms of the lipid head groups are depicted by their van der Waals radii, and the acyl chains (cyan) are represented as sticks. *Right:* Number density profiles (arbitrary units) along the bilayer normal, *z*, averaged over 2 ns of the MD trajectory. The total density, water and hydrocarbon chain contributions are indicated, along with those from the POPC headgroup moieties. The bilayer center is located at $z = 0$.

MD simulations of fully hydrated bilayers show that the phospholipid head-groups adopt in general a preferential orientation : on average, the P-N dipoles point 30 degrees away from the membrane normal [28-30]. The organization of the phosphate ($PO_4^-$) and the choline ($N(CH_3)_3^+$) groups on one hand, and of the

carbonyl (C=O) groups on the other hand result in the formation of a permanent dipole. The solvent molecules bound to the lipid head group moieties tend therefore to orient their dipoles to compensate the lipid headgroup dipoles.

Such dipole organization gives rise to an electric potential difference across lipid/water interfaces, more positive on the hydrocarbon side when compared to water [31]. The absolute value of this "dipole potential" has been very difficult to measure or predict, and estimates obtained from various methods and various lipids (see [32-34]) range from ~ 200 to 1,000 mV. More recent and direct measurement based on Cryo-EM imaging [34] and AFM [35] techniques show that the dipole potential can be "measured" in a non invasive manner and estimate its value to few hundred mV.

The EP profile along the lipid bilayer normal ($Z$) may be estimated from MD simulations using Poisson's equation and can be derived directly from simulations as the double integral of $\rho(Z)$, the molecular charge density distributions

$$\Delta\phi(Z) = \phi(Z) - \phi(0) = \frac{1}{\varepsilon_0}\int_0^Z\int_0^{Z'}\rho(Z'')dZ''dZ' \qquad (1)$$

while neglecting the explicit electronic polarization [36, 37].

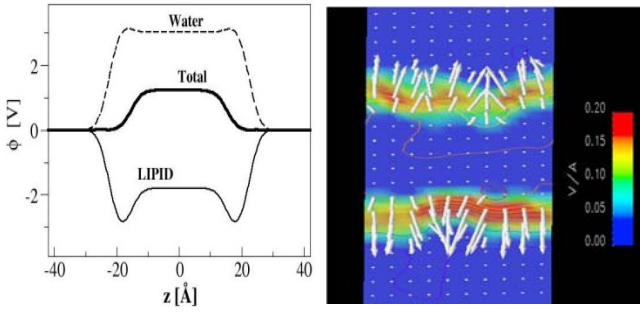


***Figure 2:*** *Left:* Electrostatic potential profile across the lipid bilayer/water interface estimated from MD simulations of a hydrated POPC lipid bilyer using Eq. (2). *Right:* Cross–sectional view of the three–dimensional map of the electric field derived as the gradient of the EP obtained by solving the Poisson equation [38]. The arrows indicate the direction and strength of the field.

Most simulations of fully hydrated lipid bilayers are found in qualitative agreement with experiments, showing that the EP profile monotonically increases across the membrane/water interface to a value of few hundred mV inside the membrane. Concerning the absolute values of the dipole potential, a wide spread range of values, ranging from 500 to 1200 mV, has been reported. This stems from the diversity of lipid studied and more significantly on the force fields used (see [33, 34, 39-42]).

Hence, both experimental and theoretical investigations clearly show that, for the purpose of describing membrane electrostatics, each lipid water interface can be thought of as a planar array of dipoles whose negative ends point toward the water. This molecular organization gives rise to a local electric field pointing outwards toward the water (Fig. 2).

## MEMBRANE ELECTROPORATION: EARLY MD STUDIES

The often used strategy in MD simulations of membranes to induce a transmembrane (TM) voltage ΔV is to apply a constant electric field $\vec{E}$, perpendicular to the membrane plane [43, 44]. In practice, this is done by adding a force $\vec{F} = q_i \vec{E}$ to all the atoms bearing a charge $q_i$. Molecular dynamics (MD) simulations adopting such an approach have been used so far to study membrane electroporation [45-48] and lipid externalization [49], to activate voltage-gated $K^+$ channels [50] and to determine transport properties of several ion channels [38, 51, 52].

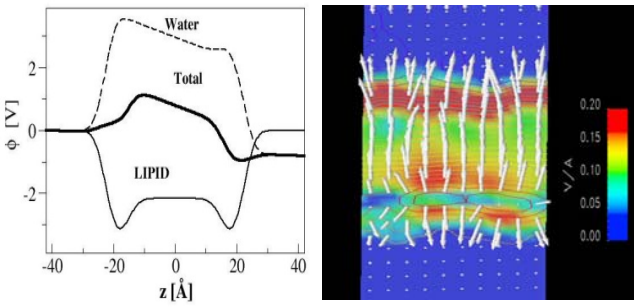


***Figure 3:*** *Left:* Electrostatic potential profile across the lipid bilayer/water interface estimated from MD simulations of a hydrated POPC lipid bilyer subject to a constant electric field perpendicular to the membrane plan and pointing upwards. *Right:* Cross–sectional view of the corresponding three–dimensional map of the electric field derived as the gradient of the EP (see Fig.2).

For pure lipid bilayers, MD trajectories [45, 46] (cf. Fig. 3) show that ΔV increases quasi-immediately (in absolute value) when the electric field is applied. A second much slower increase occurs later on and is more pronounced for the highest applied fields. The short time scale component is identified as a result of the reorientation of water molecules. The reorientation of the headgroups of the lipids on the other hand, caused by the action of external field on their dipole and responsible for the slowest change in ΔV, takes place on a longer time scale and depends of course on the field intensity.

It is very important to note here that, because of the MD simulation setup (and the use of PBCs), $\vec{E}$ induces a voltage difference $\Delta V \approx \left|\vec{E}\right| . L_z$ over the whole system, where $L_z$ is the size of the simulation

box in the field direction. This implies that it is not the electric field strength that should be compared to that of experiments but the ΔV it induces. In the example shown in the figure below, the system size in the *Z* direction is ~ 10 nm. The electric field (0.1 $V.nm^{-1}$) applied to the POPC bilayer induces ΔV of ~ 1V.
Under constant electric field, the reorientation of the water dipoles at the membrane interfaces completely reshapes the EP across the whole bilayer. Fig. 3 reports the local electric field corresponding to the sole contribution of the lipid and water dipoles and shows a drastic change in the properties of membrane water interface, with observable consequences on its integrity. Indeed, under potentials large enough, within the course of the simulation, "fingers" of water molecules begin to penetrate the hydrophobic interior of the membrane, from either side of the membrane, apparently regardless of the field orientation [45, 46]. Ultimately these fingers join up to form water channels that span the membrane. Within few nanoseconds, lipid headgroups migrate from the membrane-water interface to the interior of the bilayer, stabilizing hydrophilic pores that increase in size as the electric field is maintained. Repeated simulations at higher field strengths indicate that both the formation of the water fingers and the migration of the headgroups are speeded up.

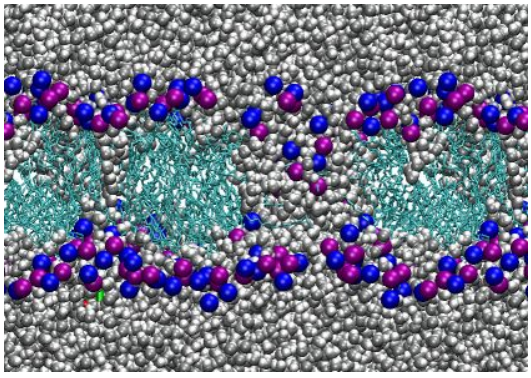

***Figure 4:*** Configuration taken from an MD simulation of a large POPC bilayer, subject to an electric field generating a transmembrane potential of ~1.5V, after a 2 ns run. Note the simultaneous presence of water wires and of large water pores stabilized by lipid head groups.

MD simulations using the electric field to trigger membrane rupture have provided a significant insight into the processes taking place at the molecular scale. In one study [46], a set of simulations has allowed modeling of the formation of pre-pores, large hydrophilic pores, subsequent partial transport of a large molecule (DNA plasmid) across the membrane, and for a simple bilayer resealing of a hydrophilic pore after switching off the applied electric field. Simulations have also shown, as predicted by Lewis [53], that the electric field induces a significant lateral stress, of the order of 1 $mN.m^{-1}$, on the bilayer. The time scales involved in all the above processes were ~ few nanoseconds. It is unclear whether these time scales are reasonable or much faster than in real samples. Note that the applied TM voltage (mainly to speed up the process in the simulations) were on the order or 1-3 Volts, *i.e.* slightly less than a 10 fold those applied in experiments.

Despite the satisfactory insight provided, the electric method suffers from many shortcomings. First, each charged particle in the system "feels" both the electric field generated by the presence of the other "charges" and the applied electric field ($\vec{F} = q_i \vec{E}$). It is very difficult to estimate the incidence of such supplementary force on the process. Second, despite the apparent modeling of the effect of an electric field on a membrane, the method is far from "reproducing" a realistic experiment. Indeed, the application of millisecond to nanosecond electrical pulses produce transient, elevated, TM potentials that charge the membrane due to ion flow, and create a local electric field. The above mentioned studies did not include the presence of ions. Their incidence on the process is however crucial, as ions of the electrolytes may contribute significantly to the collapse of the trans-membrane potential when hydrophilic pores are created during the membrane electroporation. In simulations where no ions were considered, it was impossible to witness such a scenario. As a consequence, while the electric field was maintained, the result was that the pores expanded monotonically.

## TRANSMEMBRANE VOLTAGE INDUCED BY IONIC SALT CONCENTRATION GRADIENTS

Regardless of how an electric field is applied, the ultimate step is the charging of the membrane due to ion flow. The resulting ionic charge imbalance between both sides of the lipid bilayer is locally the main effect that induces the TM potential. In a classical set up of membrane simulations, due to the use of 3d PBCs, the TM voltage cannot be controlled by imposing a charge imbalance Q across the bilayer, even when these are present in the electrolytes.

Recently, a method allowing simulations of realistic TM potential gradients across bilayers has been proposed [39]. TM voltages are obtained using a unit cell consisting of three salt-water baths separated by two bilayers and full three-dimensional periodicity [54, 55] inducing therefore an electric field generated by explicit ion dynamics. We have since introduced a variant of the method where the double layer is not needed, avoiding therefore the over-cost of a large and asymmetrical system [56]. The method consists in

simulating a unique bilayer surrounded by electrolyte baths, each terminated by an air/water interface.

First, we consider a simple lipid (here POPC) bilayer (Fig. 5). Constant pressure and constant temperature MD simulations are performed in order to equilibrate the system at a given salt concentration using 3d PBCs. Air water interfaces are then created at both sides of the system by extending the length of the original box. Further equilibration is then undertaken at constant volume, maintaining therefore a separation between the upper and lower electrolyte. As shown in Fig. 6, for large enough baths (few tens of Å), the air/water interfaces characteristics do not perturb the bilayer, and the latter can be considered as embedded in infinite baths whose characteristics are those of the modeled finite solution.

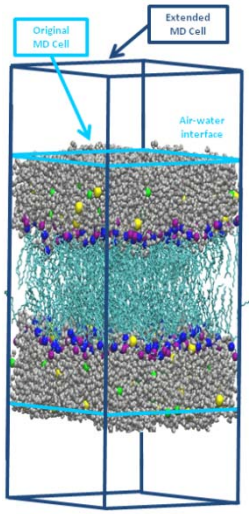


***Figure 5:*** Configuration of a hydrated POPC lipid bilayer, embedded in a 1 M NaCl electrolyte solution in the setup allowing to induce a TM voltage by imposing a net charge imbalance across the membrane. The cyan box represents the simulation box used to generate the 3d periodic boundary conditions, and the blue box the one allowing modeling of air water interfaces. Same color code as in Fig. 1; Na+ (yellow) and Cl- (green)

In Fig. 6 we report the EP profiles along the normal to the membrane generated from MD simulations of the pure bilayer in contact with salt water baths. In practice, charge imbalances (*Qs*), varying here from 0 to 8e), are generated by simply displacing at time t=0 an adequate number of ions from one slab to the other. For all simulations, the electrostatic potentials show plateau values in the aqueous regions and an increasing potential difference between the two electrolytes indicative of a TM potential ΔV. Note that the EPs are perturbed when approaching the air-water interface both due to water dipoles and to the non-uniform distribution of ions at this interface. The effect of the latter can be neglected as far as this interface is at more than 25-30 Å from the lipid.

*Membrane capacitance*

The linear variation of ΔV as a function of *Qs* reported in Figure 3 shows that, as expected, the lipid bilayer behaves as a capacitor. Its capacitance *C*, estimated considering $\Delta V = Qs / C$, amounts here to 0.85 μF.cm$^{-2}$. The capacitance values extracted from similar simulations would depend on the lipid composition (charged or not) and on the force field parameters used. Interestingly enough, in our case, it is close to the value usually assumed in the literature *e.g.* 1.0 μF.cm$^{-2}$[39, 57]. Note that this original setup considering separated baths allows direct measurement of membrane capacitance and therefore constitute a supplementary way of checking the accuracy of the parameters of lipid force fields.

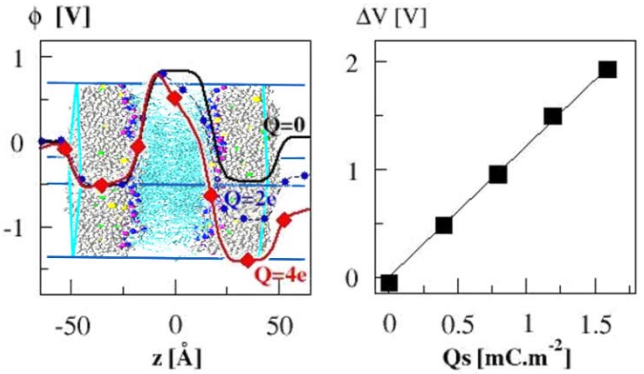


***Figure 6:*** *Left:* Electrostatic potential across a POPC lipid bilayer for different net charge imbalances Qs between the upper and lower electrolytes from MD simulations considering the setup of Fig. 5. ϕ(z), is estimated as an in-plane average of the EP distributions (Eq. 1). As a reference it was set to zero in the lower electrolyte. *Right:* TM potential ΔV as a function of the charge imbalance Qs par unit area. The capacitance of the bilayer can be derived from the slope of the curve.

*Membrane electroporation*

In this section we report preliminary results obtained from MD simulations of a large POPC lipid bilayer (144 lipids per layer), surrounded by a 1M NaCl electrolyte and subject to a TM potential of ~1V induced by a charge imbalance.

During the simulation run that spanned over 20 ns, the following processes took place:

(1) Within 2-3 nanoseconds, water fingers started forming inside the hydrophobic core.
(2) Few water wires connecting both sides of the membrane appeared within 4 ns.
(3) Lipid headgroups started to migrate along one wire and formed a connected pathway of irregular shape (see Fig. 7) large enough to conduct ions.
(4) Interestingly, as ions started migrating through the hydrophilic stable pore, water molecules forming the other wires migrated back to the bulk solution, leaving the membrane with one single ionic conduction pathway.

(5) Both cations and anions exchanged between the two baths, the former at a much higher frequency; the overall flux of charges within the hydrophilic pathway having a tendency to compensate for the remaining charge imbalance.
(6) When the charge imbalance reached a level where the TM potential was ~ 100 mV, the hydrophilic pore collapsed (closed).
(7) The final topology of the pore toward the end of the simulation remained stable for over 10 ns, probably because, as reported in previous simulations [46], the membrane complete recovery requires a much longer time scale.

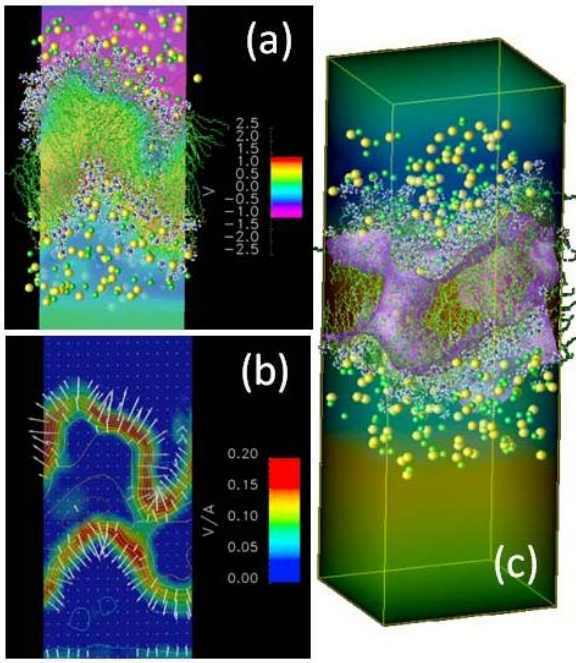


***Figure 7:*** Electrostatic properties of a POPC membrane during an electroporation process triggered by a TM voltage of ~1V resulting from a charge imbalance. (a) Cross–sectional view of the three–dimensional EP map. (b) Corresponding 2d map of the electric field derived as the gradient of the EP and a selected iso potential highlighting the contour of the hydrophilic pore. (c) 3d map of the EP. The electrostatic properties were calculated from an average over 150 ps from configurations selected around step 3 described in the main text.

One of the interesting features revealed in the present simulation is the hydrophilic pore closure following the collapse of the TM voltage set in the beginning of the trajectory. This is expected given the simulation conditions, but can be prevented if $\Delta V$ was maintained constant. In order to do so, one needs to maintain the initial charge imbalance by "injecting" charges (ions) in the electrolytes at a paste equivalent to the rate of ions translocation through the hydrophilic pore. The protocol is hence shown to allow us to perform simulations under constant voltage or constant current conditions and to compare obtained results to appropriate experiments.

The above protocol evidently presents many advantages over the electric field method, and is believed to better describe the processes taking place during electroporation of systems alike *e.g.* BLMs (black lipid membranes).

## CONCLUSION

Large computer simulations, in particular molecular dynamics simulations, are now able to provide a novel insight into membrane electroporation processes, thereby serving as an additional, complementary source of information to the current arsenal of experimental tools. At rest, *i.e.* before the membrane breakdown, many characteristics of the bilayer (hydrophobic core thickness, area per lipid, intrinsic dipole potential, capacitance) are in satisfactory agreement with experiment, which indicate that the force fields used are rather well optimized. The membrane breakdown voltage (electroporation threshold), in the order of one Volt, is also within the bulk value of measurements[3]. Of course, much more sampling through repeated simulations and under various voltage conditions is necessary before reaching quantitative agreement.

There are several points that need further investigation before fully characterizing the phenomena at the molecular level. Perhaps the most important one of these is the interplay between pore densities (number of hydrophilic pores per unit area that can form) and ionic transport rate that can be maintained at a given imposed voltage or current condition. Combined experimental/theoretical (MD) studies on the same systems should allow one to check and tune in, in a self consistent manner, these parameters that can hardly be controlled independently. Only then can we be confident in determining with accuracy the length and time scales involved in membrane electroporation, and start investigating the cascade of events involved in much more complex events such as electropulsation and transport of molecules.

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## NOTES

# *In vitro* Cell Electropermeabilization

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**Abstract:** Electropulsation is one of the most successful methods to introduce foreign molecules in living cells *in vitro*. This lecture describes the factors controlling electropermeabilization to small molecules (< 4 kDa). The description of *in vitro* events brings the attention of the reader on the processes occurring before, during and after electropulsation of cells. The role of the different electrical parameters (Field strength, pulse duration, delay between pulses) is delineated. The kinetic of the processes affecting the cell surface is described outlining that most of the exchange across the membrane takes place after the pulse during the so called resealing. Cell contribution to this critical step is tentatively explained.

## INTRODUCTION

The application of electric field pulses to cells leads to the transient permeabilization of the plasma membrane (electropermeabilization). 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* [1, 2].

One of the limiting problems remains that very few is known on the physicochemical mechanisms supporting the reorganisation of the cell membrane. Electropermeabilization is not simply punching holes in a one lipid bilayer. The physiology of the cell is controlling many parameters. The associated destabilisation of the membrane unpermeability is a stress for the cells and may affect the cell viability.

This lecture explains the factors controlling electropermeabilization to small molecules (< 4 kDa). The events occurring before, during and after electropulsation of cells are described.

## A- A biophysical description and a biological validation

### A-1 The external field induces membrane potential difference modulation

An external electric field modulates the membrane potential difference as a cell can be considered as a spherical capacitor [3]. The transmembrane potential difference induced by the electric field after a (capacitive) charging time, $\Delta\Psi_i$ is a complex function $g(\lambda)$ of the specific conductivities of the membrane ($\lambda_m$), the pulsing buffer ($\lambda o$) and the cytoplasm ($\lambda_i$), the membrane thickness, the cell size (r) and packing. Thus,

$$\Delta\Psi_i = f \cdot g(\lambda) \cdot r \cdot E \cdot \cos\theta \qquad (1)$$

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, 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 potential difference sensitive fluorescent probes [4-6]. This is valid with dilute cell suspensions. In dense systems, self shielding in the cell population affects the local field distribution and reduces the local (effective) field distribution [7]. When the resulting transmembrane potential difference (i.e. the sum between the resting value of cell membrane $\Delta\Psi o$ and the electroinduced value $\Delta\Psi i$) reaches locally 250 mV ($\Delta\Psi i, perm$), that part of the membrane becomes permeable for small charged molecules [3, 8].

### A-2 Parameters affecting electropermeabilization

#### A-2-1 Electric field parameters

Permeabilization is controlled by the field strength. Field intensity larger than a critical value ($E_{p,r}$) must be applied to the cell suspension. From Eq. (1), permeabilization is then obtained for θ close to 0 or π. $E_{p,r}$ is such that:

$$\Delta\Psi_{i,perm} = f \cdot g(\lambda) \cdot r \cdot E_{p,r} \qquad (2)$$

Permeabilization is therefore a local process on the cell surface. The extend of the permeabilized surface of a spherical cell, Aperm, is given by:

$$A_{perm} = A_{tot} \frac{\left(1-\frac{E_{p,r}}{E}\right)}{2} \qquad (3)$$

where $A_{tot}$ is the cell surface and E is the applied field intensity. Increasing the field strength will increase the part of the cell surface, which is brought to the electropermeabilized state.

These theoretical predictions are experimentally directly supported on cell suspension by measuring the leakage of metabolites (ATP) [9] or at the single cell level by digitised fluorescence microscopy [10,

11]. 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. In other words, the cell obeys the physical predictions! 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 [12]. Changing the field orientation between the different pulses increases the fraction of the cell surface which is permeabilized.

Experimental results obtained either by monitoring conductance changes on cell suspension [13] or by fluorescence observation at the single cell level microscopy [10, 11] shows that the density of the local alterations is strongly controlled by the pulse duration. An increase of the number of pulses first leads to an increase of local permeabilization level.

The field strength controls the geometry of the part of the cell which is permeabilized. Within this cap, the density of defects is uniform and under the control of the pulse(s) duration.

**A-2-2 Cell size**

The induced potential is dependent on the size of the cell (Eq (1)). The percentage of electropermeabilized cells in a population, where size heterogeneity is present, increases with an increase in the field strength. The relative part of the cell surface which is permeabilized is larger on a larger cell at a given field strength [13]. Large cells are sensitive to lower field strengths than small one. Plated cells are permeabilized with Ep value lower than when in suspension. Furthermore large cells in a population appear to be more fragile. An irreversible permeabilization of their subpopulation is observed when low field pulses (but larger than Ep) are applied [14].

**B- Practical aspects of electropermeabilization**

**B-1 Sieving of electropermeabilization**

Electropermeabilization allows a post-pulse free-like diffusion of small molecules (up to 4 kDa) whatever their chemical nature. Polar compounds cross easily the membrane. But the most important feature is that this reversible membrane organisation is nevertheless long-lived in cells. Diffusion is observed during the seconds and minutes following the ms pulse. Most of the exchange took place after the pulse [10, 11]. Resealing of the membrane defects and of the induced permeabilization is a first order multistep process, which appears to be controlled by protein and organelles reorganisation.

**B-2 Associated transmembrane exchange**

Molecular transfer of small molecules (< 4 kDa) across the permeabilized area is mostly driven by the concentration gradient across the membrane. Electrophoretic forces during the pulse may contribute [10]. During the pulse, transport is delayed after the structural alteration. Free diffusion of low weight polar molecules after the pulse can be described by using the Fick equation on its electropermeabilized part [9]. This gives the following expression for a given molecule S and a cell with a radius r:

$$\phi(S,t) = 2\pi r^2 \cdot P_S \cdot \Delta S \cdot X(N,T)\left(1-\frac{E_{p,r}}{E}\right)\exp(-k\cdot(N,T)\cdot t) \quad (4)$$

where Φ(S, t) 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 difference of S across the membrane. Ep depends on r (size). 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 [9]. A strong control by the temperature is observed. The cytoskeletal integrity should be preserved [16]. Resealing of cell membranes is a complex process which is controlled by the ATP level. Starved cells are fragile. An open question is to know if it is a self resealing or other compoments of the cell are involved.

**B-3 Cellular responses**

Reactive oxygen species (ROS) are generated at the permeabilized loci, depending on the electric field parameters [17]. These ROS can affect the viability.
When a cell is permeabilized, an osmotic swelling may result, leading to an entrance of water into the cell. This increase of cell volume is under the control of the pulse duration and of course of the osmotic stress [18].

There is a loss of the bilayer membrane asymmetry of the phospholipids [19].

When cells are submitted to short lived electric field pulses, a leakage of metabolites from the cytoplasm is observed which may bring a loss in viability. This can occur just after the pulse (short term death) or on a much longer period when membranes have resealed (long term death).

**CONCLUSION**

All experimental observations on cell electropermeabilization are in conflict with a naive model where it is proposed to result from “pores” i.e; holes punched in a lipid bilayer (see [20] as a critical review). Structural changes in the membrane organization supporting permeabilization remains

poorly characterized. New informations are provided by simulations and molecular dynamics. Nevertheless it is possible by a careful cell dependent selection of the pulsing parameteres to introduce any kind of polar molecules in a mammalian cell while preserving its viability.

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## NOTES

# Gene transfection *in vitro*: Where are we?

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**Abstract:** DNA uptake is much more complex than the simple and rapid transfer of small hydrophilic molecules into the cytosol. Small soluble molecules can freely cross the permeabilised membrane for a time much longer than the time during which the electric pulse is applied, i.e. the membrane remains permeable to these molecules for a significant time after the field is cut. However DNA transfer requires that the DNA is present during the application of the electric field pulses and involves complex steps, presumably occurring over relatively large time scales. As will be described in the lecture, these steps include the initial interaction with the electropermeabilised membrane, the crossing of the membrane, and transport within the cell and finally gene expression.

## INTRODUCTION

The use of electrotropermeabilization to deliver a wide range potentially therapeutic agents including drugs, proteins and nucleic acids in a wide range cells and tissues has been rapidly developed over the last decade [1-3]. This strategy is promising for the systemic secretion of therapeutic proteins. Vaccination and oncology gene therapy are also major fields of application of electrotransfer. Clinical trials of gene electrotransfer are currently under investigation. A phase I dose escalation trial of plasmid interleukin electroporation has been carried out in patients with metastatic melanoma and has shown encouraging results [4]. But the safe and efficient use of this physical method for clinical purposes requires the knowledge of the mechanism underlying that phenomenon of electropermeabilization. Despite the fact that the pioneering work on plasmid DNA electrotransfer in cells was initiated 25 years ago [5], many of the mechanisms underlying DNA electrotransfer remain to be elucidated [3,6,7]. One has to notice that even if *in vitro* electrotransfer is usually efficient in almost all cell lines, *in vivo* gene delivery and expression faces some problems. For instance in tumors, efficiency remains low with only a few percent of transfected cell.

## MECHANISMS OF DNA MOLECULES DELIVERY

*1) DNA/Membrane interaction*

Single-cell microscopy and fluorescent plasmids can be used to monitor these different steps of electrotransfection [6]. DNA molecules, which are negatively charged, migrate electrophoretically when submitted to the electric field. Under electric fields which are too small to permeabilise the membrane, the DNA simply flows around the membrane in the direction of the anode. However, beyond a critical field value, above which cell permeabilisation occurs, the DNA interacts with the plasma membrane. This interaction only occurs at the pole of the cell opposite the cathode and this demonstrates the importance of electrophoretic forces in the initial phase of the DNA/membrane interaction. When the DNA-membrane interaction occurs, one observes the formation of "*microdomains"* whose dimensions lie between 0.1 and 0.5 μm. Also seen are clusters or aggregates of DNA which grow during the application of the field. However once the field is cut the growth of these clusters stops. DNA electrotransfer can be described as a multi-step-process by respect with Electro-Pulsation: the negatively charged DNA migrates electrophoretically towards the plasma membrane on the cathode side where it accumulates. For electric field values above a threshold, the plasma membrane is permeabilised allowing the accumulated plasmid DNA to be inserted into it. This interaction, which is observed for several minutes, lasts much longer than the duration of the electric field pulse. Translocation of the plasmid from the plasma membrane to the cytoplasm and its subsequent passage towards the nuclear envelope take place with a kinetics ranging from minutes to hours. When plasmid has reached the nuclei, gene expression can take place and this can be detected up to several weeks later (Figure 1).

Until now, the dynamics of this process has been poorly understood because direct observations have been limited to time scales that exceed several seconds. We studied experimentally the transport of two types of molecules into cells (plasmid DNA, propidium iodide) which are relevant for gene therapy and chemotherapy with a temporal resolution of 2 ms allowing the visualization of the DNA/membrane interaction process during pulse application. DNA molecules interact with the membrane during the application of the pulse. At the beginning of the pulse application plasmid complexes or aggregates appear at sites on the cell membrane. The formation of plasmid complexes at fixed sites suggests that membrane

domains may be responsible for DNA uptake and their lack of mobility could be due to their interaction with the actin cytoskeleton. New lines of research are now necessary to characterize the membranes domains observed during electrotransfer and the involvement of cytoskeleton.

*2) DNA expression.*

Once the first stage of gene electrotransfection, i.e. migration of the plasmid DNA towards the electropermeabilised plasma membrane and its interaction with it, becomes well understood we will be able to give guidelines to improve this first step in gene electrotransfer protocols. However, successful expression of the plasmid depends on its subsequent migration into the cell. Therefore, the intracellular diffusional properties of plasmid DNA, as well as its metabolic instability and nuclear translocation, represent other cell limiting factors that must be taken into account [8].

The cytoplasm is composed of a network of microfilament and microtubule systems, along with a variety of subcellular organelles present in the cytosol. The mesh-like structure of the cytoskeleton, the presence of organelles and the high protein concentration means that there is substantial molecular crowding in the cytoplasm which hinders the diffusion of plasmid DNA. These apparently contradictory results might be reconciled by the possibility of a disassembly of the cytoskeleton network that may occur during electropermeabilisation, and is compatible with the idea that the cytoplasm constitutes an important diffusional barrier to gene transfer. In the conditions induced during electropermeabilisation, the time a plasmid DNA takes to reach the nuclei is significantly longer than the time needed for a small molecule. Therefore, plasmid DNA present in the cytosol after being electrotransferred can be lost before reaching the nucleus, for example because of cell division. Finally, after the cytoskeleton, the nuclear envelope represents the last, but by no means the least important, obstacle to the expression of the plasmid DNA. The relatively large size of plasmid DNA (2-10 MDa) makes it unlikely that the nuclear entry occurs by passive diffusion.

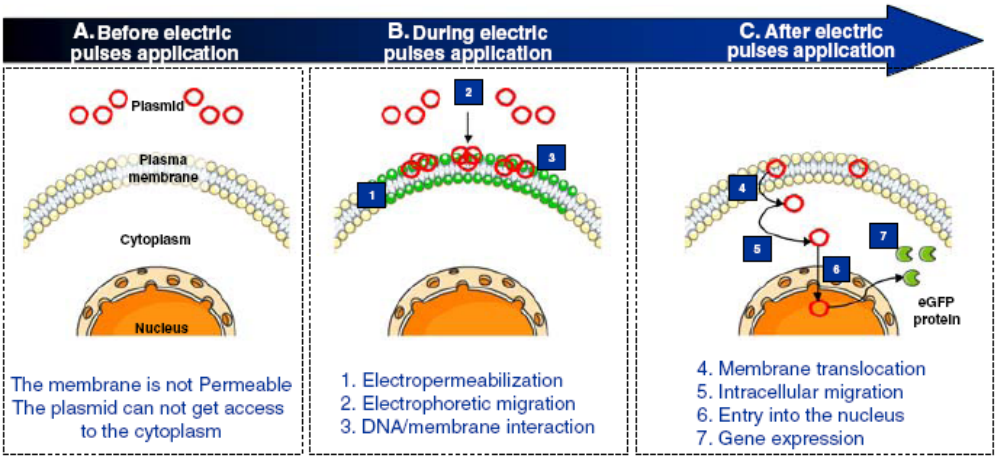


***Figure 1:*** Electrotransfection mechanism as a multistep process (from [3]).

*3) New developments.*

Clear limits of efficient gene expression using electric pulses are due to cytoplasmic crowding and transfer through the nuclear envelope. A key challenge for electro-mediated gene therapy is to pinpoint the rate limiting steps in this complex process and to find strategies to overcome these obstacles. As mentioned above, the dense latticework of the cytoskeleton impedes free diffusion of DNA. Electrotransferred plasmid DNA, containing specific sequences could then use the microtubule network and its associated motor proteins to move through the cytoplasm to the nucleus [9].

Another alternative concerns nanosecond pulsed electric fields. Studies indicate that very short (10-300 ns) but high pulses (up to 300 kV/cm) induce effects that primarily affect intracellular structures and functions. As the pulse duration is decreased, below the plasma membrane charging time constant, plasma membrane effects decrease and intracellular effects predominate [10,11]. A possible idea, to improve transfection success, is to perform classical membrane permeabilisation allowing plasmid DNA electrotransfer, and then after, when DNA has reached the nuclear envelope, to specifically permeabilise it using these short strong nanopulses. Thus, when used in conjunction with classical electropermeabilisation, nanopulses could be used to increase gene expression yields.

New lines of research are now necessary to characterize the membranes domains observed during electrotransfer. For that purpose, we used giant unilamellar vesicles to study the effect of permeabilizing electric fields in simple membrane models. Experiments showed a decrease in vesicle radius which is interpreted as being due to lipid loss during the permeabilization process. Three possible mechanisms responsible for lipid loss were directly observed and will be presented: pore formation, vesicle formation and tubule formation, which may be involved in molecules uptake [12].

However, a lipid bubble is not a cell and a tissue is not a simple assembly of single cells. Therefore, in the last part of the lecture, our new data for the understanding of the DNA electrotransfer process in tissues, obtained on multicellular tumor spheroids as an ex vivo model of tumor, will be presented. Upon growth, spheroids display a gradient of proliferating cells. These proliferating cells are located in the outer cell-layers and the quiescent cells are located more centrally. This cell heterogeneity is similar to that found in avascular microregions of tumors. We used confocal microscopy to visualize the repartition of permeabilized cells in spheroids submitted to electric pulses. Our results reveal that if small molecules can be efficiently transferred into cells, including the ones present inside the spheroids, gene expression is limited to the external layers of cells [13]. Taken together, these results, in agreement with the ones obtained in tumors, indicate that the spheroid model is more relevant to an in vivo situation than cells cultured as monolayers. The problem of the access of DNA to the internal layers of cells still remains. A possible solution is the application of very low intensity but long pulses to electrophoretically push the DNA towards the center of the tumors, before applying standard electric pulses.

## CONCLUSIONS

Classical theories of electropermeabilisation present some limits to give a full description of the transport of molecules through membranes. Certain effects of the electric field parameters on membrane permeabilisation, and the associated transport of molecules, are well established but a great deal of what happens at the molecular level remains speculative. Molecular dynamics simulations are now giving interesting new insight into the process. However, a cell membrane is highly complex and can not be considered as the simple assembly of one or two classes of lipids.

Electroinduced destabilisation of the membrane includes both lateral and transverse redistribution of lipids and proteins, leading to mechanical and electrical modifications which are not yet fully understood. One may suggest that such modifications can be involved in the subsequent transport of molecules interacting with them such as the DNA molecules. Experimental verification of the basic mechanisms leading to the electropermeabilisation and other changes in the membrane remain a priority given the importance of these phenomena for processes in cell biology and in medical applications.

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**Marie-Pierre Gorsse Rols** was born in Decazeville, the "gueules noires" city of the Duc Decazes, France, in 1962. She received a Masters in Biochemistry, a Ph.D. in Cell Biophysics and the Habilitation à Diriger les Recherches from the Paul Sabatier University of Toulouse in 1984, 1989 and 1995, respectively. She is currently Director of Research at the IPBS-CNRS laboratory in Toulouse and Vice-President of the GEIMM French group on molecule-membrane interactions, secretary of the GTRV French group on vectorisation. Her main research interests lie in the fields of membrane electropermeabilization in vitro and in vivo. In the last years, she has focused on the mechanism of DNA electrotransfer. Marie-Pierre Rols is the author of 64 articles in peer-reviewed journals. In 1993 she received the Galvani Prize of the Bioelectrochemical Society, in 2006 a joined prize of the Midi-Pyrénées region.

## NOTES

# Electropermeabilization *in vivo*

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**Abstract:** Tissues are complex assemblies of various types of cells. Moreover, for the main cell type of the tissue, both cell size, cell shape and cell-cell contacts are very different from tissue to tissue. Thus, as a function of the various tissues, there are large differences in the electrical parameters that allow achieving cell electropermeabilization *in vivo*. Usual methods for detecting cell electropermeabilisation *in vivo* are reported here. Finally, other important points are the electrodes type and their positioning, which influence electric field distribution in the tissue as well as electropermeabilization level and extent in the tissue.

## INTRODUCTION

A tissue is a complex structure. It contains the cells that characterize this tissue, with their own physiological, but also geometrical properties. For example, the fibers of the skeletal muscle are not only very long, almost cylindrical, cells, but their diameter is also much larger than that of all the other cells of the organism. Besides their characteristic cells, tissues also contain vessels (thus endothelial cells, smooth muscle cells, blood cells …) nerves, fibroblasts …

Moreover, other tissue specific properties can also considerably modify the behaviour of the cells in a tissue in response to the delivery of given electric pulses. For example, while tumor cells can often be considered as individual cells, hepatocytes in liver are electrically connected between them by means of the gap junctions that allow the free flow of molecules up to 2000 daltons between the connected cells. Thus it was expected that large differences could be observed between the different tissues and the present data confirm these expectations.

While electropermeabilisation achievement in tumors has been actually demonstrated (for example using cytotoxic molecules as described here below), quantitative data concerning tumor permeabilisation are difficult to obtain, as compared to other tissues. Indeed, tumors are very heterogeneous tissues, the tumor cells being also very irregular, as well as the vasculature of the tumor nodules.

In summary, analysis of tissue electropermeabilisation is much less easy than that of the cells in culture.

## METHODS FOR DETECTING *IN VIVO* CELL ELECTROPORATION

The delivery of appropriate electric pulse alone can be sufficient to detect irreversible cell electropermeabilization. Indeed, cell death, the natural consequence of irreversible cell electropermeabilization, can be detected several hours/days after the pulses delivery by conventional histological and immunocytochemical microscopic procedures.

To detect reversible cell electropermeabilisation, whether *in vitro* or *in vivo*, it is necessary to use a non permeant marker that will (almost) exclusively enter (and thus label or modify) the electropermeabilized cells. If this marker molecule does not bind to (or interact with) intracellular targets, then it allows to simultaneously check cell electroper¬meabilisation and cell membrane resealing, which is the first step to maintain cell survival. Indeed, if cells do not reseal, not only they will die but moreover they will loose the marker molecule, which will leak out of the cells. Then both reversible and irreversible electropermeabilisation threshold can be determined, for example as a function of the ratio of the applied voltage to electrodes distance (in V/cm).

*In vivo*, there are much more constraints than in the *in vitro* experiments. Indeed, as outlined previously, tissues are compact structures and the permeabilisation markers, even if they have a very low molecular weight, will not diffuse until the core of a piece of tissue ex vivo, for example by just placing the piece of tissue in a baker containing the permeabilisation marker. Similarly, the marker cannot usually be injected directly into the piece of tissue because the distribution of the marker will be quite inhomogeneous, forbidding quantitative and even qualitative analysis. Moreover sometimes it is quite hard to inject tissues because of either their fragility or their compactness, which may be a real problem in the case of some tumor types. Moreover, tumors are not limited by a physiological physical barrier (like the fascia in muscle or the capsule in liver) and leaks can easily occur after intratumor injections.

Thus, for an as much homogeneous as possible and efficient distribution of the marker, it is necessary to inject it *in vivo*, intravenously if possible. Of course, this is only possible if the marker is very "potent" (that is, if a limited number of molecules is sufficient to label or to modify the electropermeabilized cells, e.g. bleomycin or 51Cr-EDTA, because intravenous injection results in a large dilution of the injected marker). Then, after the injection, it is necessary to

wait for the redistribution of the marker from the vascular compartment to the tissular compartment, that is until the marker will be actually in the vicinity of the cells of the tissue. Depending on the marker, optimal time window for electric pulses delivery depends on parameters such as marker size, but also on heart beating rate. This time window of course is comprised between the end of the marker distribution from the blood into the tissues and the beginning of the decrease of the tissue concentration due to excretion (through kidneys to the urine) or metabolisation of the marker.

Therefore, marker must be an injectable product that will not be toxic for the laboratory animal, at least in the absence of the electric pulse delivery (indeed, as shown here below, bleomycin has been used as electropermeabilization marker). Of course, this marker molecule has to have a property that allows to trace the molecule itself or the consequences of its internalisation into the electropermeabilized cells, as described here below for each of them.

At least the following molecules have been used:

**BLEOMYCIN**

Bleomycin has been used to quantitatively and qualitatively analyse *in vivo* cell electropermeabilization. The qualitative use of bleomycin [1] was based on morphological changes of nucleus appearance induced by bleomycin biological effects on DNA (achievement of DNA double strand breaks, [2,3]) at high bleomycin doses. The interest of the test is that a topological information can be obtained, indicating thus electric field distribution in the tissue if bleomycin is homogenously distributed in the tissue (after intravenous injection of the drug). The quantitative use of bleomycin is based either on the injection of radioactive bleomycin (the 57Cobalt-bleomycin is a very stable complex [4] that allows to follow bleomycin distribution in the body using e.g. gamma cameras; 118Indium-bleomycin has also been used, with the interest that half life of 118Indium is short allowing to inject higher specific activities than using 57Cobalt; however, stability of 118Indium-bleomycin is lower than that of the 57Cobalt-bleomycin. In the case of the 57Cobalt-bleomycin, strict experimental precautions must be taken for animal handling because of the long half-life of the 57Cobalt gamma emitters (270 days).

Using 57Cobalt-bleomycin, Belehradek and colleagues showed a 4 times increased retention of radioactive bleomycin in tumors exposed to permeabilizing electric pulses as compared to unexposed tumors [5]. This factor was equivalent to the one observed *in vitro* [6] using cells in suspension exposed to external concentrations of radioactive bleomycin similar to those measured in mice blood at the time of tumor exposure to the electric pulses. Cell electropermeabilization *in vivo* was also demonstrated using the huge increase in bleomycin cytotoxicity when the electric field intensity is above the treshold necessary to achieve cell permeabilisation [5]. Indeed, using an appropriate drug concentration (like the rather low therapeutic concentrations of bleomycin used in the clinical application termed antitumor electrochemotherapy), all the unpermeabilized cells remain alive in spite of the external presence of bleomycin, while all the permeabilized dividing cells are killed by the internalized bleomycin. Electric pulses of various field intensities were applied to pieces of tumors removed from mice three minutes after bleomycin injection to the animal. Cell killing due to the permeabilization-facilitated uptake of bleomycin was then determined. The existence of a threshold intensity demonstrated the occurrence of cell permeabilisation in tissues [5]. It is noteworthy that the threshold in tumor tissue was inferior to the threshold found with the same tumor cells in suspension exposed to the same electric pulses.

**51Cr-EDTA**

51Cr is also a gamma emitter but its half-life is very short and the 51Cr-EDTA complex is very rapidly secreted from the organism. It is used regularly in clinics for scintigraphic examinations. This product is thus easily available. Usually electric pulses must be delivered at a short, precise time after the intravenous injection of the 51Cr-EDTA. One hour after the pulses delivery, a difference in the retention of the radioactivity between the muscle exposed to reversibly permeabilizing electric pulses and the contralateral non exposed muscle can already be observed in the skeletal muscle [7]. If the mouse is sacrificed 24 hours after the electric pulses delivery, the control unpulsed muscles do not contain any radioactivity and less animals can be used to have the same number of experimental samples (exposure to the electric pulses of the two contralateral muscles) [8]. The quantitative 51Cr-EDTA test for the evaluation of the *in vivo* electropermeabilisation level has already allowed :

- to determine reversible and irreversible thresholds [7,9];
- to show differences between internal and external electrodes [10];
- to show differences between pulses of different durations thresholds [7,9];
- to show similarities between the same tissue in different species thresholds [7,9,10];
- to show differences between different tissues [10].

**PROPIDIUM IODIDE**

As *in vitro*, Propidium Iodide has also been used to show *in vivo* permeabilisation achievement, based on the increase of fluorescence of this molecule when it can enter the cells and bind to DNA [11]

**(99m)Tc-DTPA**

Radiolabelled diethylenetriaminepentaacetic acid (DTPA) was used to trace the distribution and internalisation of a hydrophilic drug after *in vivo* electropermeabilization [12]. Skeletal muscle tissue in rat was treated with permeabilising electric pulses before or after intravenous administration of (99m)Tc-DTPA. The drug accumulation in the treated volume was subsequently evaluated with a scintillation camera.

**ELECTROPORATION OF CELLS IN TISSUES**

Permeabilization has been demonstrated and evaluated using the methods described in the first part of this chapter. As main trends, it is important to highlight that:

- the range of voltages between the thresholds for the reversible and irreversible permeabilization are much larger *in vivo* than for the cells exposed *in vitro*. For example, in the skeletal muscle exposed to 8 transcutaneous pulses of 100 µs, the reversible threshold was found at 450 V/cm, while the irreversible one was 800 V/cm [7]. Usually, in cells in culture, using the same type of electric pulses, the irreversible permeabilisation threshold is always much more smaller than a value twice of that of the reversible threshold. In an ex vivo experiment, using slices of tumors prepared from mice having received an intravenous injection of bleomycin (see above), reversible permeabilisation was achieved at voltages as low as 350 or 550 V/cm (depending on the individual tumors considered) while the irreversible threshold was above 1200V/cm (determined by the absence of cell killing by the electric pulses alone) [5]. Moreover the comparison was done with the electropermeabilisation of same cells in suspension instead that in the tissue. For the cells in suspension, the permeabilisation threshold was at 700 V/cm, a value higher than the one found on tissue slices treated ex-vivo (350 or 550 V/cm) [5]. This example shows how much the structure of the tissue can affect the permeabilisation of the cells within that tissue.
- the duration of the permeabilized state is longer that the duration that could be expected from experiments *in vitro* on isolated cells. Indeed, *in vitro*, resealing time depends on the temperature and, at about 37°, cells become impermeable in less than one minute. *In vivo*, muscle, fibers remain at a high level of permeabilisation for more than 5 minutes after one single HV of 100 µs [8] and between 7 and 15 minutes after 8 pulses of 100 µs [7].
- there is a transient vascular lock in the volume exposed to the electric pulses. A temporary arrest of the blood flow in the treated volume of tissue has been described in all the electropermeabilized tissues [13], partly due to a physiological, histamine dependent reaction, and partly due to the permeabilisation of the cells, including the permeabilisation of the endothelial vascular cells. Interestingly, this vascular lock is much more pronounced in the tumors [14], maybe due to their irregular vasculature, where it last for hours instead than for a few minutes. This vascular lock prevents the washing of the drugs from the electropermeabilized tissue and can help in the uptake of the anticancer drugs by the tumor cells.
- for the skeletal muscle, the same thresholds were found between the mouse and the rat [7 and D. Cukjati et al. in preparation], showing that differences between various tissues are larger than the differences between the same tissue from different species.

**MODELS OF TISSUE ELECTROPERMEABILISATION**

Several models of tissue electropermeabilization have been published and will not be compared in detail here since they are basically dependent on the electrodes geometry. Only a few general features wil be recalled.

A two-dimensional model [1] was used in 1999 to compare two types of electrodes: plate electrodes and rows of needle electrodes (two kind of electrodes largely used in ulterior experiments). A good fit was found between the percentages of tissue exposed to fields of strength above a given value and the 51Cr-EDTA uptake values at different field strengths. Thus the first precise value of the reversible permeabilization threshold could be determined in the skeletal muscle.

A numerical three dimensional model was proposed in 2000, and it was topologically validated using the bleomycin qualitative test described here above [1]. This model has been quite important to define electrodes geometry since it showed that in the case of needle electrodes, the diameter of the needles is of the utmost importance to have a more or less heterogeneous distribution of the electric field between the electrodes (and these differences could then be experimentally demonstrated). The model has been refined: it has been possible to made a numerical model of the dynamics of tissue permeabilisation *in vivo* [15]. Indeed, the permeabilisation of the part of the tissue exposed to the highest electric field strengths changes the electrical properties of this part

of the tissue, and therefore changes the electric field distribution and thus the tissue volume that will be actually exposed to fields above the permeabilisation threshold. Model has also allowed giving instructions to the physicians applying the electrochemotherapy antitumor treatment for the correct use of the various types of available electrodes (plate electrodes or needle electrodes [16,17]. Indeed the placement of the electrodes with respect to the tissues is very important to get a rather uniform and enough intense local electric field in the tissue: as a general rule, the larger the contact surface between the electrode and the tissue, the better [18]. The use of appropriate conductive gels is also recommended in some situations [19].

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Lluis M. Mir was one of the pioneers of the research of electropermea-bilization (electroporation) and the applications of

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## NOTES

# DNA electrotransfer *in vivo*: An efficient non-viral approach for gene therapy Application of electroporation in gene transfection

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**Abstract:** At the end of the 90's, several publications from various laboratories reported efficient in vivo electrotransfer of plasmids coding for several reporter genes. This was the natural evolution resulting from the finding that Eberhard Neumann and colleagues published in 1982 [1] that reported successful DNA electrotransfer into living cells in vitro by means of electric pulses. Mechanisms of gene electrotransfer in vivo have been studied in detail. For example, in skeletal muscle, electropermeabilisation of the muscle fibers is mandatory, but efficacy is determined by an electrophoretic effect of the electric pulses on the DNA injected in the muscle. Optimal electric parameters differ from tissue to tissue, mainly imposed by the tissue characteristics. Nowadays clinical trials of this non-viral gene therapy method are in progress with different purposes, including cancer treatment and vaccination.

## INTRODUCTION

The development of DNA electrotransfer and its progression towards its application in the clinics is also the result of the general developments concerning the *in vivo* use of electric pulses to electropermeabilize solid tumors after the delivery of non permeant or low permeant cytotoxic drugs (this combination was termed electrochemotherapy) [2,3]. It seemed thus possible to transfer plasmid DNA to cells *in vivo* by appropriate electric pulses (DNA electrotransfer).

Very efficient DNA transfer has been shown in the last ten years, particularly to skeletal muscle in a number of animal species including cattle [4,5]. Efficiency can approach that of the viral methods. However biological safety is much higher because there is no virus manipulation at all. The easiness and security of DNA preparation is also an important issue that pleads in favor of the electrogenetherapy. As discussed below, efficacy is proven in several tissues, particularly in the skeletal muscle. Finally, appropriate equipment is available that is based on the two distinct roles of the electric pulses in DNA electrotransfer, that is the targeted cell electropermeabilization and the electrophoretic transport of the DNA towards or across the electropermeabilized membranes. Thus DNA electrotransfer actually appears to be an appealing non viral approach for gene therapy.

## DNA ELECTROTRANSFER IN SKELETAL MUSCLE

A search for optimised conditions using trains of similar square wave pulses was performed by Mir and colleagues in 1999 [4]. The main conclusions were that, with respect to the injection of naked DNA (plasmid DNA alone in saline or phosphate buffer), DNA electrotransfer allowed to achieve a 200 times increase in gene expression and a large reduction in the variability of gene expression when 8 consecutive pulses of 200 V/cm and 20 ms were delivered to the muscle at a repetition frequency of 1 Hz after DNA injection [4]. The same group showed that, using these conditions, expression of a reporter gene (in this particular work, coding for the firefly luciferase) is maintained for at least 9 months in the skeletal muscle [6]. These conditions are largely used nowadays, even though other pulse conditions were also proposed [7,8]. In particular these conditions are interesting since they allow the co-transfer of several plasmids coding separately for a protein of interest (for example a "therapeutical" protein) and for factors allowing the regulated expression of the "therapeutical" protein [9]. Moreover, it has been shown that these conditions induce the expression of the endogenous gene coding for metallothionein I, opening new ways for both gene transfer AND expression control [10].

## DNA ELECTROTRANSFER MECHANISMS ANALYSED IN MUSCLE

The mechanisms of DNA electrotransfer have been analysed in the skeletal muscle using combinations of high voltage short duration pulses (HV; 100 µs and voltage such as the ratio of applied voltage to electrodes distance is comprised between 800 and 1300 V/cm, as a function of the tissue treated and of the electrodes used) and of low voltage long duration pulses (LV; 50 to 400 ms and several tens of V/cm, that is of a strength below the electropermeabilisation threshold of the tissue) [11]. It has been shown that, as expected, the electric pulses must "permeabilize" the targeted cells. This can be obtained even with a single HV pulse, that does not result in a very high level of muscle fiber permeabilisation [12] as measured using the 51Cr EDTA uptake test [13]. The electric pulses have a second role: to electrophoretically move the DNA towards or across the "electropermeabilized"

membrane. Moreover, DNA does not need to be present at the time of the cell electropermeabilisation [11] but it is mandatory to inject it before the electrophoretic LV pulse [4, 11]. Actually, the LV component is the real responsible for plasmid transfer to muscle fibers since it efficiently pulls the DNA towards the membrane still altered after the delivery of the electroporating pulse. It is not possible to say towards the "electroporated" membrane because high levels of permeabilisation, under the same experimental conditions, only last for 300 seconds (5 minutes)[11]. The precise structure of the membrane during this period of time (between 5 and 50 minutes after the delivery of the HV) is not known but this kind of observations might argue in favor of the electropermeabilisation theory. Nevertheless, it is possible to conclude that target cell electropermeabilisation is mandatory, but that electrotransfer efficacy is determined by the EP electrophoretic component [11, 14, 15]. Safety of the procduree was also demonstrated as only minor perturbations of muscle fibers physiology were reported [16].

## DNA ELECTROTRANSFER IN LIVER

DNA transfer in liver, using short pulses, was described in 1996 [17] (this was the second paper relating DNA electrotransfer in vivo, after the article by Titomirov et al in 1991 [18], in which exogenous myc and ras genes were expressed in a few of the skin cells exposed in vivo to the DNA and the electric pulses). However much care is necessary in experiments dealing with gene transfer in liver. Indeed, hepatocytes in vivo are easily transfected by simple hydrostatic pressure [19]. Recent data indicates that using long LVs (for example 4 LV of 100 ms) at field strengths even rather low (for example as low as 20 V/cm), there is no need for an HV. The exact reasons for such behaviour are not yet understood [15].

## DNA ELECTROTRANSFER IN TUMORS

The first tissue to which DNA was transferred by means of long electric pulses were tumors transplanted in the flank of mice [20]. A clear increase in the efficacy of DNA transfer was shown. DNA has been transferred to various types of tumors. However, the results are much less reproducible than in the case of plasmid DNA transfer to the skeletal muscle. The main reason for such variability lies on the structure of the tumors themselves: tumors are heterogeneous tissues, not limited by a physiological physical barrier (like the fascia in muscle or the capsule in liver). Injection is more or less easy, reproducible and complete depending on the consistence of the tumor (for example, experimental melanomas like the B16 melanoma are soft, inflatable tissue while fibrosarcoma is a hard, breakable one). Injection often results in a very heterogeneous distribution of the fluid and thus of the DNA. Nevertheless DNA transfer has been achieved both using trains of similar 20 ms squave wave pulses (but the voltage was adapted to obtain a ratio of the voltage applied to the electrodes distance of 600 V/cm) [21] or using combinations of HV and LV [15].

## PERSPECTIVES

### DNA electrotransfer to non accessible targets

In preclinical studies most of the experiments dealt with the electrotransfer of DNA to the skeletal muscle, using external non invasive electrodes. However other tissues like liver have been exposed to the electric pulses after open surgery of the laboratory animals. In larger animals, as well as in clinical trials, it is possible to foresee the use of electrodes for minimally invasive electrochemotherapy, such as the treatment of organs reachable through endoscopes. This kind of electrodes is under development. Similarly, electrodes on balloon catheters were tested in animals for DNA electrotransfer in situ to the wall of vascular trunks, in order to establish the feasibility of a new treatment of the restenosis.

### DNA electrotransfer combined to ECT

DNA electrotransfer uses electric pulses, like the electrochemotherapy. Some attempts have been performed to deliver genes and drugs either simultaneously or successively. When using bleomycin in electrochemotherapy, most of the published work has been performed by the group of R. and L. Heller. The DNA electrotransferred coded for either the IL-2 or the GM-CSF. To obtain an increase of the ECT efficacy due to an appropriate stimulation of the immune system, GM-CSF gene must be transferred to the tumor cells the day before the ECT, while IL-2 gene must be transferred to the dying tumor cells (and most probably to the stromal and surrounding normal cells) the day after the ECT [22]. No beneficial effect of the combination was found if bleomycin and these genes were transferred simultaneously.

Interesting studies have been performed on horses affected by sarcoids, a skin tumor. ECT using cisplatin has been combined with the electrotransfert of IL-12 genes to the tissues around the treated tumors. Because of the immune response mediated by the IL-12, the authors of this study have termed this approach electro-chemo-immuno-gene-therapy [23].

## Painless approaches or methods to control the sensations

Animals are treated after inducing general anaesthesia using standard laboratory protocols. However, the translation of the DNA electrotransfer to humans requires an extensive analysis of the analgesia or sedation needs. Indeed, it is convenient to avoid, as much as possible, unnecessary patient's anaesthesia. The sensations caused by HV pulses alone are well known since they are used to treat solid tumors in patients with electrochemotherapy. It has been reported that electrochemotherapy provokes disagreeable sensations linked to the passage of the electrical current and there is indeed an "immediate" pain if these sensations are too intense. However there is never long term pain since sensations stop immediately when current passage ceases (except in cases where bleomycin dosage was too high).

The Standard Operating Procedures for the Electrochemotherapy of cutaneous and subcutaneous tumor nodules provide the physicians with the rules to avoid pain during ECT application [24]. The same procedures have been applied before delivering genes in humans using HV + LV combinations. Pain was prevented, which means that the same procedures seem valid for Electrogenetherapy (G. Sersa and J. Gehl, personal communication).

## Clinical perspectives

Currently clinical trials are ongoing, with different genes, using trains of identical long pulses in muscles, or trains of identical short pulses or HV+LV combinations in melanoma [25]. One of the trials is already closed. It reported good antitumor effects [26].

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## NOTES

# Gene expression regulation by siRNA electrotransfer

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**Abstract:** In a number of evolutionary-distant organisms the introduction of a double-stranded RNA in the cell induces the degradation of homologous messenger RNA. This phenomenon, discovered in 1998 and called RNA interference (RNAi), through the use of siRNAs, represents a powerful tool for reverse genetics in mammalians and possibly for gene therapy in humans. The major hurdle to the therapeutic development of RNAi strategies remains however the efficient delivery of the RNAi-inducing molecules, the short interfering RNAs (siRNAs) and short hairpin RNAs (shRNAs), to the target tissue. Electroporation can provide siRNA delivery in various tissues. We have investigated the contribution of electrically-mediated delivery of siRNA into muscles and into tumors stably expressing a green fluorescent protein (EGFP) target reporter gene. The silencing of EGFP gene expression was quantified over time by fluorescence imaging in the living animal. Our results indicate that electric field can be used as an efficient method for RNAi delivery and associated gene silencing into cells of muscle and solid tumors in vivo.

## INTRODUCTION

Since its discovery [1], a tremendous number of data have been reported on the phenomenon of RNA interference and our understanding of the molecular mechanisms and intracellular function of RNAi, although still incomplete, has been considerably improved. RNA interference has been described and extensively characterized in a number of organisms [2-4].

The identification of the short interfering RNAs (siRNAs) involved in this process and their use for sequence specific gene silencing has offered a new approach for molecular therapeutics by taking advantages of the progress in genomics [5, 6].

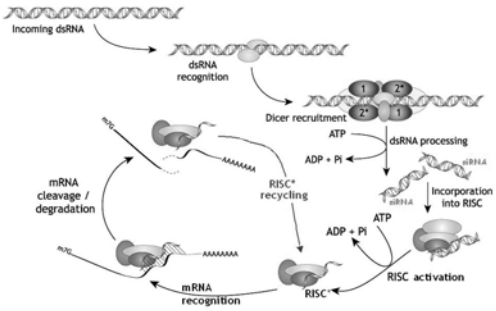


***Figure 1:*** Current model for RNAi-induced mRNA degradation

Chemically-synthesized siRNAs have also been proposed as powerful reagents for therapeutic gene silencing owing to their great specificity and efficiency, their apparent lack of toxicity and their usually moderate propensity to trigger non specific gene silencing and/or interferon-related responses in the cell (the so-called "off-target" effects) [7-9]. This technique is an attractive alternative to the use of antisense oligonucleotides and ribozymes for therapies based on the inhibition of target genes. Several applications of siRNA therapeutics are currently under clinical development [7,10].

Basically, two non exclusive therapeutic RNAi strategies can be applied. The first one will involve the specific introduction in the target cells or tissues of DNA sequences allowing the sustained production of inhibitory RNAs such as shRNAs and microRNAs. This approach, related to gene therapy, will have to face the difficulties encountered with this technique and associated with the use of viral vectors. The second approach will be based on the direct *in vivo* treatment with either siRNAs, native or chemically modified and/or conjugated with other compounds facilitating their delivery. siRNAs which can now be produced at moderate costs represent indeed a very attractive new type of small molecule drugs.

This development requires, however, new safe and efficient *in vivo* siRNA delivery methods. SiRNAs appear as a very promising new therapeutic agent but besides the problem of delivery, an unanswered problem is to know how long its effect lasts after a single dose delivery [11]. Different complementary approaches are currently developed in rodents in order to increase the *in vivo* stability of the siRNA molecule, to target it to the correct tissue and to facilitate its cellular uptake. These include chemical modification of the silencing RNA [10] packaging into protective particles [12,13] and conjugation to cell-specific ligands or antibodies [14,15]. Most of the *in vivo* published results were obtained by "hydrodynamic transfection", but other methods were described where a systemic or a localized (portal vein injection) delivery was obtained by adding different chemical compounds to the siRNA solution [16,17].

SiRNA gene silencing could be obtained *in vivo* on reporter as well as endogenous genes. This remains a

critical issue for the development of siRNA as an effective therapeutic.

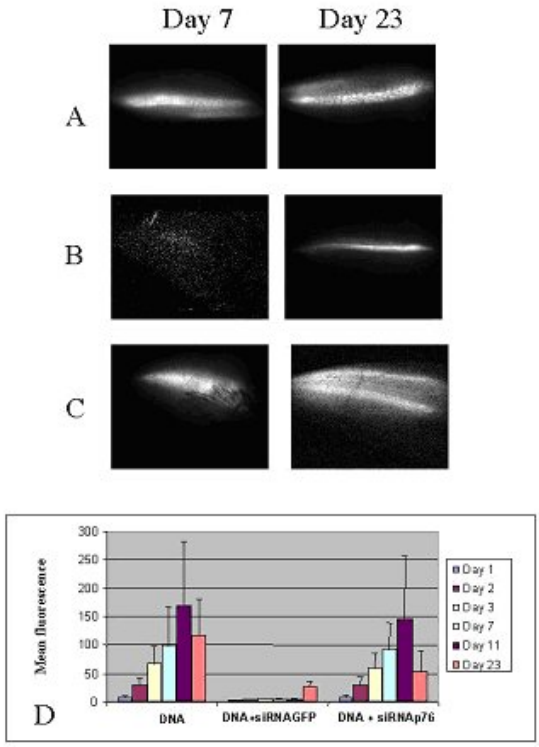


***Figure 2:*** RNA interference in 9 weeks old C57Bl/6 mouse leg muscle. Representative images of the GFP fluorescence of the mouse leg are shown. (Each image is 1 cm wide).
A- GFP expression resulting from plasmid alone electrotransfer as observed on days 7 and 23 in the same leg.
B- GFP expression silencing as observed on days 7 and 23 in the same leg when the plasmid was cotransferred with the specific egfp22 siRNA.
C- GFP expression remained unaffected when an unrelated siRNA (p76) was cotransferred with the plasmid.
D- Changes in the mean fluorescence emission with time. Sample standard deviations are shown. (n=4).

The demonstration in 1998 of drug and plasmid electrotransfer and gene expression in tumors [18,19] led to the proposal that *in vivo* electropulsation was a promising tool for delivery of exogenous agents [20]. Furthermore it was observed that a very efficient *in vivo* electroloading of large molecules other than plasmids was obtained for proteins [19], dextran [21], and antisense oligonucleotides [22]. Electrically mediated gene transfer had been shown to be effective on many tissues: liver [23], skin [24], muscle [21,25] and heart [26]. Delivery is targeted to the volume where the field pulse is applied, *i.e.* under the control of the electrode localization. This technology allows delivery to almost all tissues, after a small surgery when needed. Impressive results were described in the case of muscles where treatment with non-invasive contact electrodes brought a long lasting expression of therapeutic genes [27].

Recent developments in optical imaging provide continuous monitoring of gene delivery and expression in living animals [28]. Indeed, reporter gene activity can be accurately followed of the same animal as a function of time with no adverse effects either on the reporter gene product or on the animal itself. This increases the statistical relevance of a study, while decreasing the number of animals required. Exogenous gene expression of fluorescent reporter proteins such as GFP can be detected by the associated emission using a highly sensitive CCD camera**.**

Plasmid electrotransfer and expression in muscles are known to be very efficient. Up to 70-80 % of the fibers can be transfected after injection of 25 µg of a GFP coding plasmid and by using adequate electrical conditions. Emission of the green protein was high 7 days after the electrical treatment. When the specific siRNA was electro-transferred (DNA +AntiGFP), significant decrease of the GFP expression was observed. Our fluorescence analyses also led us to conclude that inhibition of gene expression lasts more than 11 days. SiRNA delivery therefore occurs in almost all fibers.

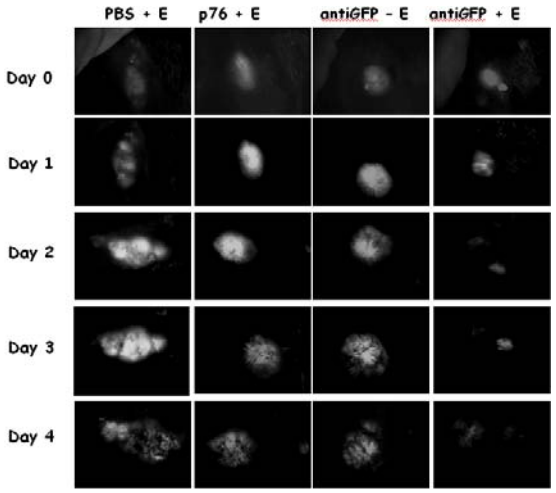


***Figure 3:*** Time dependence of mean fluorescence intensity of the B16F10 GFP tumors in tumors treated with; electric field : PBS + E, unrelated siRNA : p76 + EP, 12 µg of egfp22 siRNA alone : AntiGFP - E and egfp22 siRNA electro-transferred : AntiGFP + E.

*In vivo* imaging was used to follow fluorescent tumors and fluorescence intensity as a function of time. Tumor growth was not affected by the treatment (neither siRNA intra-tumoral injection nor electric pulses i.e. E). When the specific siRNA was electro-transferred (AntiGFP + E), significant decrease of the GFP expression was observed within 2 days following the treatment. As shown in the pictures of the figure 3, the fluorescence associated to the tumor disappeared in the treated group (AntiGFP + E) whereas the

fluorescence remained the same in the different control groups.

More and more studies investigated the effectiveness of electropulsation for the localized delivery of siRNA in adult mice (muscle, tumor, testis …) [29-34].

For example, it has successfully been used by others to silence the Mitf gene in mice tumors leading to reduction in the outgrowth of subcutaneous melanoma [35].

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**Muriel Golzio** was born in Montauban, France, in 1970. She received a Ph.D. in the "Universite Paul Sabatier Toulouse III" in 1999. Since 2002, she is Researcher at the "Institut de Pharmacologie et de Biologie Struscturale" of the "Centre National de la Recherche Scientifique" in Toulouse, France. Her main research interests lie in the field of *in vivo* nucleic acids transfer by electroporation visualized by mean of optical imaging on live small animals.

**NOTES**

# DNA vaccine by electrogenetransfer

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**Summary:** DNA vaccination is a new vaccine approach used to induce an immune response to an antigen protein expressed in vivo. It is based on the injection of a purified DNA plasmid encoding for the polypeptide sequence. The resulting in situ protein synthesis involves biosynthetic processing and post-translational modifications.

The effectiveness of DNA vaccines has been demonstrated in many animal models. Cell-mediated immunity (Th1 and Th2 responses) and humoral immunity can be obtained.

Various approaches to boost the immune response have been studied, including co-administration of cytokines, co-stimulation with specific genes, and addition of targeting molecules. DNA vaccine and Gene therapy techniques are similar. DNA vaccines have been introduced into animal tissues by a number of different methods and one of the most promising method is "electroporation".

Research with animal models has shown that DNA electrovaccines are safe and efficient. Deleterious immune responses, such as autoimmunity and development of tolerance in response to persistent expression of a foreign antigen, have not been observed.

Phase I and Phase II clinical trials with DNA vaccines have been conducted for HIV, HBV, HVC, HSV, tuberculosis, and malaria. Clinical trials are also in hand for cancer and the treatment of allergies. Moreover, DNA electrovaccination offers new hope because of its low cost and manufacturing stability at ambient temperature.

## INTRODUCTION

The development of vaccination against harmful pathogenic microorganisms represents an important advancement in the history of modern medicine. Traditional vaccination has relied on two specific types of microbiological preparations to produce material for immunization and generation of a protective immune response (living infectious material that has been manufactured in a weaker state or inert, inactivated, or subunit preparations).

In 1990, it was first demonstrated that direct injection of naked plasmid DNA encoding a foreign antigen into mouse myocytes resulted in uptake of the DNA by host cells and subsequent expression of the foreign antigen [1]. Thereafter, animal studies clearly demonstrated that DNA vaccines were capable of eliciting humoral [2] and cellular immune responses against the encoded vaccine antigen [3,4]. Following these studies, plasmid DNA vaccine strategies have been evaluated in a wide variety of animal models [5,6]. DNA vaccines are less efficient in large animal maybe because of inefficient uptake of DNA and subsequent immune responses [6]. To overcome this problem, electroporation or electro-permeabilization (EP) have been used to increase in vivo transfection and therefore associated immune responses [7,8,9,10,11]

In this paper, the principle and the mechanism of DNA vaccines is described. Then, the various approaches to boost the immune response we be analysed. Finally clinical trials cases involving DNA vaccines and electropermeabilization are cited.

## DNA VACCINES OR GENETIC IMMUNIZATION

DNA-based immunization has now become a novel approach to vaccine development. Direct injection of naked plasmid DNA induces strong immune responses to the antigen encoded by the gene vaccine. Once the plasmid DNA construct is injected, the host cells take up the foreign DNA, expressing the viral gene and producing the corresponding antigen protein inside the cell. This form of antigen presentation and processing induced both MHC class I and class II restricted cellular and humoral immune responses [12,13].

## VARIOUS APPROACHES TO BOOST THE IMMUNE RESPONSE

Both better engineering of the DNA plasmid and improved expression may enhance antibody response to the gene products and expand the applications of the gene vaccines. Therefore, DNA vaccine and Gene therapy techniques are similar.

***Administration:*** Several possible routs of plasmid delivery have been tested. Successful immunization has been demonstrated after delivery of plasmids through intra-muscular, intradermal injection [15, 9]. The skin and mucous membranes are considered the best site for immunization due to the high concentrations of dendritic cells (DC), macrophages and lymphocytes.

The plasmid DNA can be diluted in distilled water, saline or sucrose, or mixed with gold particles that act as an attractant for immune cells [16].

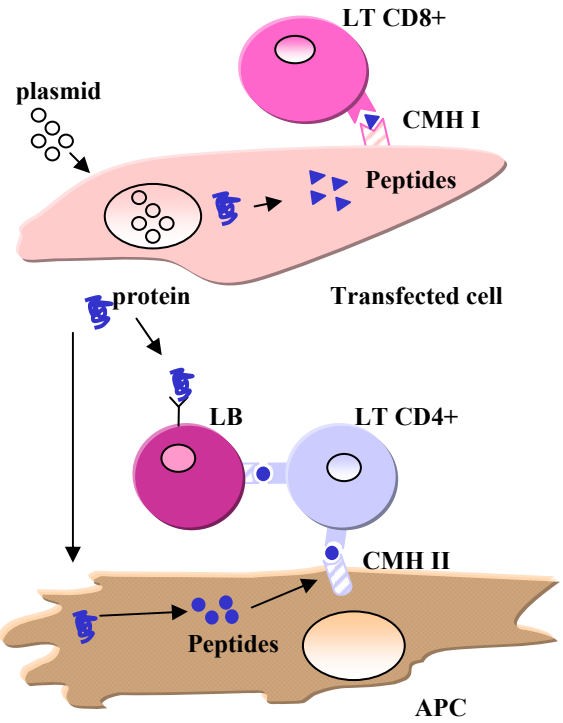


***Figure 1:*** DNA vaccine Mechanism: A plasmid vector that expresses the protein of interest (e.g. viral protein) under the control of an appropriate promoter is injected into the skin or muscle of the host. After uptake of the plasmid, the protein is produced endogenously and intracellularly processed into small antigenic peptides by the host proteases. The peptides then enter the lumen of the endoplasmic reticulum (E.R.) by membrane-associated transporters. In the E.R., peptides bind to MHC class I molecules. These peptides are presented on the cell surface in the context of the MHC class I. Subsequent CD8+ cytotoxic T cells (CTL) are stimulated and they evoke cell-mediated immunity. CTLs inhibit viruses through both cytolysis of infected cells and noncytolysis mechanisms such as cytokine production [12]. The foreign protein can also be presented by the MHC class II pathway by APCs which elicit helper T cells (CD4+) responses. These CD4+ cells are able to recognize the peptides formed from exogenous proteins that were endocytosed or phagocytosed by APC, then degraded to peptide fragments and loaded onto MHC class II molecules. Depending on the the type of CD4+ cell that binds to the complex, B cells are stimulated and antibody production is stimulated. This is the same manner in which traditional vaccines work [14].

***Construction:*** DNA vaccines are composed of bacterial plasmids. Expression plasmids used in DNA-based vaccination normally contain two unites: the antigen expression unit composed of promoter/enhancer sequences, followed by antigen-encoding and polyadenylation sequences and the production unit composed of bacterial sequences necessary for plasmid amplification and selection [14].

Tissues specific promoter [17] or several (2 to 3) antigen-encoding sequences [18] can be used. The number of antigens is defined by the size of the plasmid and its delivery in the host tissue [18, 19].

As the immunogenicity of DNA plasmids relies, to the large extent, on the presence of CpG motifs as built in adjuvants, plasmids used for vaccination purposes may be enriched in immunostimulatory sequences [20].

Co-delivery of cytokines [21, 22] or viral sequences [23] can enhance the immune responses after DNA vaccine electrotransfer.

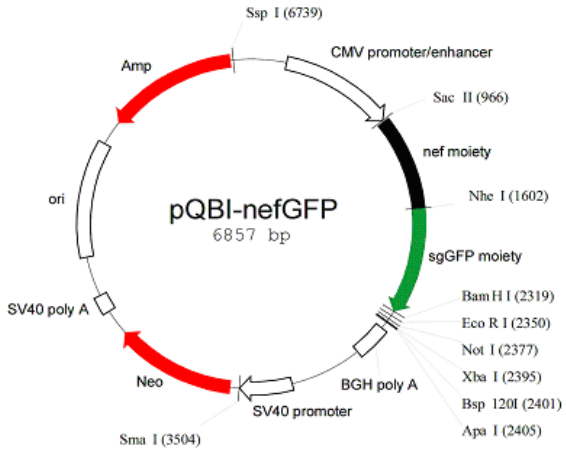


***Figure 2:*** DNA vaccine plasmid

***Delivery:*** The method of delivery determines the dose of DNA required to raise an effective immune response. The two first used approaches were injection of DNA in saline, using a standard hypodermic needle, and gene gun delivery. But since the last decade, EP appears to be a convenient method to promote DNA vaccine after saline injection. Injection in saline is normally conducted intramuscularly (IM) in skeletal muscle [24], or intradermally (ID) [25, 26].

Indeed saline injections require variable amounts of DNA, from 10 µg-1 mg, whereas EP deliveries require 10 to 100 times less DNA than intramuscular saline injection to raise an effective immune response. A single immunization prolong primary immune response [27].

EP enhances gene transfer of the tissue. EP can be affected by many factors, including electrode type, speed of injection, volume of injection, muscle type, and age, sex and physiological condition of the animal being injected [28].

As an additional mechanism, mild tissue damage may be induced by EP that could provoke an influx of APC's [29], even when the plasmid is injected after EP [30].

***Advantages and Limitations:*** DNA immunization offers many advantages over the traditional forms of vaccination. DNA vaccination may provide an important tool for stimulating an immune response in HBV, HCV and HIV patients which cannot be obtained by other traditional vaccines [12]. These

advantages and limitations are briefly reviewed in Table 1.

Table 1. Advantages And limitation Of DNA Vaccines

| Advantages | Limitations |
|---|---|
| In vivo expression ensures protein more closely resembles normal eukaryotic structure, with accompanying post-translational modifications | Limited to protein immunogens (not useful for non-protein based antigens such as bacterial polysaccharides) |
| Antigen presentation by both MHC class I and class II molecules; | Risk of affecting genes controlling cell growth |
| Able to polarise T-cell help toward type 1 or type 2 | Possibility of inducing antibody production against DNA |
| Long-term persistence of immunogen | Possibility of tolerance to the antigen (protein) produced |
| Immune response focused only on antigen of interest | Potential for atypical processing of bacterial and parasite proteins |
| Subunit vaccination with no risk for infection | |
| Ease of development and production; | |
| Stability of vaccine for storage and shipping; | |
| Cost-effectiveness | |
| Obviates need for peptide synthesis, expression and purification of recombinant proteins and the use of toxic adjuvants | |

## PRECLINICAL STUDIES

There are numerous preclinical studies that have investigated EP for DNA vaccination in several species including mice, guinea pigs, rabbits, farm ruminants, and rhesus macaques. Moreover, this has been observed against quite a wide variety of antigens from intracellular pathogens involved in disease such as AIDS, Hepatitis B and C; herpes virus; tuberculosis; malaria [[9], [31], [32], [33], [34], [8], [10], [24]]

Few examples can be mentioned, for instance in rhesus macaques, Otten et al., 2004 found that EP enhanced DNA vaccination to both the gag and env proteins of HIV. Later [[11]] showed that EP led to an expansion of antigen-specific CD4+ and CD8+ T cells of both central and effector memory phenotype compared to direct injection alone.

In case of cancer, most of the tumor-associated antigens (TAAs) are poorly immunogenic self molecules. As DNA vaccine break tolerance and EP improved antigen expression, vaccination against melanoma-associated antigens, HER2/neu (c-ErB2) and carcinoembryonic antigen (CEA) have studies [[32],[23],[35],[36]].

## CLINICAL TRIALS

Clinical trials are currently under way. Their goals are to evaluate (http://clinicaltrials.gov):

- Safety and Immunogenicity of a Melanoma DNA Vaccine Delivered by Electroporation
- Dose Finding Study of a DNA Vaccine Delivered With Intradermal Electroporation in Patients With Prostate Cancer
- Study of a Potential Preventive Vaccine Against HIV in Healthy Volunteers
- Phase I of Human Papillomavirus (HPV) DNA Plasmid (VGX-3100) + Electroporation for CIN 2 or 3
- Phase I/IIa Dose Ranging CHRONVAC-C® Study in Chronic HCV Patients
- A Study of V934/V935 in Cancer Patients With Selected Solid Tumors (Merck)

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## NOTES

# Skin and Transdermal drug delivery by electroporation

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## STRUCTURE OF THE SKIN

Skin is composed of three primary layers: the epidermis, which provides waterproofing and serves as a barrier to infection; the dermis, which serves as a location for the appendages of skin; and the hypodermis (subcutaneous adipose layer).

The epidermis consists of stratified squamous epithelium. The epidermis contains no blood vessels, and cells in the deepest layers are nourished by diffusion from blood capillaries extending to the upper layers of the dermis. The main type of cells which make up the epidermis are keratinocytes, with melanocytes and Langerhans cells also present. The main barrier to drug permeation is the stratum corneum, the outermost layer of the skin made of corneocytes embedded in a multiple lipid bilayers.

The dermis is the layer of skin beneath the epidermis that consists of connective tissue and cushions the body from stress and strain. The dermis is tightly connected to the epidermis by a basement membrane. It also harbors many nerve endings that provide the sense of touch and heat. It contains the hair follicles, sweat glands, sebaceous glands, apocrine glands, lymphatic vessels and blood vessels. The blood vessels in the dermis provide nourishment and waste removal to its own cells as well as the Stratum basale of the epidermis. The dermis is structurally divided into two areas: a superficial area adjacent to the epidermis, called the papillary region, and a deep thicker area known as the reticular region.

## TRANSDERMAL AND TOPICAL DRUG DELIVERY

The easy accessibility and the large area of the skin make it a potential route of administration. Despite these potential advantages for the delivery of drugs across or into the skin, a significant physical barrier impedes the transfer of large molecules. First, transdermal transport of molecules is limited by the low permeability of the stratum corneum, the outermost layer of the skin. Only potent lipophilic low molecular weight (<500) drugs can be delivered by passive diffusion at therapeutic rates. Hence, the transdermal penetration of hydrophilic and/or high molecular-weight molecules, including DNA, requires the use of methods to enhance skin permeability and/or to provide a driving force acting on the permeant. Both chemical (e.g. penetration enhancer) and physical (e.g. iontophoresis, electroporation, or sonophoresis) methods have been used.

## TRANSDERMAL DRUG DELIVERY BY ELECTROPORATION

It has been demonstrateded that application of high voltage pulses permeabilize the stratum corneum and enhance drug transport. Electroporation of skin was shown to enhance and expedite transport across and/or into skin for many different compounds. Within a few minutes of high-voltage pulsing, molecular transport across skin increased by several orders of magnitude.

*In vitro*, the transport of several conventional drugs (e.g., fentanyl, β blockers, peptides (e.g., LHRH or calcitonine) was shown to be enhanced. Few *in vivo* studies confirm the increased transport and rapid onset of action.

The parameters affecting the efficacy of transport have been extensively studied. The electrical parameters (voltage, number and duration of the pulses), the formulation parameters (ionic strength…) allow the control of drug delivery.

The mechanism of drug transport is mainly electrophoretic movement and diffusion through newly created aqueous pathways in the stratum corneum created by the “electroporation” of the lipid bilayers.

The alterations in skin induced by high-voltage pulsing are relatively minor (decrease in skin resistance, hydration, lipid organisation) and reversible. However, light sensation and muscle contraction that can be reduced by developing better electrode design, have been observed.

## TOPICAL DRUG DELIVERY BY ELECTROPORATION

Besides the permeabilization of the stratum corneum and the subsequent corneum and the subsequent increased skin permeability, electroporation also enhances the permeability of the viable cells of the skin and the subcutaneous tissue. Hence, it is an efficient method to deliver molecules into the skin when these molecules are applied topically or more efficiently for macromolecules including DNA when they are injected intradermally.

As the skin is an immunocompetent organ, DNA delivery in the skin by electroporation seems particularly attractive for DNA vaccination.

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## NOTES

# Electroporation in skin gene delivery

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## SKIN GENE DELIVERY

The skin represents an attractive site for the delivery of nucleic acids-based drugs for the treatment of topical or systemic diseases and immunisation. It is the most accessible organ and can easily be monitored and removed if problems occur. It is the largest organ of the body (15% of total adult body weight) and delivery to large target area could be feasible. However attempts at therapeutic cutaneous gene delivery have been hindered by several factors. Usually, except for viral vectors, gene expression is transient and typically disappears with 1 to 2 weeks due to the continuous renewal of the epidermis. Moreover, DNA penetration is limited by the barrier properties of the skin, rendering topical application rather inefficient.

The potential use of DNA-based drugs to the skin could be: (i) gene replacement by introducing a defective or missing gene, for the treatment of genodermatosis (ii) gene therapeutic by delivering a gene expressing protein with a specific pharmacological effect, or suicidal gene, (iii) wound healing, (iv) immunotherapy with DNA encoding cytokines and (v) DNA vaccine. The gene encoding the protein of interest can be inserted in a plasmid that carries this gene under the control of an appropriate eukaryotic promoter (e.g., the CMV promoter in most cases). Alternatively, it can be inserted in viral vectors.

Effective gene therapy requires that a gene encoding a therapeutic protein must be administered and delivered to target cells, migrate to the cell nucleus and be expressed to a gene product. DNA delivery is limited by: (i) DNA degradation by tissues or blood nucleases, (ii) low diffusion at the site of administration, (iii) poor targeting to cells, (iv) inability to cross membrane, (v) low cellular uptake and (vi) intracellular trafficking to the nucleus.

Epidermal gene transfer has been achieved with *ex vivo* approaches. Genes of interest have been introduced, mainly with viral vectors, in keratinocytes or fibroblasts and then grafted on nude mice or patients. Permanent expression can be achieved. *In vivo* approaches, which are more patient-friendly, less invasive, less time consuming and less expensive, are more attractive and will gradually replace the ex vivo gene transfer protocols.

The methods developed for gene transfer into the skin are based on the methods developed for gene transfection *in vitro* and in other tissues *in vivo* as well as methods developed to enhance transdermal drug delivery. They include (i) topical delivery, (ii) intradermal injection, (iii) mechanical methods, (iv) physical methods and (v) biological methods.

Topical application of naked plasmid DNA to the skin is particularly attractive to provide a simple approach to deliver genes to large areas of skin. However, the low permeability of the skin to high molecular weight hydrophilic molecules limits the use of this approach. Gene expression after topical delivery of an aqueous solution of DNA on intact skin has been reported to induce gene expression but the expression is very low. Hence, topical DNA delivery into the skin can only be achieved if the barrier function of the stratum corneum is altered. The selection of appropriate vector or method to promote the penetration of DNA through and/or into the skin has been shown to be paramount.

One of the simplest ways of gene delivery is injecting naked DNA encoding the therapeutic protein. In 1990, Wolff et al. observed an expression during several months after injection of naked DNA into the muscle. Expression following the direct injection of naked plasmid DNA has been then established for skin. The epidermis and the dermis can take up and transiently express plasmid DNA following direct injection into animal skin. However, the expression remains low and physical and/or mechanical methods have been developed to enhance gene expression.

## ELECTROPORATION IN SKIN GENE DELIVERY

Electrotransfer has been widely used to introduce DNA into various types of cells *in vitro* and is one of the most efficient non-viral methods to enhance gene transfer in various tissues *in vivo*. Electrotransfer involves plasmid injection in the target tissue and application of short high voltage electric pulses by electrodes. The intensity and the duration of pulses and the more appropriate type of electrodes must be evaluated for each tissue. It is generally accepted that the electric field plays a double role in DNA transfection: it transiently disturbs membranes and increases cells permeability and promotes electrophoresis of negatively charged DNA.

Electrotransfer may be used to increase transgene expression 10 to 1000-fold more than the injection of naked DNA into the skin. Local delivery combined with electrotransfer could resulted in significant

increase of serum concentrations of a specific protein. Neither long-term inflammation nor necroses are generally observed.

After direct intradermal injection of plasmid, the transfected cells are typically restricted to the epidermis and dermis. However, when high voltage pulse are applied after this intradermal injection, other cells, including adipocytes, fibroblasts and numerous dendritic-like cells within the dermis and subdermal layers were transfected. After topical application of plasmid on tape stripped rat skin followed by electrotransfer, GFP expression was also reported but was very low and restricted to the epidermis.

Duration of expression after electrotransfer depends on the targeted tissue. In contrast to the skeletal muscle where expression lasts for several months, gene expression is limited to only of few weeks into the skin. For example, after intradermal electrotransfer of plasmid coding erythropoietin, the expression persisted for 7 weeks at the DNA injection site, and hematocrit levels were increased for 11 weeks. With reporter gene, shorter expressions were reported, probably due to an immune response.

Several authors tried to increase the effectiveness of the electrotransfer into the skin. By co-injecting a nuclease inhibitor with DNA, transfection expression was significantly increased. The use of a particulate adjuvant (gold particles) enhanced the effectiveness of DNA vaccination by electrotransfer. For the skin, combination of one high-voltage pulse and one low-voltage pulse delivered by plate electrodes has been proven to be efficient and well tolerated. The design of electrodes and injection method can also be optimised.

Electrotransfer has no detrimental effect on wound healing. A single injection of a plasmid coding keratinocyte growth factor coupled with electrotransfer improved and accelerated wound closure in a wound-healing diabetic mouse model.

Vaccination is another interesting application of electrotransfer into the skin. Intradermal electrotransfer enhanced DNA vaccine delivery to skin and both humoral and cellular immune responses have been induced. Hence, it could be developed as a potential alternative for DNA vaccine delivery without inducing any irreversible change.

Electrotransfer of DNA in melanoma is currently tested in clinical trials.

**NOTES**

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# Other Physical and Mechanical Methods in Skin Gene Delivery

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## INTRODUCTION

Physical methods and mechanical methods such microneedles or sonophoresis developed to enhance transdermal and topical delivery of conventional drugs and to extend its field of application have been reported to enhance DNA transfer into the skin and into cutaneous cells.

## MICRONEEDLES

The most direct permeation enhancement relies on physical/mechanical disruption of the stratum corneum. Recently, the ability of microneedles to disrupt the stratum corneum and create microchannels (10 to 20 μm diameter) has been reported. Microneedles have been widely used to deliver conventional drugs but only proof of principle of DNA delivery has been reported.

Arrays of micron scale silicon projection (microenhancer arrays) that were dipped into a solution of naked plasmid DNA and scraped across the skin of mice enabled topical gene transfer resulting in reporter gene activity up to 2,800-fold above topical controls and topical immunisation inducing stronger and less variable immune responses than via needle-based injections. In a human clinical study, these devices effectively breached the skin barrier, allowing direct access to the epidermis with minimal associated discomfort and skin irritation. Preliminary gene expression studies confirmed that naked DNA plasmid can be locally expressed in excised human skin following disruption of the stratum corneum barrier with longer silicon microneedles.

In contrast to solid microneedles, hollow microneedles offer the possibility of transporting drugs by diffusion or by pressure-driven flow. A variety of hollow microneedles have been fabricated but only limited work has been published on their possible use to deliver nucleic acids into the skin. Following microneedle-assisted delivery of pDNA hydrogels to human skin expression of the pCMVbeta reporter gene was demonstrated in the viable epidermis proximal to microchannels.

## SONOPORATION

Sonoporation is the ultrasound-mediated enhancing of cell permeability.

Biological effects are mainly due to two mechanisms, cavitation and heating. Acoustic cavitation is the nonthermal interaction between a propagating pressure wave and a gaseous inclusion in aqueous media responsible of mechanical perturbation, collapse and implosion of gas bubbles. The importance of this phenomenon depends on ultrasound intensity and frequency. It might lead to a release of a sufficient energy to permeabilize cell membranes and to enhance drug or gene delivery from the microbubbles surface into cells and tissue. Ultrasound could also generate heat. When a beam is focused down to a small size in target tissue, the thermal energy per area is high. This energy can be absorbed by the tissue, resulting in increased temperature which might perturb biological systems. Thermal effect varies with the exposure time and ultrasound intensity. It has only a minor role in the ultrasound-induced increase in permeability.

The first result of sonoporation gene transfer was obtained *in vitro* in the middle of the nineties. Since this time, this technique has been used in wide variety of tissues such as muscle, tumor, and recently into living skin equivalents transplanted onto nude mice after the ultrasound mediated gene transfer.

The use of ultrasound contrast microbubbles might improve transfection. These microscopic (1 to 3 μm) microbubbles contain air or an inert gas with a shell composed of proteins, lipids or polymers. An example of microbubble that has been proved very effective in sonoporation research is Optison® (perfluoropropane encapsulated in a human albumin sphere). Gene vectors mixed with microbubbles can be injected locally or systemically before the application of ultrasound on the target area. It is also possible to use polymer-coated microbubbles that can bind and protect the DNA or microbubbles encapsulating DNA.

## MICROCHANNELS

Transient microconduits can be created in human skin by arrays of radiofrequency microelectrodes without impinging underlying blood vessels and nerve endings. The transient microconduits of approximately 30 μm diameter, 70 μm depth allow topical DNA delivery and result in gene expression (βgal for example) within the viable epidermal cells surrounding the microchannels. The staining was higher when ViaDerm™ (the radiofrequency-microchannel generator) was applied both prior to and immediately following the topical application of the DNA formulation.

## LASER IRRADIATION

Laser irradiation is another method to transfer DNA into cells either *in vitro* or *in vivo*. The beam is emitted by a laser source, for example neodymium yttrium-aluminium-garnet (YAG) or argon ion laser, and is focused by a lens. The exact mechanism remains unknown but the permeability of the cellular membrane is increased, probably by a thermal effect, sufficiently to permit the entry of DNA into the cell. Direct transfer of the neomycin gene by YAG laser was reported for the first time in 1987 *in vitro* .

Ogura et al. reported levels of luciferase activities after laser irradiation two orders of magnitude higher than those after injection of naked DNA into the skin. No major side effects were observed. Luciferase activity levels were sustained even 5 days after gene transfer. Fentosecond Laser irradiation was used *in vivo* to transfer genetic material into the muscle and into the skin and achieve long term expression. The development of laser gene transfer is limited by the high cost and the size of the laser.

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### NOTES

# Tumor Biology

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**Abstract:** Cancer arises within a single cell as a result of accumulation of mutations or other changes within the DNA of that cell. When these changes affect key genes *e.g.* those involved in control of the cell cycle, apoptosis, DNA repair etc., it can lead to uncontrolled cell growth and eventually to a cell type that has acquired the ability to invade and metastasise. Due to the high variability of cancer types, the treatments given for cancer are highly variable and dependent on a number of factors, including the type, location, amount of disease and the health status of the patients. A short overview of carcinogenesis, tumour progression, types of cancer and treatment options is presented.

## INTRODUCTION

Cancer is a class of diseases characterized by uncontrolled cell division and the ability of these cells to invade other tissues, either by direct growth into adjacent tissue (invasion) or by migration of cells to distant sites (metastasis). This unregulated growth is caused by damage to DNA, resulting in mutations to vital genes that control cell division, among other functions. One or more of these mutations, which can be inherited or acquired, can lead to uncontrolled cell division and tumour formation. Tumour ("swelling" in Latin) refers to any abnormal mass of tissue, but may be either malignant (cancerous) or benign (noncancerous). Only malignant tumours are capable of invading other tissues or metastasizing.

## CARCINOGENESIS

Carcinogenesis (meaning literally, the creation of cancer) is the process by which normal cells are transformed into cancer cells.

Cell division (proliferation) is a physiological process that occurs in almost all tissues and under many circumstances. Normally homeostasis, the balance between proliferation and programmed cell death, usually in the form of apoptosis, is maintained by tightly regulating both processes to ensure the integrity of organs and tissues. Mutations in DNA that lead to cancer, disrupt these orderly processes by disrupting the regulation of the processes of cell division and cell death.

Carcinogenesis is caused by mutation of the genetic material of normal cells, which upsets the normal balance between proliferation and cell death. This results in uncontrolled cell division and tumour formation.

Carcinogenesis is divided into two steps: initiation and promotion. Initiation is rapid and irreversible and affects DNA directly (mutation). Promotion can be reversible, it's not necessary that it affects DNA directly (mutation), in this case the development of cancer requires prolonged exposure to the promoting agent (Figure 1).

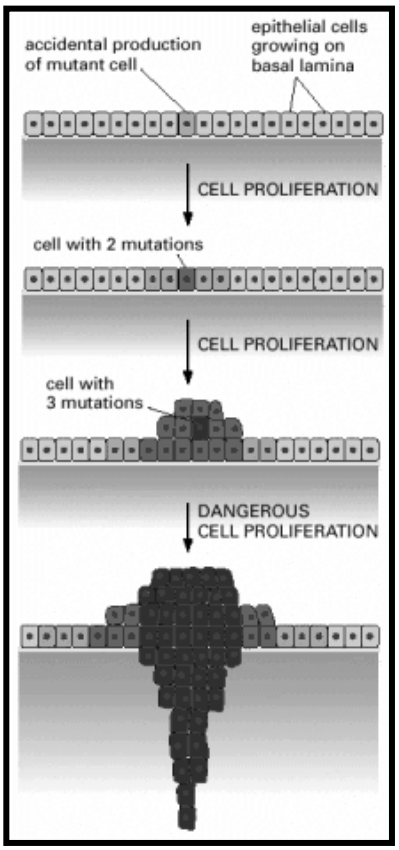


***Figure 1:*** Development of cancer requires more than one mutation.

In most cases, more than one mutation is necessary for carcinogenesis. In fact, a series of several mutations to certain classes of genes or parts of DNA is usually required before a normal cell will transform into a cancer cell. Only mutations in those certain types of genes or parts of DNA that control these genes, which play vital roles in cell division, cell death, and DNA repair, will cause a cell to lose control of its proliferation. The typical features of cancer are listed in Figure 2: the order of acquired

capabilities is not uniform and each particular cancer can obtain them in a different sequence.

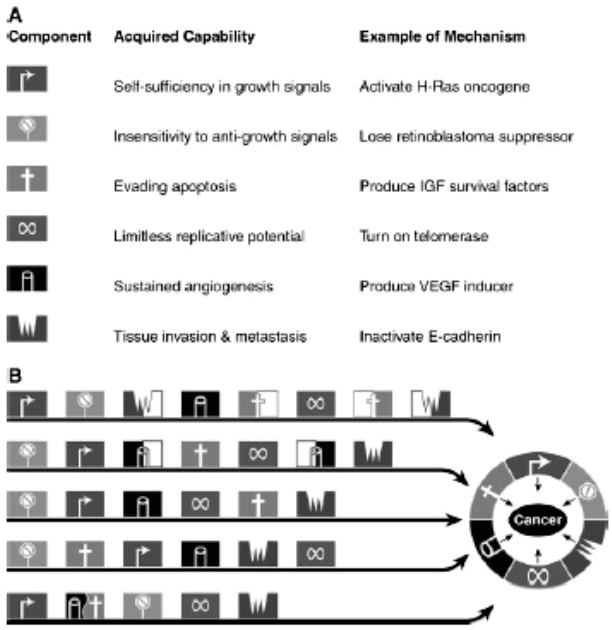


***Figure 2:*** *Parallel pathways of carcinogenesis. From Hanahan (Cell 2000).*

## MECHANISMS OF CARCINOGENESIS

Cancer is, ultimately, a disease of genes. Typically, a series of several mutations is required before a normal cell transforms into a cancer cell. The process involves proto-oncogenes, tumour suppressor genes and recently discovered microRNA (miRNA) genes.

*Proto-oncogenes* are involved in signal transduction by coding for a chemical "messenger", produced when a cell undergoes protein synthesis. These messengers send signals based on the amount of them present to the cell or other cells, instructing them to undergo mitosis in order to divide and reproduce. When mutated, they become (are called) oncogenes and overexpress the signals to divide, giving cells a higher chance to divide excessively. The chance of cancer cannot be reduced by removing proto-oncogenes from the human genome as they are critical for growth, repair and homeostasis of the body. It is only when they become mutated or their regulation is changed that the signals for growth become excessive. Their role in cancer progression is “dominant”, as mutation in only one of the alleles produces excessive cell growth and division.

Protooncogenes can be thus growth factors or growth factors receptors, such as Her2/neu (erbB2), PDGF, signal transduction molecules, such as ras and src, transcription factors, such as myc, fos and jun, and others coding for antiapoptotic proteins bcl2 or mdm2 (Figure 3).

*Tumour suppressor* genes code for chemical messengers that command cells to slow or stop mitosis in order to allow DNA repair (Figure 2). This is done by special enzymes, which detect any mutation or damage to DNA, such that the mistake is not carried on to the next generation. Tumour suppressor genes are usually triggered by signals that DNA damage has occurred. In addition, they can code for the enzymes themselves that repair DNA, or code for signals that activate such enzymes. However, a mutation can damage the tumour suppressor gene itself or the signal pathway, which activates it, "switching it off". The invariable consequence is that DNA repair is hindered or inhibited by every such event. Damage is originally checked by the tumour suppressor genes, but accumulates and becomes more abundant as more tumour suppressor genes succumb to mutation. With repair functions disabled, this inevitably leads to cancer. According to their function in the cells, their role in cancer progression is recessive, which means that mutations on both alleles have to occur to diminish the function of tumour suppressor proteins. Examples of tumour suppressor genes are p53, Rb1, APC and BRCA1 (Figure 3).

MicroRNA genes are part of DNA that code for so-called miRNAs (also called oncomirs), which are a class of small non-coding RNAs. The role of miRNAs is a post-transcriptional silencing of target genes, by binding to mRNA of target genes. Since the first discovery, more than 650 miRNA are now known and it is estimated that these miRNA regulate about 30% of all human transcripts. miRNAs are involved in the regulation of multiple cellular processes, such as proliferation, apoptosis, cell-cycle regulation, and differentiation, all hallmarks of cancer. MiRNAs can act as tumour suppressors when they regulate expression of oncogenes and their expression in tumours is downregulated or as oncogenes, when they target tumour suppressor gene and their expression is upregulated. Examples of miRNA genes involved in carcinogenesis are miRNA genes of let-7 family and miR-15a, miR-16-2, and miR-21.

In general, mutations in all three types of genes are required for cancer to occur. For example, a mutation limited to one oncogene would be suppressed by normal mitosis control (the Knudson or 1-2-hit hypothesis) and tumour suppressor genes. A mutation to only one tumour suppressor gene would not cause cancer either, due to the presence of many "backup" genes that duplicate its functions. It is only when enough proto-oncogenes have mutated into oncogenes, and enough tumour suppressor genes deactivated or damaged, and enough miRNA genes are either activated or deactivated that the signals for

cell growth overwhelm the signals to regulate it and cell growth quickly spirals out of control.

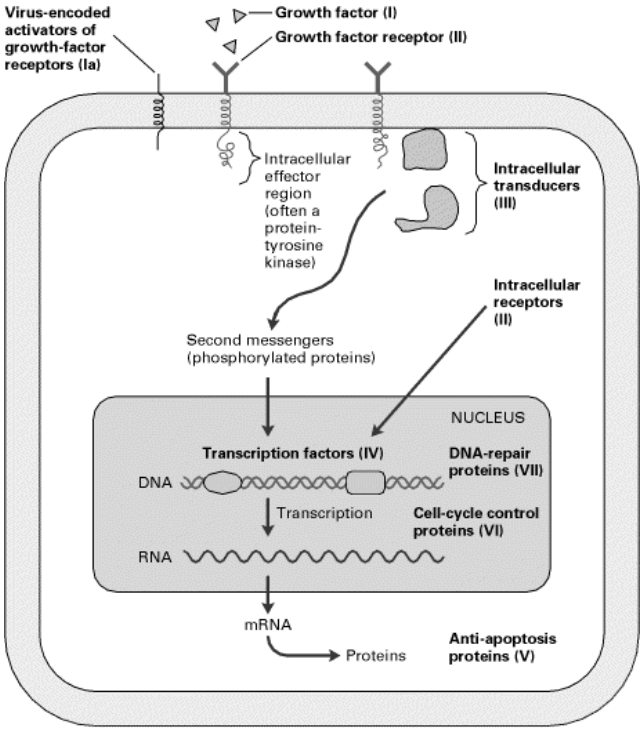


***Figure 3:*** *Mutation in the genes coding for proteins shown on the figure can lead to cancer development.*

Accumulated damage is generally theorised by most cancer researchers to build up exponentially later in life. Originally at youth defences against DNA damage are strong, but as more mutations to tumour suppressor genes occur, the rate of damage accumulation rises, causing exponential accumulation, a "death spiral" of sorts. This is further supported by the fact that the chance of acquiring cancer increases exponentially with age, rather than linearly. The average accumulated damage sampled from cancer cells tend to be immense - nearly all of the chromosomes have been mutated in some way, such as four copies of the same chromosome, trisomy, monosomy, or even completely missing chromosomes in the cell.

Mutations can be caused by various factors. In principle they can be divided into physical, such as UV and ionizing radiation, chemical, such as free radicals and benzene and biological factors, such as viruses. Particular causes have been linked to specific types of cancer. Tobacco smoking is associated with lung cancer. Prolonged exposure to radiation, particularly ultraviolet radiation from the sun, leads to melanoma and other skin malignancies. Breathing asbestos fibres is associated with mesothelioma. In more general terms, chemicals called mutagens and free radicals are known to cause mutations. Other types of mutations can be caused by chronic inflammation, as neutrophil granulocytes secrete free radicals that damage DNA. Chromosomal translocations, such as the Philadelphia chromosome, are a special type of mutation that involve exchanges between different chromosomes. Among biological factors, viruses play the biggest role as some types of viruses can cause mutations. They play a role in about 15% of all cancers. Tumour viruses, such as some retroviruses, herpesviruses and papillomaviruses, usually carry an oncogene, or a gene that inhibits normal tumour suppression. Examples are papilloma viruses, which are involved in development of cervical cancer, hepatitis B and C viruses in hepatic cancer and HIV-1, which is involved in development of Kaposi's sarcoma.

Many mutagens are also carcinogens, but some carcinogens are not mutagens and act as promoting agents. Examples of carcinogens that are not mutagens include alcohol and estrogen. These are thought to promote cancers through their stimulating effect on the rate of cellular mitosis (promoting phase of carcinogenesis). Faster rates of mitosis increasingly leave less window space for repair enzymes to repair damaged DNA during DNA replication, thus increasing the likelihood of a genetic mistake. A mistake made during mitosis can lead to the daughter cells receiving the wrong number of chromosomes, which leads to aneuploidy and may lead to cancer.

Mutations can also be inherited. Inheriting certain mutations in the BRCA1 gene, a tumour suppressor gene, renders a woman much more likely to develop breast cancer and ovarian cancer. Mutations in the Rb1 gene predispose to retinoblastoma, and those in the APC gene lead to colon cancer, listing just a few examples.

It is impossible to tell the initial cause for any specific cancer. However, with the help of molecular biological techniques, it is possible to characterize the mutations or chromosomal aberrations within a tumour, and rapid progress is being made in the field of predicting prognosis based on the spectrum of mutations in some cases. For example, up to half of all tumours have a defective p53 gene, a tumour suppressor gene also known as "the guardian of the genome". This mutation is associated with poor prognosis, since those tumour cells are less likely to go into apoptosis (programmed cell death) when damaged by therapy. Telomerase mutations remove additional barriers, extending the number of times a cell can divide. Other mutations enable the tumour to grow new blood vessels to provide more nutrients, or to metastasize, spreading to other parts of the body.

## PROPERTIES OF MALIGNANT CELLS

Cells capable of forming malignant tumours exhibit many properties, which distinguish them from the cells of healthy tissue.

- They have an uncontrolled ability to divide (or, they are immortal), and they often divide at an increased rate.
- They evade apoptosis ("programmed" cell death).
- These cells are self-sufficient with respect to growth factors.
- They are insensitive to antigrowth signals, and contact inhibition is suppressed.
- These cells may exhibit altered differentiation.
- More aggressive malignant cells may also show additional abilities.
- They have the ability to invade neighbouring tissues, usually through the secretion of metalloproteinases that can digest extracellular matrix material.
- They can form new tumours (metastases) at distant sites. They secrete chemical signals that stimulate the growth of new blood vessels (angiogenesis).

Nearly all cancers originate from a single cell, but a cell that degenerates into a tumour cell does not usually acquire all these properties at once. With each carcinogenic mutation, a cell gains a slight selective advantage over its neighbours, resulting in a process known as *clonal evolution*. This leads to an increased chance that the descendents of the original mutant cell will acquire extra mutations, giving them even more selective advantage. Cells, which acquire only some of the mutations necessary to become malignant, are thought to be the source of benign tumours. However, when enough mutations accumulate, the mutant cells will become a malignant tumour.

Recently, a hypothesis that cancer can originate only from the cells that have stem cells properties has evolved. These, so called cancer stem cells, have all stem cells properties, including self-renewal, tumourigenicity and multi-lineage differentiation capacity. Currently, most of the research is focused on finding the validated markers for cancer stem cells, which would lead to development of new treatment strategies.

## STAGES OF TUMOUR PROGRESSION

Cancer has different morphology when investigated under the microscope. Cancer cells/tissue differ from normal in many features including variation in nuclear size and shape, variation in cell size and shape, loss of specialized cell features, loss of normal tissue organization, increase in cell division and a poorly defined tumour boundary. On the basis of patohistological examination some solid cancers can be distinguished from hyperplasia, dysplasia, carcinoma *in situ* and invasive cancer. Imunnohistochemistry and other molecular methods are used to determine specific tumour markers, which may aid to diagnosis and prognosis of the disease.

*Hyperplasia* can be described by altered cell division in uncontrolled manner; but cell have a normal appearance and the process is considered to be reversible.

*Dysplasia* occurs when additional genetic changes in the hyperplastic cells lead to the even more abnormal growth. Cells no longer look normal, tissue may became dis-organised (loss of normal tissue organisation and cell structure). Very often cells regain their normal behaviour, but in some cases, they revert to malignant.

Severe dysplasia can be considered as *carcinoma in situ*. (Latin “in situ” means “in place”). Cells become more dedifferentiated or anaplastic. However, cells are still contained within the initial location and have not yet crossed the basal lamina.

*Cancer* is characterised by invasion into surrounding tissues and/or spread (metastasis) to areas outside the local tissue.

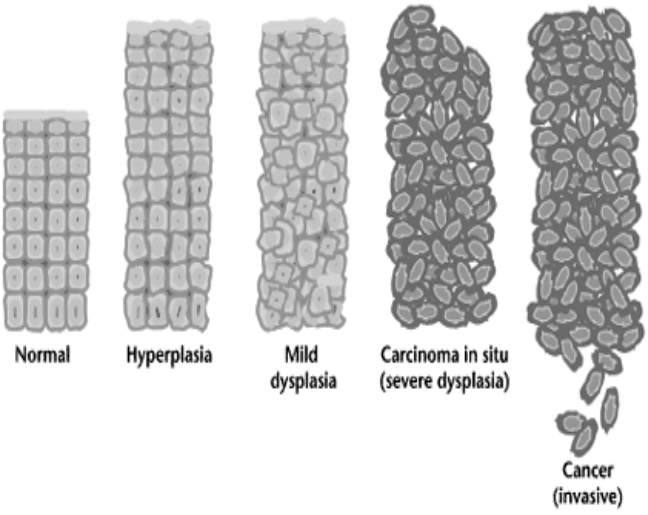


***Figure 4:*** *Stages of tumour progression.*

## TYPES OF CANCER

The uncontrolled and often rapid proliferation of cells can lead to benign tumours; some types of these may turn into malignant tumours (cancer). *Benign tumours* do not spread to other parts of the body or

invade other tissues, and they are rarely a threat to life unless they compress vital structures or are physiologically active (for instance, producing a hormone). *Malignant tumours* (cancer) can invade other organs, spread to distant locations (metastasize) and become life threatening.

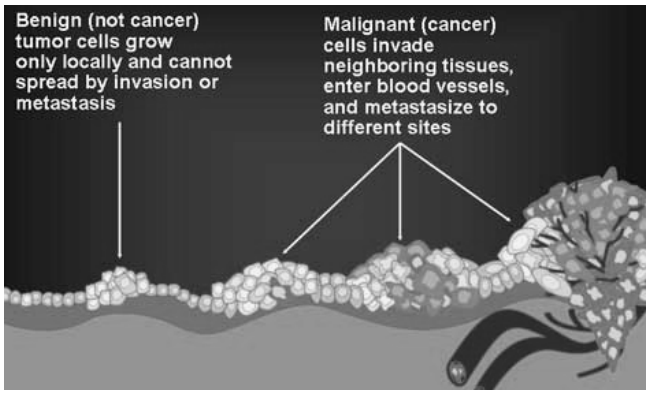


***Figure 5:*** *Difference between benign and malignant tumours.*

Cancer cells within a tumour are the descendants of a single cell, even after it has metastasized. Hence, a cancer can be classified by the type of cell in which it originates and by the location of the cell.

*Carcinomas* originate in epithelial cells (e.g. the digestive tract or glands). *Haematological malignancies*, such as leukaemia and lymphoma, arise from blood and bone marrow. *Sarcoma* arises from connective tissue, bone or muscle. *Melanoma* arises in melanocytes. *Teratoma* begins within germ cells.

## TREATMENT OF CANCER

Although extensive cancer research led to development of many new drugs and treatments for cancer there are still only three major cancer therapies; two being local: surgery and radiotherapy and a systemic chemotherapy. The choice of therapy depends upon the location and grade of the tumour and the stage of the disease, as well as the general state of the patient (performance status). A number of experimental cancer treatments are also under development.

Complete removal of the cancer without damage to the rest of the body is the goal of treatment. Sometimes this can be accomplished by surgery, but the propensity of cancers to invade adjacent tissue or to spread to distant sites by microscopic metastasis often limits its effectiveness.

Radiotherapy is the use of ionizing radiation to kill cancer cells and shrink tumours. Radiation therapy injures or destroys cells in the area being treated (the "target tissue") by damaging their DNA through direct action on DNA or indirect action via free radicals that atack DNA, making it impossible for these cells to continue to grow and divide. Although radiation damages both cancer cells and normal cells, most normal cells can recover from the effects of radiation and function properly. The goal of radiation therapy is to damage as many cancer cells as possible, while limiting harm to nearby healthy tissue. Radiation therapy may be used to treat almost every type of solid tumor, including cancers of the brain, breast, cervix, larynx, lung, pancreas, prostate, skin, spine, stomach, uterus, or soft tissue sarcomas. Radiation can also be used to treat leukemia and lymphoma (cancers of the blood-forming cells and lymphatic system, respectively). Radiation dose to each site depends on a number of factors, including the type of cancer and whether there are tissues and organs nearby that may be damaged by radiation.

Chemotherapy is the systemic treatment of cancer with drugs (chemotherapeutic or anticancer drugs) that can destroy cancer cells. The drugs interfere with cell division in various possible ways, e.g. with the duplication of DNA or affecting the mitotic spindle. Most forms of chemotherapy target all rapidly dividing cells and are therefore not specific for cancer cells. Hence, chemotherapy has the potential to harm healthy tissue, especially those tissues that have a high replacement rate (e.g. intestinal lining). These cells usually repair themselves after chemotherapy. Because some drugs work better together than alone, two or more drugs are often given at the same time. This is called "combination chemotherapy"; most chemotherapy regimens are given in a combination. In most cases, therapy for cancer includes the combination of all three standard treatments.

New, so-called biological treatment strategies include hormonal therapy, immunotherapy, gene therapy and others. The most promising drugs so far are either small drugs, such as tyrosine kinase inhibitor imatinib, which is used for treatment of chronic myeloid leukaemia and monoclonal antibodies such as bevacizumab and cetuximab, which are used for treatment of colon cancer, transtuzumab for treatment of breast cancer, rituximab for treatment of non-Hodgkin’s lymphoma, and others.

Because "cancer" refers to a class of diseases, it is unlikely that there will ever be a single "cure for cancer".

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She is the author of more than 80 articles in peer-reviewed journals.

## NOTES

# Electroporation in Electrochemotherapy of Tumors

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**Abstract:** Electrochemotherapy consists of chemotherapy followed by local application of electric pulses to the tumor to increase drug delivery into tumor cells in tumors. Drug uptake can be increased by electroporation only for drugs having impeded transport through the plasma membrane. Among many drugs which have been tested so far, only bleomycin and cisplatin have found their way from preclinical testing to clinical trials. *In vitro* studies demonstrated a several-fold increase of their cytotoxicity by electroporation of cells. *In vivo*, electroporation of tumors after local or systemic administration of either of the drugs *i.e.* electrochemotherapy, proved to be an effective antitumor treatment. Electrochemotherapy studies using either bleomycin or cisplatin in several tumor models elaborated treatment parameters for effective local tumor control. In veterinary medicine, electrochemotherapy proved to be effective in primary tumors in cats, dogs and horses. In clinical studies, electrochemotherapy was performed on accessible tumor nodules of different malignancies in progressive disease. All clinical studies provided evidence that electrochemotherapy is an effective treatment for local tumor control in patients with different types of cancer. The perspectives of electrochemotherapy are also in combination with other established treatment modalities, like irradiation, and newcomers, like gene therapy. Since application of electric pulses to the tumors induces transient reduction of tumor perfusion and oxygenation, it can be exploited in several other treatment combinations such as with bioreductive drugs and hyperthermia.

## INTRODUCTION

Treatments for cancer may be divided into different categories based on their goals and mode of action. Very often, the different types of treatment are used in combination, either simultaneously or sequentially. In general, cancer treatment includes three major treatment modalities: surgery and radiation, which are local treatment modalities and chemotherapy, which is a systemic treatment modality.

Chemotherapy, a systemic treatment modality for cancer, is effective for drugs which readily cross the plasma membrane and are cytotoxic once they reach their intracellular targets. However, among the chemotherapeutic drugs which are very cytotoxic, there are some having hampered transport through the plasma membrane. These drugs are good candidates for electrochemotherapy. Electrochemotherapy is a local treatment combining chemotherapy and application of electric pulses to the tumor. In electrochemotherapy, the optimal anti-tumor effectiveness is achieved when electric pulses are given at the time of the highest extracellular concentration of the hydrophilic chemotherapeutic drug, thereby increasing its transport through the plasma membrane towards the intracellular targets [1-4].

## PRECLINICAL DATA

### *In vitro studies*

Electroporation proved to be effective in facilitating transport of different molecules across the plasma membrane for different biochemical and pharmacological studies. However, when using chemotherapeutic drugs, this facilitated transport increases intracellular drug accumulation with the aim to increase their cytotoxicity. Since electroporation can facilitate drug transport through the cell membrane only for molecules which are poorly or non-permeant, suitable candidates for electrochemotherapy are limited to those drugs that are hydrophilic and lack a transport system in the membrane. Several chemotherapeutic drugs were tested *in vitro* for potential application in combination with electroporation of cells. Among the tested drugs, only two were identified as potential candidates for electrochemotherapy of cancer patients. The first was bleomycin, which is hydrophilic and has very restricted transport through the cell membrane, but its cytotoxicity can be potentiated up to several 1000 times by electroporation of cells. A few hundred internalized molecules of bleomycin are sufficient to kill the cell. The second is cisplatin, whose transport through the cell membrane is also hampered. Only 50% of cisplatin is transported through the plasma membrane by passive diffusion, the rest is via carrier molecules. Electroporation of the plasma membrane enables greater flux and accumulation of the drug in the cells, which results in an increase of cisplatin cytotoxicity by up to 80-fold [1-4]. This promising preclinical data obtained *in vitro* on a number of different cell lines has paved the way for testing these two drugs in electrochemotherapy *in vivo* on different tumor models.

***In vivo studies***

Bleomycin and cisplatin were tested in an electrochemotherapy protocol on animal models *in vivo* (Fig 1). Extensive studies on different animal models with different tumors, either transplantable or spontaneous, were performed. The antitumor effectiveness of electrochemotherapy was demonstrated on tumors in mice, rats, hamsters, cats and rabbits. Tumors treated by electrochemotherapy were either subcutaneous or in muscle, brain or liver, being sarcomas, carcinomas, glioma or malignant melanoma [1-4].

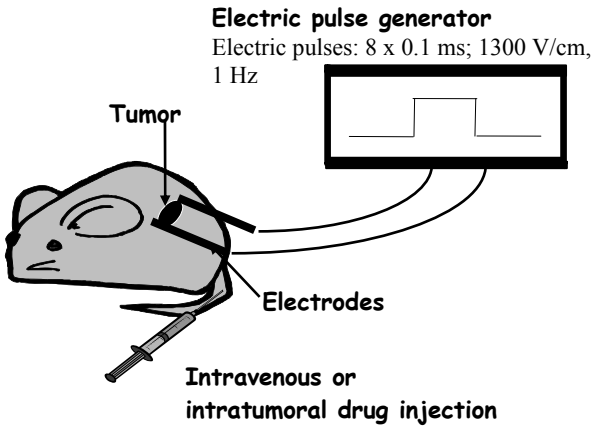


***Figure 1:*** Protocol of electrochemotherapy of experimental tumors presented schematically. The drug is injected either intravenously or intratumorally at doses which do not usually exert an antitumor effect. After an interval which allows sufficient drug accumulation in the tumors, electric pulses are applied to the tumor either by plate or needle electrodes (1300 V/cm, 100 μs, 1 Hz, 8 pulses). The electrodes are placed in such a way that the whole tumor is encompassed between the electrodes, providing good electric field distribution in the tumors for optimal electroporation of cells in the tumors.

In these studies, different factors controlling antitumor effectiveness were determined:

- The drugs can be given by different *routes of administration*, they can be injected either intravenously or intratumourally. The prerequisite is that, at the time of application of electric pulses to the tumor, a sufficient amount of drug is present in the tumor. Therefore, after intravenous drug administration into small laboratory animals (4 mg/kg of cisplatin or 0.5 mg/kg bleomycin), only a few minutes interval is needed to reach the maximal drug concentration in the tumors. After intratumoural administration, this interval is even shorter and the application of electric pulses has to follow the administration of the drug as soon as possible (within a minute) [1-4].
- Good antitumor effectiveness may be achieved by good tissue electroporation. Electroporation of the plasma membrane is obtained if the cell is exposed to a sufficiently high electric field. This depends on the *electric field distribution in the tissue* which is controlled by the electrode geometry and tissue. The electric field distribution in the tissue and cell electroporation can be improved by rotating the electric field. Surface tumours can be effectively treated by plate electrodes, whereas appropriate electric field distribution in the deeper parts of the tumour is assured by using needle electrodes [5-7].

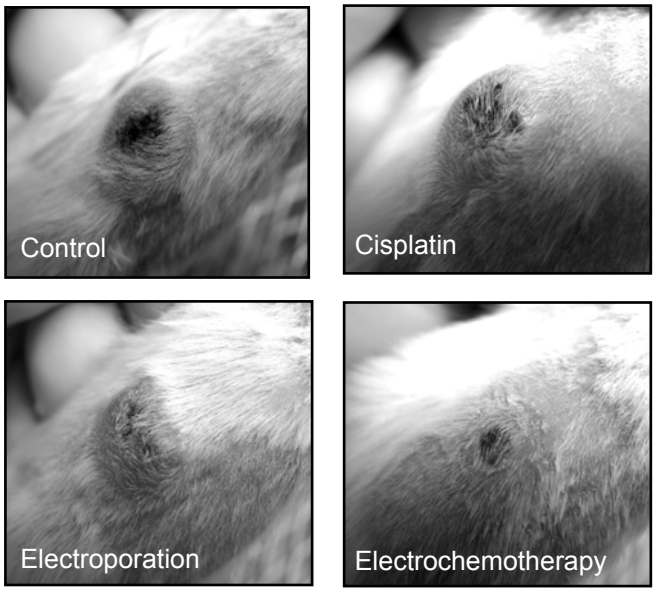


***Figure 2:*** Example of good antitumor effectiveness on SA-1 tumors after electrochemotherapy with cisplatin. Cisplatin was given intravenously (4 mg/kg), 3 min thereafter 8 electric pulses were applied to the tumor with plate electrodes. Electric pulses were applied in two directions; 4 pulses in one and the other 4 in the perpendicular direction. Eight days after the treatment good antitumor effectiveness of electrochemotherapy with cisplatin is evident, compared to the single treatments with cisplatin or electric pulses.

- The antitumor effectiveness is dependent on the *amplitude, number and duration of the electric pulses applied*. Several studies in which parallel plate electrodes were used for surface tumors showed that an amplitude over distance ratio above 1000 V/cm is needed for tumor electroporation, and that above 1500 V/cm, irreversible changes in the normal tissues adjacent to the tumor occur. So, the window for effective and safe electrochemotherapy is between 1000-1500 V/cm. In most studies, the amplitude over distance ratio of 1300 V/cm induced good antitumor effectiveness without sub-optimal electroporation of the tissue or damage to the tissue due to irreversible cell permeabilisation [6]. For other types of electrodes, the electric field distribution and thus, also the necessary amplitude of electric pulses, need to be determined by

numerical calculations. *Repetition frequencies of the pulses* for electrochemotherapy are either 1 Hz or 5 kHz. The minimal number of pulses used is 4; most studies use 8 electric pulses of 100 μs [1,4,6-8].

All the experiments conducted *in vivo* on animals provided sufficient data to demonstrate that electrochemotherapy with either bleomycin or cisplatin is effective in the treatment of solid tumors, using drug concentrations which have no or minimal antitumor effect without application of electric pulses. A single treatment by electrochemotherapy already induces partial or complete regression of tumors, whereas treatment with bleomycin or cisplatin alone or application of electric pulses alone has no or minimal antitumor effect (Figure 2).

### *Mechanisms of action*

The principal mechanism of electrochemotherapy is *electroporation* of cells in the tumors, which increases the drug effectiveness by enabling the drugs to reach the intracellular targets. This was demonstrated in studies which measured the intratumoural drug accumulation and the amount of drug bound to DNA. Basically, the amounts of bleomycin and cisplatin in the electroporated tumours were up to 2-4 fold higher than in those without application of electric pulses [1-4].

Besides membrane electroporation, which facilitates drug transport and its accumulation in the cell, other mechanisms that are involved in the antitumour effectiveness of electrochemotherapy were described. The application of electric pulses to tissues induces a transient, but reversible *reduction of blood flow* [9]. Restoration of the blood flow in normal tissue is much faster than that in tumors [10]. The vascular lock in the tumor induces *drug entrapment* in the tissue, providing more time for the drug to act.

The cytotoxic effect of electrochemotherapy is not limited only to tumor cells in the tumors. Electrochemotherapy also acts on stromal cells, including endothelial cells in the lining of tumor blood vessels, which undergo apoptosis [11]. Consequently, by vascular-disrupting action of electrochemotherapy, a cascade of tumor cell death occurs due to long-lasting hypoxia in the affected vessels. This represents yet another mechanism involved in the antitumor effectiveness of electrochemotherapy, i.e. a *vascular-disrupting effect* [12,13]. This vascular-disrupting action of electrochemotherapy is important in clinical situations where haemorrhagic tumor nodules need to be treated [14].

A difference in the antitumor effectiveness of electrochemotherapy was observed between immunocompetent and immunodeficient experimental animals, indicating involvement of the *immune response* in antitumour effectiveness [15]. Due to massive tumor antigen shedding in organisms after electrochemotherapy, systemic immunity can be induced and up-regulated by additional treatment with biological response modifiers like IL-2, GM-CSF and TNF-α [16-18].

To sum up, the electrochemotherapy protocol was optimized in preclinical studies *in vitro* and *in vivo*, and basic mechanisms were elucidated. In addition to the electroporation of cells, tumor drug entrapment, a vascular- disrupting effect and involvement of the immune response were also demonstrated. Based on all this data, electrochemotherapy with bleomycin and cisplatin was promptly evaluated in clinical trials.

## PERSPECTIVES

Knowledge about the mechanisms involved in the antitumor effectiveness of electrochemotherapy opened new possibilities for the application of electric pulses or electrochemotherapy in the treatment of cancer.

The chemotherapeutic drugs which increase effectiveness of radiation therapy are radiosensitizing drugs. These include bleomycin and cisplatin. Since drug delivery induced by electroporation is site-specific, it could be used for tumor-specific delivery of radiosensitizing drugs. By increased radiosensitizing drug delivery into tumors and not in the surrounding normal tissue, the therapeutic index of tumor irradiation is increased. In our studies, we combined electrochemotherapy with bleomycin or cisplatin with radiotherapy and demonstrated a good potentiation of the tumor radiation response: 1.9-fold for electrochemotherapy with bleomycin and 1.6- fold for electrochemotherapy with cisplatin [19, 20]. The radiosensitizing effect of electrochemotherapy with bleomycin was also demonstrated in a fractionated radiation regime which makes this treatment potentially available also in the clinic [21].

The application of electric pulses was shown to modulate tumor blood flow. Both reduced blood flow and lowered partial oxygen pressure ($pO_2$) in the tumors are consequences of the applied electric pulses [22]. The reduced $pO_2$ can activate bioreductive drugs to exhibit a cytotoxic effect on hypoxic cells [23]. In well- oxygenated cells, the drug remains inactive. On the other hand, tumor hypoxia induced by application of electric pulses can improve therapeutic conditions for the use of hyperthermia since tumor cells are more sensitive to heat in sub-optimal physiological conditions [24].

Electrochemotherapy with cisplatin or bleomycin was also successfully used in veterinary medicine. It was used to treat different tumors, such as mammary adenocarcinoma, fibrosarcoma, cutaneous mast cell tumor, hemangioma, hemangiosarcoma, perianal tumors, neurofibroma and sarcoids in dogs, cats, hamsters, rabbits and horses. Recent reports demonstrated successful treatment of different neoplasms in companion animals and sarcoids in horses [25-28]. Hopefully, electrochemotherapy will be broadly used in veterinary medicine for the treatment of different malignancies, both in primary and metastatic disease.

Electrochemotherapy is an effective cytoreductive treatment; however, its curative effect is dependant on the permeabilisation of possibly all cells in the tumour. Since permeabilisation of every single cell in the tumour is virtually impossible, electrochemotherapy could be combined with other cytoreductive treatments. Another approach is a combination of electrochemotherapy with electrogene therapy. The first promising reports and data are already available, supporting the effectiveness of this concept [29-30].

In conclusion, electroporation in electrochemotherapy has already been very well exploited; however, there are new biomedical applications of electroporation in cancer treatment that still need testing and development.

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## NOTES

# Clinical electrochemotherapy

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**Abstract:** Electrochemotherapy consists of chemotherapy followed by local application of electric pulses to a tumor to increase drug delivery into tumor cells. Due to selective drug uptake in the area of application of electric pulses, the therapeutic index for bleomycin and cisplatin is increased, resulting in a good local potentiation of drug effectiveness and minimal systemic and local side effects. Electrochemotherapy is an effective local treatment for cutaneous and subcutaneous tumor nodules of different malignancies, with a 60-80% objective response of the tumors. With the development of new electric pulse generators and electrodes, as well as standard operating procedures, electrochemotherapy has become a standard treatment. This is the result of efforts of the European consortium gathered in the CLINOPORATOR and ESOPE projects.

## INTRODUCTION

Electrochemotherapy protocols were optimized in preclinical studies *in vitro* and *in vivo*, and the basic mechanisms elucidated, such as electroporation of cells, tumor drug entrapment, a vascular-disrupting effect and involvement of the immune response. Based on all this data, electrochemotherapy with bleomycin and cisplatin was promptly evaluated in clinical trials.

## CLINICAL STUDIES

The results of several clinical studies have confirmed the preclinical data: high antitumor effectiveness of electrochemotherapy with bleomycin and cisplatin on cutaneous and subcutaneous tumor nodules with different histologies was demonstrated.

The first clinical study was published in 1991 on head and neck tumor nodules [1], which was thereafter followed by several others [2-38]. These clinical studies demonstrated the antitumor effectiveness of electrochemotherapy using either bleomycin or cisplatin, given intravenously or intratumorally. Successful treatment of cutaneous and subcutaneous tumor nodules by electrochemotherapy was reported also from the Sydney Melanoma Unit as well as several Italian cancer centers [39-62]. In addition to single or multiple cutaneous or subcutaneous melanoma nodules, a response was demonstrated in breast and head and neck cancer nodules, as well as Kaposi's sarcoma, hypernephroma, chondrosarcoma and basal cell carcinoma. However, these clinical studies were performed with slightly variable treatment protocols, different electrodes and different electric pulse generators. Thus, there was a need for a prospective multi-institutional study, which was conducted by a consortium of four cancer centres gathered in the ESOPE project funded under the European Commission's 5$^{th}$ Framework Programme. In this study, the treatment response after electrochemotherapy according to tumor type, drug used, route of administration and type of electrodes, was tested [40]. The results of this study can be summarized as follows:

- An objective response rate of 85% (73.7% complete response rate) was achieved for electrochemotherapy-treated tumor nodules, regardless of tumor histology and drug or route of administration used (Figure 1).
- At 150 days after treatment, the local tumor control rate for electrochemotherapy was 88% with bleomycin given intravenously, 73% with bleomycin given intratumorally and 75% with cisplatin given intratumorally, demonstrating that all three approaches were equally effective in local tumor control.
- Electrochemotherapy was equally effective, regardless of tumor type.
- Side effects of electrochemotherapy were minor and tolerable (muscle contractions and pain sensation).

In all clinical studies reported, including the ESOPE study, 288 patients were treated: 782 tumor nodules were treated by electrochemotherapy with bleomycin and 398 tumor nodules were treated by electrochemotherapy with cisplatin. The results of the ESOPE study confirmed previously reported results on the effectiveness of electrochemotherapy and Standard Operating Procedures (SOP) for electrochemotherapy were prepared [41].

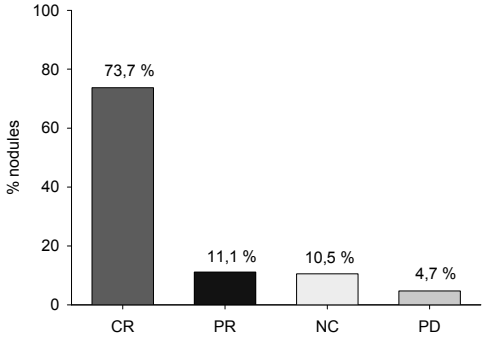


***Figure 1:*** Treatment response of tumor nodules treated by electrochemotherapy in the ESOPE project.

After the ESOPE study, several clinical studies were published confirming the results of the ESOPE study and elaborating on the treatment effectiveness of repetitive electrochemotherapy on bigger tumor nodules [57,58].

## TREATMENT PROCEDURES FOR ELECTROCHEMOTHERAPY

Electrochemotherapy is used for the treatment of cutaneous and subcutaneous tumor nodules of different malignancies. The treatment advantages and clinical uses for electrochemotherapy can be summarized [52].

- Easy and effective treatment of single or multiple tumor nodules of any histology in cutaneous and subcutaneous tissue.
- Treatment that increases quality of life in patients with progressive disease.
- Treatment of choice for tumors refractory to conventional treatments.
- Neoadjuvant treatment in the form of cytoreductive therapy before conventional treatment.
- Organ-sparing and function-saving treatment.
- Treatment of hemorrhagic or painful nodules, since it reduces bleeding and, in some cases, pain level.

The treatment procedure is as follows: based on SOP, tumor nodules can be treated by electrochemotherapy with injection of bleomycin intravenously or intratumorally and by electrochemotherapy with cisplatin given intratumorally. The choice of the chemotherapeutic drug in not based on tumor histology, but depends on the number and size of the nodules. After drug injection, the tumor nodules are exposed to electric pulses. The interval between intravenous drug injection and application of electric pulses is 8-28 min, and after intratumoral injection, as soon as possible. Different sets of electrodes are available for application; plate electrodes for smaller tumor nodules and needle electrodes for the treatment of larger (3 cm) and thicker tumor nodules. The treatment can be performed in a single session or can be repeated in case of newly emerging nodules or on those nodules which relapsed in some regions which were not treated well in the first treatment [41].

Electrochemotherapy does not induce side effects due to chemotherapeutic drugs since the drug dosage is very low. However, the application of electric pulses to the tumors induces contraction of the underlying muscles. For electroporation, square wave electric pulses with an amplitude over distance ratio of 1000-1300 V/cm, duration of 100 μs, frequency 1 Hz or 5 kHz are used. These muscle contractions are painful, but the pain dissipates immediately after electric pulse application. Nevertheless, in SOP, the procedures for alleviating pain by local anaesthesia or by general anaesthesia in case of treating multiple nodules, are also described [41].

The treatment after a single electrochemotherapy session in most cases results in complete tumor eradication. When necessary, treatment can be repeated at 4-8 week intervals with equal antitumor effectiveness. The treatment has a good cosmetic effect without scarring the treated tissue

## CONCLUSION

Electrochemotherapy is now a standard approach in the palliative treatment of cutaneous and subcutaneous tumor nodules of different malignancies. However, the development of electrochemotherapy will proceed with development of new electrodes which will enable treatment of bigger tumors and tumors in internal organs. Consequently, indications for electrochemotherapy will broaden.

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## NOTES

# Development of devices and electrodes

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**Abstract:** Since first reports on electroporation more than thirty years ago, a number of electroporation based biotechnological and biomedical applications has been developed. The necessary pulse generators are characterized by the shape of the pulses and their characteristics: pulse amplitude and duration. In addition, the electrodes are the important "connection" between the cells/tissue and pulse generator. The geometry of the electrodes together with the cell/tissue sample properties determine the necessary output power and energy that the electroporators need to provide. The choice of electroporator – the pulse generator depends on biotechnological and biomedical application but has to be linked also to the electrode choice.

## INTRODUCTION

Since first reports on electroporation (both irreversible and reversible) more than thirty years ago, a number of applications has been developed and list of applications which are based on electroporation is still increasing. First pulse generators have been simple in construction and have provided an exponentially decaying pulse of up to several thousands of volts. Also the electrodes were very simple in their design – usually parallel plate electrodes with couple of millimeters distance was used, and cells in suspension were placed in-between. Later, new pulse generators were developed which were/are able to provide almost every shape of pulse, and also electrodes which can be bought are extremely diverse. It is important to note that most often nowadays devices that generate rectangular pulses are being used.

The amplitude of pulses and their duration depend strongly on biotechnological/biomedical application. For electrochemotherapy most often a number of 1000 V pulses of 100 μs duration are needed. For effective gene transfection longer pulses 5-20 ms pulses but of lower amplitude (e.g. 200 V), or a combination of short high- and longer low-voltage pulses are used. For other applications like tissue ablation by means of irreversible electroporation, or liquid-food or water sterilization, thousand of volts and longer ms pulses are needed. In addition to the pulse amplitude and duration, an important parameter to be taken into account is also the power and energy that need to be provided by the generator.

The energy that needs to be provided is governed by the voltage, current and pulse duration and/or number of pulses. The current if the voltage is set is governed by the load, and this is determined by the geometry of the load, and the load is determined by geometry of the tissue/cell sample and its electrical conductivity. The geometry of the tissue to be exposed to electric pulses are predominantly determined by the shape of the electrodes, the distance between them, depth of electrode penetration/immersion into the sample and their electrical connections to the generator if more than two are active at the same time. Tissue/cell suspension electrical conductivity depends on tissue type or cell sample properties and can be considerably increased while tissue/cells are being exposed to electrical pulses of sufficient amplitude.

Based on the above considerations not a single pulse generator will fit all applications and all needs of a researcher. One can either seek for a specialized pulse generator which will only provide the pulses for this specific biotechnological or biomedical application, or for a general purpose pulse generator which will allow to generate "almost" all what researcher may find necessary in his/her research. Irrespective of the choice, it has to be linked also to the electrodes choice.

## THERAPEUTIC AND TECHNOLOGICAL APPLICATIONS OF ELECTROPORATION:

Nowadays electropermeabilization is widely used in various biological, medical, and biotechnological applications. Destructive applications relying on irreversible electroporation are less than a decade old, but their efficacy is promising especially in the field of water treatment where efficacy of chemical treatment is enhanced with electropermeabilization, in food preservation where electropermeabilization has proven, in some cases, to be as effective as pasteurization or in tissue ablation. In contrast, applications based on reversible electroporation are currently more widespread and established in different experimental and/or practical protocols. Probably the most important of them is the introduction of definite amount of small or large molecules to cytoplasm through the plasma membrane. Furthermore, slight variation of electric field parameters results in an application where molecules can be directly inserted into the plasma membrane or cells can be effectively fused.

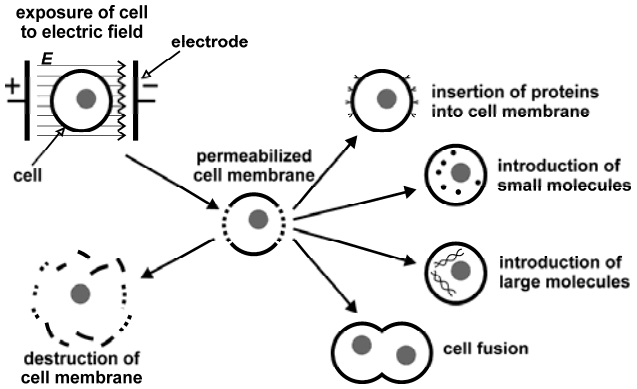


***Figure 1:*** Exposure of a cell to an electric field may result either in permeabilization of cell membrane or its destruction. In this process the electric field parameters play a major role. If these parameters are within certain range, the permeabilization is reversible; therefore it can be used in applications such as introduction of small or large molecules into the cytoplasm, insertion of proteins into cell membrane or cell fusion. Originally published in Wiley Encyclopedia of Biomedical Engineering: Electroporation by Miklavčič and Puc 2006.

## ELECTROCHEMOTHERAPY

The most representative application of delivery of small molecules through electroporated membrane is electrochemotherapy. It was demonstrated in several preclinical and clinical studies, both on humans and animals, that electrochemotherapy can be used as treatment of choice in local cancer treatment. Most often a number of short rectangular 100 μs long pulses with amplitudes up to 1000 V, are applied.

## GENE ELECTROTRANSFECTION

Exogenous genetic material can be delivered to cells by using non-viral methods such as electropermeabilization. Electrotransfection can be achieved using: exponentially decaying pulses; square wave pulses with superimposed RF signals; or only long square wave pulses up 20 ms and with amplitudes ranging from 200 to 400 V. In general, it can be stated that longer pulses are used in gene transfection than in electrochemotherapy. Furthermore, recently two distinct roles of electric pulses were described. In experiments where several short high voltage pulses (e.g. 8 × 100 μs of 1000 V) were followed by long low voltage pulses (e.g. 1 × 100 ms of 80 V). It was suggested that short high voltage pulses are permeabilizing the membrane while the longer lower voltage pulses have an electrophoretic effect on DNA itself facilitating interaction of plasmid with the membrane.

## ELECTROINSERTION

To achieve uptake of ions or molecules through cell plasma membrane to the cytosol with electroporation electric field intensity must exceed critical value. If the field intensity is just below the critical value it is possible to insert proteins directly into the cell plasma membrane. Further studies have shown that electric field intensity plays crucial role in process of membrane protein insertion. Electric field intensity should be just below the critical value of permeabilization if insertion is done on the red blood cells, i.e. non-nucleated cells, but in a case of nucleated cells the field intensity must trigger membrane permeabilization in order to achieve effective insertion.

## ELECTROFUSION

So far we have presented applications of electroporation that are used to introduce different molecules either to the cytosol or to the cell plasma membrane. But electroporation of cell plasma membrane can also result in fusion of cells. This process has been termed electrofusion. First reports of *in vitro* electrofusion of cells date back into 1980s. In the reports it has been shown that fusion between two cells can proceed only if the cells are in contact prior or immediately after electroporation. The contact between the cells can be achieved either by dielectrophoretic collection of neighboring cells, which is followed by electropermeabilization or by centrifugation of cell suspension after exposure to electric field. In both cases cells must be reversibly permeabilized, otherwise they lose viability and there is no electrofusion. Electrofusion in *in vitro* environment is possible due to high possibility of cell movement while cells in tissues are more or less fixed, nevertheless *in vivo* electrofusion has been observed in B16 melanoma tumors as well as cells to tissue fusion.

## ELECTROSTERILIZATION

Irreversible electroporation can be used in applications where permanent destruction of microorganisms is required, i.e. food preservation and water treatment. Still, using irreversible electropermeabilization in these applications means that substance under treatment is exposed to a limited electric field since it is desirable that changes in treated substance do not occur (e.g. change of food flavor) and that no by-products emerge due to electric field exposure (e.g. by-products caused by electrolysis).

## TISSUE ABLATION

The ablation of undesirable tissue through the use of irreversible electroporation has recently been suggested as a minimally invasive method for tumor removale but could also be used in cardiac tissue

ablation instead of RF heating tissue ablation or other tissue ablation techniques.

## ELECTRIC FIELD DISTRIBUTION *IN VIVO*

In most applications of tissue permeabilization it is required to expose the volume of tissue to E intensities between the two thresholds i.e. to choose in advance a suitable electrode configuration and pulse parameters for the effective tissue permeabilization. Therefore electric field distribution in tissue has to be estimated before the treatment, which can be achieved by combining results of rapid tests with models of electric field distribution. However, modeling of electric field distribution in tissue is demanding due to heterogeneous tissue properties and usually complex geometry. Analytical models can be employed only for simple geometries. Usually they are developed for 2D problems and tissue with homogenous electrical properties. Therefore in most cases numerical modeling techniques are still more acceptable as they can be used for modeling 3D geometries and complex tissue properties. For that purpose mostly finite element method and finite difference method are applied. Both numerical methods have been successfully applied and validated by comparison of computed and measured electric field distribution. Furthermore, a few advanced numerical models were build, which took into consideration also tissue conductivity increase due to tissue or cell electroporation. These advanced models consist of a sequence of static models (steps), which describe E distribution in discrete time intervals during permeabilization. In this way models present dynamics of electroporation since in each step the tissue conductivity is changed according to distribution of electric field intensities from the previous step.

## ELECTRODES FOR IN VITRO AND IN VIVO APPLICATIONS

Effectiveness of electroporation in *in vitro*, *in vivo* or clinical environment depends on the distribution of electric field inside the treated sample. Namely, the most important parameter governing cell membrane permeabilization is local electric field exceeding critical threshold. To achieve this we have to use an appropriate set of electrodes and an electroporation device – electroporator that generates required voltage or current signals. Although both parts of the mentioned equipment are important and necessary for effective electroporation, electroporator has a substantially more important role since it has to be able to deliver the required signal to its output loaded by impedance of the sample between electrodes.

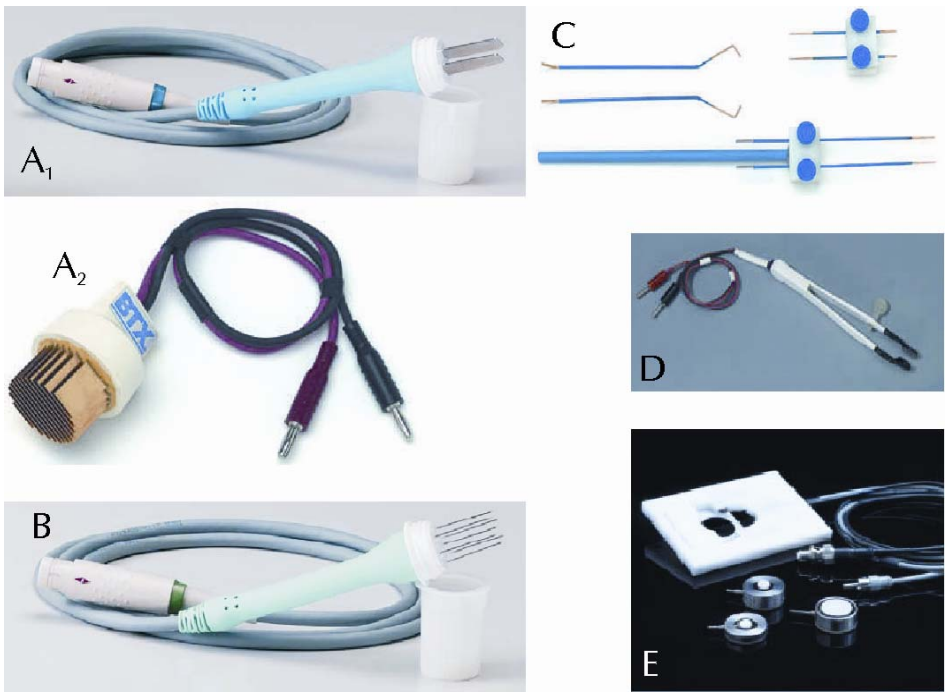


***Figure 2:*** Examples of commercially available electrode for electropermeabilization. Electrodes belong to the following group: $A_1$ and $A_2$ – to parallel plate electrodes, B – needle arrays, C – wire electrodes, D – tweezers electrodes and E – coaxial electrodes. Electrodes $A_1$ and B are produced by IGEA, Italy and are used for clinical applications of electrochemotherapy and electrotransfection. Electrodes $A_2$, C and E are used for different in vitro applications and are produced by: E – Cyto Pulse Sciences, U.S.A.; $A_2$, C and also D that are used for in vivo applications, are produced by BTX Hardware division, U.S.A.

Nowadays there are numerous types of electrodes that can be used for electroporation in any of the existing applications. According to the geometry, electrodes can be classified into several groups, i.e. parallel plate electrodes, needle arrays, wire electrodes, tweezers electrodes, coaxial electrodes, etc (Fig. 2). Each group comprises several types of electrodes that can be further divided according to the applications, dimensions, electrode material etc. In any case selection of electrode type plays an important role in characterization of the load that is connected to the output of the electroporator. During the design of the electroporator load characterization represents the greatest engineering problem, because electrical characteristics of substance between electrodes (e.g. cell suspension, tissue, etc.) vary from experiment to experiment and even during the same experiment. In general the load between electrodes has both a resistive and a capacitive component. The value of each component is defined by geometry and material of electrodes and by electrical and chemical properties of the treated sample. In *in vitro* conditions these parameters that influence the impedance of the load can be well controlled since size and geometry of sample are known especially if cuvettes are used. Furthermore, by using specially prepared cell media, electrical and chemical properties are defined or can be measured. On the other hand, in *in vivo* conditions, size and geometry can still be controlled to a certain extent but electrical and chemical properties can only be estimated, especially if needle electrodes are used that penetrate different tissues. However, even if we manage to reliably define these properties during the development of the device, it is practically impossible to predict changes in the electrical and chemical properties of the sample due to exposure to high-voltage electric pulses. Besides electropermeabilization of cell membranes which increases electrical conductivity of the sample, electric pulses also cause side effects like Joule heating and electrolytic contamination of the sample, which further leads to increased sample conductivity.

## ELECTROPORATORS – THE NECESSARY PULSE GENERATORS

Electroporator is an electronic device that generates signals, usually square wave or exponentially decaying pulses, required for electroporation. Parameters of the signal delivered to electrodes with the treated sample vary from application to application. Therefore, it is very important that electroporator is able to deliver signals with the widest possible range of electrical parameters if used in research. If however used for a specific application only, e.g. clinical treatment such as electrochemotherapy, pulse generator has to provide exactly the required pulse parameters. Moreover, electroporator must be safe and easy to operate and should offer some possibilities of functional improvements. In principle, electroporators can be divided in several groups depending on biological applications, but from the electrical point of view only two types of electroporators exist: devices with voltage output (output is voltage signal U(t)) and devices with current output (output is current signal I(t)). Both types of devices have their advantages and disadvantages, but one point definitely speaks in favor of devices with voltage output. For example, if we perform *in vitro* experiments with parallel plate electrodes with plate sides substantially larger than the distance between them, the electric field strength E that is applied to the sample can be approximated by the voltage-to-distance ratio $U/d$, where $d$ is the electrode distance and $U$ the amplitude of applied signal obtained from an electroporator with voltage output. On the other hand, if an electroporator with current output is used, the same approximation could be used only if additional measurement of voltage difference between electrodes is performed or if the impedance $Z$ of the sample is known, measured or approximated and voltage difference between electrodes is estimated using Ohm's law $U=IZ$. Nevertheless, there are several commercially available electroporator that fulfill different ranges of parameters and can be used in different applications. A list of commercially available electrodes and electroporators has been presented in 2004 by Puc and colleagues in a paper that describes techniques of signal generation required for electropermeabilization.

Based on the studies reported in the literature it is very difficult to extract a general advice how to design experiments or treatments with electroporation. In principle we can say that pulse amplitude (voltage-to-distance ratio) should typically be in the range from 200 V/cm up to 2000 V/cm. Pulse durations should be in the range of hundreds of microseconds for smaller molecules and from several milliseconds up to several tens of milliseconds for macromolecules such as DNA fragments (in the latter case, due to the very long pulse duration, optimal pulse amplitude can even be lower than 100 V/cm). If there is any possibility to obtain the equipment that generates bipolar pulses or have a possibility to change electric field orientation in the sample, these types of pulses/electroporators should be used because bipolar pulses yield a lower poration threshold, higher uptake, and an unaffected viability compared to unipolar pulses of the same amplitude and duration. Better permeabilisation and survival can also be obtained by changing field orientation in the sample.

This general overview of electrical parameters should however only be considered as a starting point for a design of experiments or treatments. Optimal values of parameters namely also strongly depend on the cell type used, on the molecule to be introduced, and on specific experimental conditions.

## CONCLUSIONS

Electroporation has been studied extensively until now, and a number of applications has been suggested. Electrochemotherapy has been demonstrated as an effective local treatment of solid tumors and is the most mature therapeutic application right now. Electroporation for gene transfection however has been long used in *in vitro* situation. With a hold on viral vectors electroporation represents a viable non viral alternative also for *in vivo* gene transfection. Clinical applications and expansion of electrochemotherapy and tissue ablation have been hindered by the lack of adequate electroporators and their certification in Europe (CE Medical Device) and limited approval by FDA in USA. Cliniporator (IGEA, s.r.l. Carpi, Italy) was certified as a medical device and is offered on the market along with standard operating procedures for electrochemotherapy of cutaneous and subcutaneous tumors. Recently NanoKnife (AngioDynamics, Queensbury, USA) was approved by the FDA for surgical ablation of soft tissue, including cardiac and smooth muscle.

Development of new applications warrants further development of pulse generators and electrodes. Based on the above considerations however, a single pulse generator will not fit all applications and all needs of researchers. One can either seek for a specialized pulse generator which will only provide the pulses for this specific biotechnological or biomedical application, or for a general purpose pulse generator which will allow to generate “almost” all what researcher may find necessary in his/her research. Irrespective of the choice, this choice has to be linked also to the electrodes choice.

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## NOTES

# INVITED LECTURERS

# Delivery of Nanosecond, Megawatt, Pulsed Electrical Energy to Membranes, Cells, and Tissues — Biophysics and Therapeutics

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## INTRODUCTION

To utilize the diverse *effects* of electric fields on biological systems we must understand the ***causes***. In particular, we want to know the details of the ***interactions*** between electric fields and biomolecular structures. By looking at very short time scales (nanoseconds) and at single events (non-repetitive stimuli), we reduce the number of larger-scale disturbances and concentrate on reversible perturbations. The analysis is primarily in the time domain, but pulse spectral content may be important.

Of course, some important ***effects*** may be a consequence of irreversible processes driven by longer electric field exposures (microseconds, milliseconds). Short-pulse studies can help to dissect these processes.

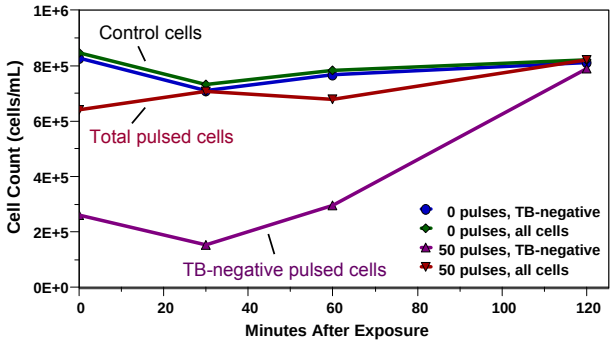


***Figure 1.*** Nanoelectropulsed Jurkat T lymphoblasts recover over 2 hrs from initial Trypan blue permeabilization after exposure to 50, 20 ns, 4 MV/m pulses at 20 Hz.

Although modeling is of necessity a significant component of nanosecond bioelectrics investigations, experimental observations are fundamental, and to conduct experiments in nanosecond bioelectrics, one must be able to generate and accurately monitor the appropriate electrical stimuli, a non-trivial engineering challenge. We will discuss cause and effect here from both **scientific and engineering perspectives**, using data from experiments and simulations It is commonplace in electrical engineering, and increasingly so in biology, to attack a problem with a combination of modeling and experimental tools. In nanosecond bioelectrics, observations (in vitro and in vivo) give rise to models (molecular and continuum), which drive experiments, which adjust and calibrate the models, which feed back again to empirical validation. This loop focuses investigations of a very large parameter space.

## NANOSECOND BIOELECTRICS

From longstanding theory that models the cell as a dielectric shell [1–4] came the notion that nanosecond-scale electric pulses could "bypass" the cell membrane, depositing most of their energy inside the cell instead of in the plasma membrane, as is the case with longer pulses. This idea was investigated experimentally beginning in the late 1990s [5–6]. Although one early report indicated that the electric field-driven conductive breakdown of membranes can occur in as little as 10 ns [7], and a more careful analysis demonstrated that pulses with field amplitudes greater than about 1 MV/m will produce porating transmembrane potentials within about 2 ns [8, and a well grounded model predicted "poration everywhere" in the nanosecond pulse regime [9], procedures used to detect electroporation of the plasma membrane (and the loss of membrane integrity in general) produced negative results for pulses with durations less than the charging time constant of a small cell in typical media (< 50 ns).

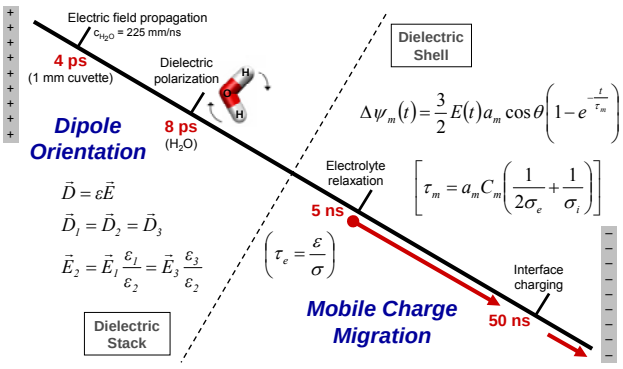


***Figure 2.*** Timeline representing the sequence of events following electrical polarization of a biological tissue or aqueous suspension of cells. The sub-nanosecond regime can be modeled by the dielectric properties of the system. For longer times the distribution of fields and potentials is dominated by the migration of charged species.

In addition to highlighting the limitations of the traditional experimental methods for observing membrane permeabilization, this apparent discrepancy between model and observation points also to inadequacies in the dielectric shell model itself at time

scales below the membrane (cell) charging time. Higher-frequency effects associated with the dielectric properties of high-permittivity aqueous media and low-permittivity biological membranes [10–13] have not received much attention until recently. For the electropermeabilizing conditions that are most commonly studied (μs, kV/m pulses) these effects are secondary and minor, but for nanosecond pulses they cannot be ignored.

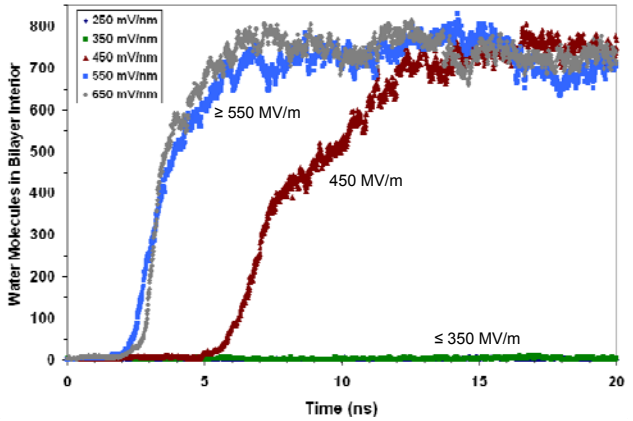


***Figure 3.*** Electric field-driven intrusion of water into a simulated lipid bilayer.

Several lines of experimental evidence indicate that nanosecond electric pulses cause changes in the integrity and organization of the cell membrane.

*Trypan blue permeabilization*. While remaining PI-negative, the cell volume of Jurkat T lymphoblasts exposed to a series of 50, 20 ns, 4 MV/m pulses increases, and they become visibly permeable to Trypan blue (TB) in the microscope (Figure 1). With increasing time after pulse exposure, these weakly TB-positive cells become again impermeable to TB. Similar observations have recently been reported for B16 murine melanoma cells exposed to sub-nanosecond (800 ps) pulses at very high fields [14].

*Nanosecond porating transmembrane potentials*. Fluorescence imaging with a membrane potential-sensitive dye shows that porating transmembrane potentials are generated during nanoelectropulse exposure [15].

*Nanoelectropulse-induced PS externalization*. Loss of asymmetry in membrane phospholipid distribution resulting from phosphatidylserine (PS) externalization occurs immediately after nanoelectropulse exposure [16], consistent with membrane reorganization driven directly by nanosecond-duration electric fields and a mechanism in which nanometer-diameter pores provide a low-energy path for electrophoretically facilitated diffusion of PS from the cytoplasmic leaflet of the plasma membrane to the external face of the cell [8].

*Simulations link PS externalization and nanoporation*. In molecular dynamics (MD) simulations of electroporation, hydrophilic pores appear within a few nanoseconds [17], and PS migrates electophoretically along the pore walls to the anode-facing side of the membrane [18–19], in silico replication of experimental observations in living cells [20].

*Nanoelectropermeabilization*. The first direct evidence for nanoelectropermeabilization was obtained by monitoring YO-PRO-1 (YP) influx [21], a more sensitive indicator of membrane permeabilization than propidium iodide (PI) [22]. Additional direct evidence comes from patch clamp experiments, which reveal long-lasting increases in membrane conductance following exposure to 60 ns pulses [23–25].

*Nanosecond activation of electrically excitable cells*. Electrically excitable cells provide a highly responsive environment for nanoelectropulse biology. Adrenal chromaffin cells [26] and cardiomyocytes [27] react strongly to a single 4 ns pulse, and muscle fiber has been shown to respond to a 1 ns stimulus [28].

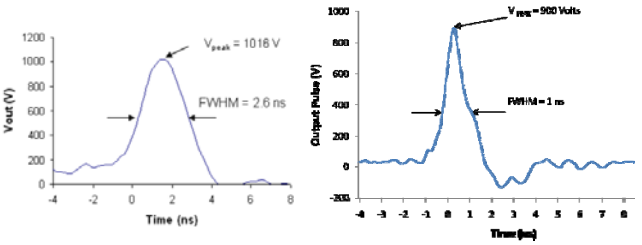


***Figure 4.*** Pulse shortened from 2.6 ns to 1 ns with avalanche diode and differentiating network.

*Nanosecond bioelectrics and the dielectric stack model*. Figure 2 depicts a time line of events in an aqueous suspension of living cells and electrolytes between two electrodes after an electric pulse is applied. Water dipoles re-orient within about 8 ps The field also alters the electro-diffusive equilibrium among charged species and their hydrating water, with a time constant that ranges from 0.5 to 7 ns. Pulses shorter than the electrolyte relaxation time do not generate enough interfacial charge to produce porating transmembrane potentials. The dielectric shell model in this regime can be replaced with a simpler, dielectric stack model, in which the local electric field depends only on the external (applied) electric field and the dielectric permittivity of each component of the system.

*Nanoelectropermeabilization and continuum models*. MD simulations at present provide the only available molecular-scale windows on electropore

formation in lipid bilayers. Current models perform reasonably well, but simulations of electroporation still contain many assumptions and simplifications. To validate these models we look for intersections between all-atom molecular assemblies, continuum representations of cell suspensions and tissues, and experimental observations of cells and whole organisms. For example, a leading continuum model assumes an exponential relation between the transmembrane potential and several indices of electropore formation [29]. The MD results in Figure 3, showing water intrusion into the membrane interior as a function of applied electric field, qualitatively demonstrate this same non-linear relation between field and poration.

## NANOSECOND PULSE GENERATION AND METROLOGY

*Pulse shortening*. To explore the nanosecond and sub-nanosecond pulse regime, engineers must provide fast-rising (and falling), high-current (>10 A), high-voltage (>1 kV) signals matched to low-impedance loads (≤50 Ω), with fast repetition rates (> 1 kHz). One approach begins with a longer pulse generated by a robust architecture and looks for ways to shorten it. Figure 4 shows how a 2.6 ns pulse was shortened to 1 ns using a combination of a reverse-biased avalance diode to shorten the leading edge and a differentiating capacitor to reduce the fall time.

*Current measurement*. Accurate dose-response studies require precise metrology, not a trivial matter for nanosecond pulses with picosecond edges. Particular care must be taken to avoid modifying the signal with the measurement probe or monitoring circuit. Practical experience, a solid theoretical foundation, and a certain amount of artistry are requirements for success in nanosecond-picosecond (GHz) metrology. How to make current measurements, and how not to make them, will serve as an illustrative example.

*Microcircuit pulse generator*. The need to drive large currents through cables and connectors into low-impedance cell suspensions can be avoided by constructing the pulse generator and output electrodes with integrated circuit, microelectromechanical systems, and microfluidics technologies, similar to microscale systems for conventional electroporation [30]. An initial device is shown in Figure 5. The nanometer scale of the elements of a microfabricated, field-enhanced electrode array, coupled with appropriate patterning and functionalization, will make it possible eventually to target cells with specific surface features and to concentrate the electrical stimulus in localized volumes of the cell, permitting the selective treatment of a sub-population of cells in a heterogeneous mixture (culture medium, blood, other tissues). These are the first steps on the path to a microelectronics-based nanoelectropulse analysis system constructed with post-processing extensions of integrated circuit technology — nanosecond bioelectrics on a chip — a contribution to the biophysical and cell biology research infrastructure.

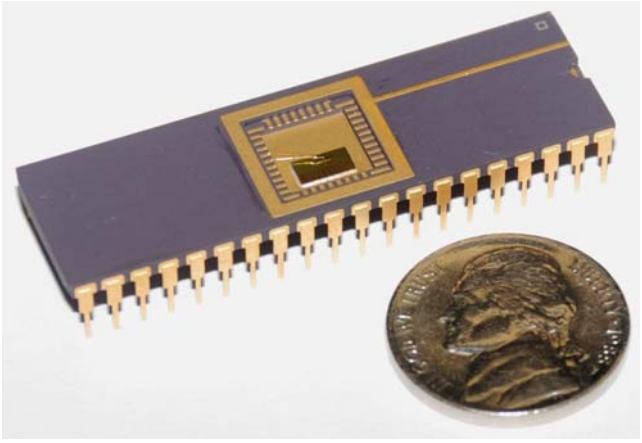

***Figure 5.*** Preliminary version of microelectronic nanoelectropulse generator in a DIP40 package.

## NANOSECOND THERAPEUTICS

*Nanoelectropulse therapy for skin cancer*. Exciting new results continue to come from the utilization of the unique properties of nanosecond, megavolt-per-meter electric pulses in the treatment of malignancies and other disease conditions. One focus of this effort has been skin cancers, specifically melanoma (in mice) and basal cell carcinoma (BCC; mouse and human) [31–33]. In vitro and in vito studies are in progress. Figure 6 shows a probe monitoring skin impedance during a treatment session.

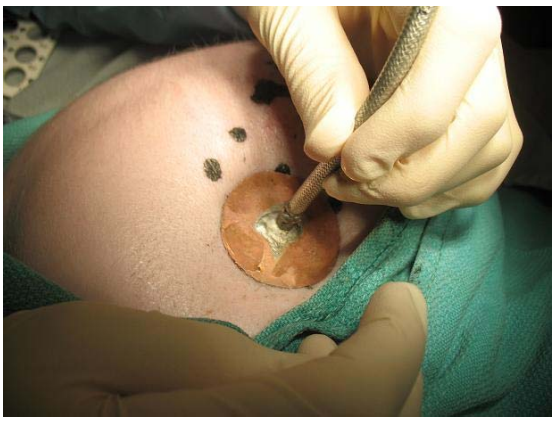

***Figure 6.*** Skin impedance measurement during nanoelectropulse therapy.

*Nanoelectrostimulation of neurosecretory and neuromuscular cells*. The sensitivity of electrically excitable cells to nanoelectropulses raises the

possibility that very low energy (nanosecond, megavolt-per-meter pulses are high power, but low total energy because of their brief duration) devices for cardiac regulation (implanted pacemakers and defibrillators), remote muscle activation (spinal nerve damage), and neurosecretory modulation (pain management) can be constructed with nanoelectropulse technology.

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Vernier received his Ph.D. in Electrical Engineering from the University of Southern California in 2004, and is a member of the American Chemical Society, American Society for Microbiology, Bioelectrochemical Society, Bioelectromagnetics Society, Biophysical Society, and Institute of Electrical and Electronics Engineers.

## NOTES

# Nanoelectropores of the cell plasma membrane

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**Abstract:** Undisturbed lipid bilayers are virtually impermeable to ions, so the transmembrane ion flows and electric potentials in living cells are controlled by specialized protein structures, namely ion channels and transporters. Chemical and physical insults such as electroporation may compromise the membrane integrity and allow leak ion currents through *de-novo* formed lipidic pores filled with water. These pores are generally thought to short-circuit the membrane and hinder ion channels' physiological function rather than to complement it. However, here we demonstrate that brief electric stimuli can trigger formation of membrane pores with specific behaviors that are traditionally considered to be unique for protein ion channels; still other behaviors of these pores distinguish them from both "conventional" electropores and any known ion channels. A single electric shock (e.g., 600-ns duration, 1 to 5 kV/cm) can cause minutes-long increase of the membrane electrical conductance due to formation of long-lived, voltage- and current-sensitive, rectifying, ion-selective pores of nanometer diameter ("nanopores"). Once induced, nanopores oscillate between open and quasi-open (electrically silent) states, followed by either gradual resealing or abrupt breakdown into larger pores, with immediate loss of nanopore-specific behaviors. Due to their extended lifetime and specific conductive properties, nanopores can have significant impact on cell function and physiology.

## INTRODUCTION: NANOSECOND ELECTRIC PULSES IN ELECTROPORATION

Compared with milli- and microsecond electric pulses, nanosecond-duration stimuli are a recent technology that was tested by biologists the first time about a decade ago. It has been proposed that shortening of the electric pulses into the nanosecond range would facilitate the electric field penetration through the plasma membrane into the cell interior, possibly giving rise to some unique and previously unknown biological effects [1-4]. The possibility to influence the structure and function of cell organs directly has motivated intensive research in this area. As of today, most nsEP studies were focused on such effects as intracellular calcium bursts [5, 6], fast translocation of phosphatidylserine residues in plasma membrane [7], cell and nuclear shrinkage [1, 8], necrotic and apoptotic cell death [9-13]. Contemplated mechanisms underlying these effects include electroporative damage of organelles, also called "supra-electroporation" [5, 14-16], free radical damage [12, 13], electrodeformation [17, 18], electro-conformational damage of membrane channel proteins [19-21], and possible mechanical damage by thermoelastic pressure transients [22, 23]. Cells exposed to nsEP showed little or no uptake of membrane integrity marker dyes such as Trypan Blue (TB) or propidium iodide (PI) [5, 7, 10-12], suggesting that, in contrast to the conventional electroporation with longer pulses [4, 24-27], the plasma membrane barrier function in nsEP-treated cells is not compromised.

At the same time, analytical models of nsEP effect on cells [14, 16, 28-30] and numerical simulations [15, 31, 32] suggested that the cell plasma membrane should not be "exempt" from poration. Instead, the anticipated effect was formation of small pores in large numbers, both in the plasma membrane and in the internal membranes of cell organs. These "nanopores" are believed to be large enough for the passage of small inorganic ions, but too small to allow dyes like TB or PI into the cell. In agreement with these predictions, an electrical breakdown of the plasma membrane within nanoseconds after the onset of the electric pulse was demonstrated by fluorescence measurements with a fast voltage-sensitive dye [33]. Furthermore, a recent study reported entry of a fluorescent dye YO-PRO-1 into nsEP-treated cells[34], although only after exposure to multiple high-voltage pulses delivered at a high rate (30 pulses, 4-ns pulse width, 60 kV/cm, at 1 kHz).

These findings motivated us to employ electrophysiological techniques for analysis of plasma membrane permeabilization by nsEP [35-37]. We developed a specialized setup for nsEP exposure of individual cells in culture and characterized it for compatibility with the whole-cell patch-clamp recording.

For the first time, these studies provided direct evidence for a profound and long-lasting (minutes) increase in whole-cell membrane electrical conductance (Gm) after a single nsEP stimulus. For example, one 60-ns pulse at 12 kV/cm increased Gm about 3-fold in GH3, PC-12, CHO, and Jurkat cells; however, HeLa cells required a more intense treatment. Further analysis established that, at different electric pulse durations, it was the absorbed dose that determined the magnitude of the biological effect. The threshold absorbed dose to produce plasma membrane effects in GH3 and CHO cells, at either 60-

or 600-ns pulse duration, was found to be at or below 10 mJ/g. Despite being determined by the dose, the nsEP effect clearly was not thermal, as the maximum heating at the threshold dose did not exceed 0.01 $^{o}$C. Importantly, the threshold for cell membrane permeabilization was substantially less than for any other known nsEP effects.

In agreement with earlier studies, nsEP-stimulated cells displayed little or no PI uptake, even after exposure to multiple pulses that caused cell swelling and blebbing. Hence, consistent with the theoretical predictions, nsEP-opened pores apparently were too small to allow for PI passage[4]. Based on the propidium cation box dimensions (13.8 x 11.5 x 5.4 A, not considering any hydration layers), the maximum diameter of propidium-impermeable pores should be less than some 12 A or 1.2 nm. Hence, nsEP-opened conductance pores in cell plasma membrane were named "nanopores," to distinguish them from "regular," propidium-permeable electropores of larger diameter.

Most surprisingly, nsEP-induced Gm increase persisted for minutes after the treatment, which is orders of magnitude longer than the expected lifetime of nanometer-diameter membrane defects. Furthermore, smaller size was not the only or the main parameter that distinguished nanopores from regular electropores. In fact, nanopores proved to be a qualitatively new phenomenon, with multiple implications for understanding cell membrane biophysics and cell physiology, and opening new avenues to deliberately manipulate these processes in living cells. Below we demonstrate that nanopores are different from plain "holes" in the lipid bilayer and exert complex behaviors that are normally expected only from sophisticated devices like protein ion channels. We also provide an explanation of a hypothetical mechanism that makes these behaviors possible. Furthermore, nanopores with minutes-long life span can profoundly affect the transmembrane potential and ionic gradients, thereby potentially triggering a wide range of physiological responses.

[4] In water solutions, PI ($C_{27}$ $H_{34}$ $N_4$ $I_2$) dissociates into a propidium cation and iodide anions. Nonetheless, numerous publications that deal with fluorescent properties of this dye and its use as a marker of membrane integrity, traditionally use terms "PI fluorescence" and "PI uptake" (instead of more appropriate "propidium fluorescence" and "propidium uptake"). However, it is the size of the propidium cation, not of the PI molecule, that limits passage of this dye through nanopores. Hence, below we will mostly use more accurate terms "propidium uptake" and "propidium staining".

## METHODS EMPLOYED TO DETECT AND STUDY NANOELECTROPORES

1. NsEP exposure. Mammalian cells (CHO, GH3, HeLa, NG108) were grown attached to a glass cover slip pre-treated with poly-L-lysine. The cover slip was placed into a glass-bottomed chamber (Warner Instruments, Hamden, CT) mounted on an IX71 inverted microscope (Olympus America Inc., Center Valley, PA), configured with an Olympus FluoView TM 300 confocal laser scanning microscope.

Nearly rectangular 600-ns pulses were generated in a transmission line-type circuit, by closing a MOSFET switch. NsEP were delivered to the selected cell by a pair of tungsten rod electrodes (0.1 mm electrode diameter, 0.14-0.2 mm gap). With a help of a micromanipulator, the electrodes were positioned touching the surface of the cover slip on the sides of the selected cell [38, 39]. Alternatively, the electrodes could be placed precisely 50 um above the cover slip surface (to avoid any mechanical shift of the cover slip with cells).

The E-field between the electrodes was determined by a 3D simulation with the finite element Maxwell equations solver Amaze-3D (Field Precision, Albuquerque, NM). The E-field at the location of the cell was practically uniform, yielding about 60 kV/cm per 1 kV amplitude of the applied pulse (or about 40 kV/cm per 1 kV when the electrodes were 50 um above the cover slip).

2. Patch clamp. This method is widely used to record and measure single-cell or even single-channel currents while applying a precisely known voltage to the membrane. A detailed description of the technique is beyond the scope of this lecture and can be found in specialized publications [40, 41]. In brief, a whole-cell recording configuration is achieved by (1) approaching the selected cell with a buffer-filled glass micropipette, (2) touching the cell with the pipette tip, to form a tight seal ("gigaseal") between the tip and the cell membrane, and (3) rupture of the membrane within the seal, to allow the pipette buffer to enter the cell. Next, voltage applied to the inside of the pipette becomes transmembrane voltage for the "patched" cell, which makes it possible to measure transmembrane currents while holding the voltage at a specified level ("voltage clamp"). Patch clamp is arguably the most sensitive and precise technique to detect nanoelectropores and explore their conductive properties. However, the patch clamp method is always invasive, and the integrity of the whole-cell configuration can be compromised by nsEP exposure.

For experiments reported below, we pulled recording pipettes from borosilicate glass (BF150-86-10, Sutter Instrument, Novato, CA) to a tip resistance of 1.5-3 MOhm using a Flaming/Brown P-97

Micropipette puller (Sutter). Electrophysiology data were acquired using a Multiclamp 700B amplifier, Digidata 1322A A-D converter, and pCLAMP 10 software (Molecular Devices, Foster City, CA). The analog signal was low-pass filtered at 2 or 5 kHz, and digitized at "oversampling" rates of 10 to 50 kHz. Typical values of the seal and access resistance were 2-8 GOhm and 4-10 MOhm, respectively.

2. Live cell microscopy. Differential uptake of fluorescent dyes by nsEP-exposed cells remains the most straightforward indication of nanopore formation, and enables fast distinction of nanopores from larger, "conventional" electropores. As formulated above, the lack of propidium uptake by nsEP-exposed cells, along with a profound increase of the cell electrical conductance, indicates that the pores formed are just too small for the passage of the propidium cation.

Recently, we proposed a method of fluorescent detection of nanopores by pre-loading cells with a $Tl^+$-sensitive fluorophore (FluxOR™ Thallium Detection Kit, Invitrogen, Eugene, OR) [38]. Since the atomic diameter of $Tl^+$ is 0.34 nm (not considering any hydration layers), this ion can penetrate through nanopores that remain completely or mostly impermeable to propidium. Therefore, an nsEP exposure that leads to nanopore formation causes a distinct surge in $Tl^+$-dependent fluorescence due to $Tl^+$ entry through nanopores, but it is not accompanied by any measurable propidium uptake (Fig. 1, 70-100 s). A more intense exposure, e.g., applying multiple pulses of the same intensity, eventually results in the opening of larger pores and propidium entry into the cell (Fig. 1, 425 s).

## FUNCTIONAL PROPERTIES OF NANOELECTROPORES

1. Inward rectification. Perhaps the most prominent feature of nanopores is inward rectification of the transmembrane current. Although the extent of rectification varies, it can be seen in most of nsEP-treated cells, in different cell lineages and different pipette/bath solutions (Fig. 2). Note that nsEP had little effects on the outward current, but drastically increased the inward current. In this cell, it took about a minute for the pores to reseal, and whole-cell conductance returned to the pre-exposure level.

Inward rectification takes place even in symmetrical solutions (when cells are loaded with the extracellular buffer, thereby making ionic concentrations inside and outside the cell essentially identical). It appears reasonable to conclude that inward rectification is an intrinsic and universal property of nsEP-opened nanopores, and points to their functional and structural asymmetry.

2. Increased electrical "noise". At high negative voltages, original current traces show profound and random fluctuations of the transmembrane current, as compared to the pre-exposure level (Fig. 3). This "noise" could reasonably be attributed to the opening and closing of nanopores, as well as fluctuations of their diameter. Contrary to classic ion channels, nanopores show no discrete open states, but smooth transitions between the electrically-silent state and

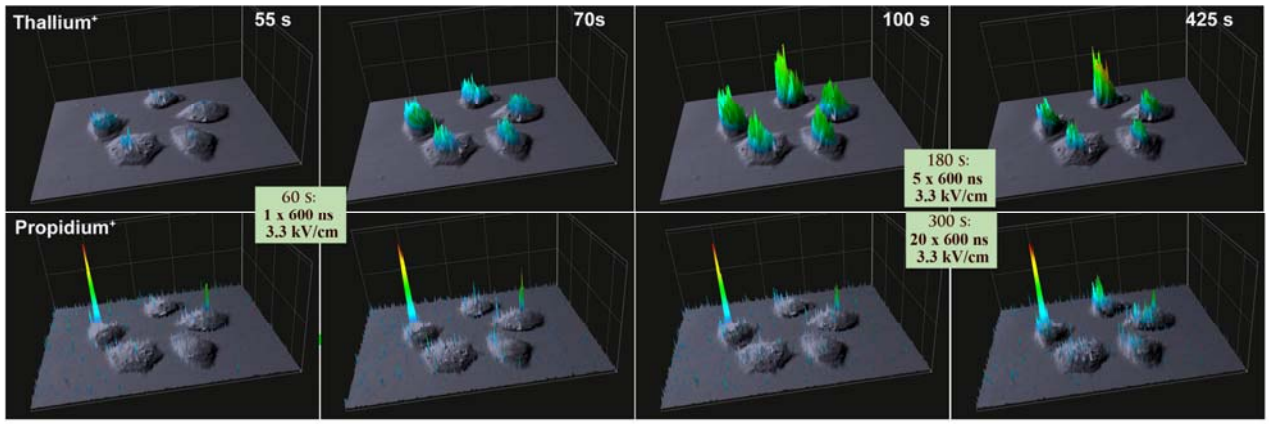


**Fig. 1. Thallium$^+$ and propidium$^+$ uptake by a group of CHO cells exposed to a single 600-ns pulse followed by multiple pulses at 3.3 kV/cm.**

CHO cells were loaded with a $Tl^+$- sensitive dye (FluxOR) and incubated in a buffer containing 16 mM $Tl^+$ and 30 μg/ml of propidium$^+$. Images were taken every 5 sec throughout the experiment; shown are representative images before nsEP exposures (55 s), after a single pulse (70 and 100 s), and after application of multiple pulses (425 s). Localization of the fluorescence signal and its intensity (arbitrary units) are coded by both the height and the color of "peaks" along Z-axis. Background fluorescence has not been subtracted; several sharp peaks of propidium$^+$ signal that are seen even before nsEP are caused by dye sticking to debris outside the cells, and are not related to cell permeabilization. Note that a single nsEP caused intense uptake of $Tl^+$, but not of propidium$^+$; in this experiment, it took 26 pulses to trigger a detectable propidium$^+$ uptake. Also note that the sensitivity of the propidium channel is set so high that isolated "sparks" of fluorescence can be seen even in the bath buffer.

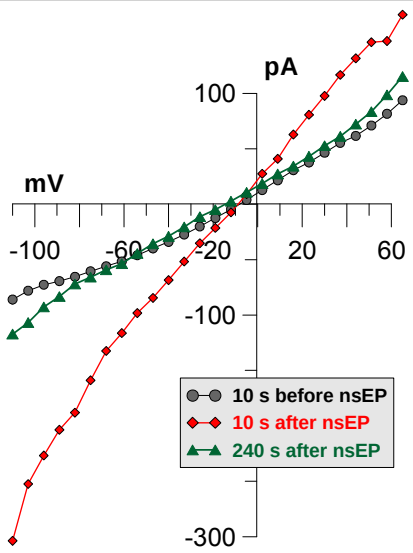


**Fig. 2. Typical effect of nsEP on whole-cell current-voltage (I-V) characteristic in a GH3 cell.**
The I-V curves were recorded by applying a step voltage protocol 10 s before nsEP treatment (one 600-ns pulse at 2.4 kV/cm), and 10 and 240 s after it. The pipette buffer contained (in mM): CsCl 140, Cs-EGTA5, $MgCl_2$ 4, HEPES 10; pH 7.4. The bath buffer contained: CsCl 25, Cs-EGTA 5, $MgCl_2$ 4, sucrose 182, HEPES 10; pH 7.4. Note profound increase of the inward current (at negative voltages) after nsEP exposure and smaller effect at positive voltages ("inward rectification"). Note also partial recovery by 240 s after the treatment.

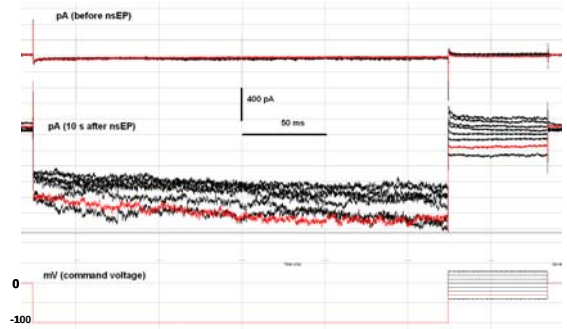


**Fig. 3. Changes in the whole-cell current caused by 600-ns pulse at 2.4 kV/cm.**
Shown are original current traces recorded 10 s before nsEP (top) and 10 s after it (middle) in response to the same voltage-step protocol (bottom). Currents evoked before the nsEP are so small that the traces overlap and appear as a single trace. The bath and pipette buffers were the same as in Fig. 2. Note the following effects of nsEP: (1) profound increase of the inward current, (2) increase of current fluctuation ("noise"), in particular during the -100 mV voltage step, and (3) gradual increase of current while the voltage is kept constant at -100 mV

various conductive states. The peak conductance and mean open time of nanopores can be estimated at about 100 pS and 1-2 ms, respectively (data not shown). Such brief open times appear inconsistent with the minutes-long increase in the whole-cell conductance after nsEP stimulation; the most likely way to explain it would be that nanopores do not close completely, but become too narrow for ion passage and turn electrically silent ("quasi-open" state). A similar quasi-open state of electropores has recently been demonstrated in planar lipid bilayers [42].

3. Current sensitivity. Another unique behavior of nanopores is their ability to gradually increase the electrical conductance at a constant voltage (Fig. 3). Without nsEP stimulation, the current induced by a hyperpolarization step is constant throughout the duration of the step. However, in nsEP-treated cells, current induced by the same step gradually increased. Apparently, the current going through nanopores further increased their conductance (positive feedback effect). This behavior resembles current trough hyperpolarization-activated channels; however, these channels are efficiently blocked by $Cs^+$ ions, whereas nanopores are highly permeable to $Cs^+$.

4. Ion selectivity. Aside from the lack of propidium uptake, the diameter of nanopores can be estimated from their permeability to ions of different sizes. As measured from the reversal potential of membrane currents under bionic conditions [43], nanopores were permeable even to relatively large (0.3-0.6 nm) ion species such as gluconate, isetheinate, tetramethylammonium, tetraethylammonium, and N-methyl-D-glucamine. Notably, nanopores were preferentially permeable to cations, suggesting that their entrance and/or inner surface are negatively charged. For example, nanopores' permeability to $Cs^+$ was 1.8 ± 0.1 times higher than to $Cl^-$ (n=15, mean ± s.e.m.), although the water mobilities of these ions are equal.

5. Pore evolution. Depending on the severity of nsEP damage, composition of the buffers, and functional state of the treated cells, nsEP-induced membrane conductance gradually recovers to its pre-exposure-level (Fig. 2), or it increases abruptly and irreversibly [38]. Restoration of the membrane conductance reflects gradual resealing of nanopores, which takes as long as 10-15 min at room temperature [35, 38]. On the contrary, the abrupt increase of the whole-cell current is best explained by a breakdown of nanopores into larger, non-rectifying "regular" aqueous pores; it is accompanied by immediate propidium uptake by the cell and the loss of inward rectification.

## SUMMARY

Our experiments established (a) the presence of long-lasting nanopores in plasma membrane of living cells, (b) the possibility to create such pores at a certain time point and location on the membrane by

nsEP stimuli, (c) that such nanopores are functionally asymmetric, and (d) they can exert complex behaviors that are normally expected only from protein ion channels with sophisticated, specialized organization. While the actual structure of nanopores remains in question, their unique functional properties can potentially be explained by asymmetrical, funnel-like shape, similar to synthetic nanopores fabricated in polymer foils [44]. Although not discussed above, it is worth mentioning that long-lasting nanopores have profound impact on the membrane potential, water balance, cell size and shape, functioning of the classic ion channels, and on multiple biochemical processes.

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**NOTES**

# Review of experimental and clinical results with non-thermal irreversible electroporation.

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## INTRODUCTION

Cell ablation with pulsed electrical fields (PEF) has been used in the food industry for breaking the cell membrane and for microorganism sterilization for almost half a century, [1,2]. Recently, it has been proposed that irreversible electroporation (IRE), the name now used to describe a specific subset of the pulsed electrical field domain of parameters, could become a minimally invasive surgery technology with unique properties when delivered to tissue *in vivo* in such a way as to avoid any other possible modes of electrical field induced damage to biological components [3]. This way of delivering IRE, which we refer to as non-thermal IRE (NTIRE) requires detailed treatment planning and when successfully delivered produces some unique results in the field of minimally invasive tissue ablation. The electrical fields required for delivering irreversible electroporation to the targeted cell membranes, in a volume of tissue, without affecting any other biological compounds, occur in a relatively narrow range of values. These must be determined from experiment studies and mathematical modelling which require the simultaneous solution of the electrical field and the bio-heat equations [4,5]. While the mathematics and treatment design are not trivial, when non-thermal irreversible electroporation is successfully delivered to a volume of tissue in such a way as to avoid damage to all the other biological molecules in the treated domain, the outcome is unique. In other minimally invasive tissue ablation techniques such as injection of alcohol, radiation therapy, radiofrequency tissue ablation, high intensity focused ultrasound, laser ablation, microwave ablation, cryosurgery the mode of tissue ablation is through non-discriminated denaturation of all biological molecules in the treated volume. Electroporation, reversible and irreversible as well as nanopulses belong to a family of new ablation modalities, which include also photodynamic therapy that can destroy the functionality of cells in a treated volume without affecting molecules that are not targeted. This means that these new modalities of tissue ablation, which can be referred to as minimally invasive molecular surgery, can be used in a different way from all other conventional ablation modalities and probably represent the future of minimally invasive surgery.

Quite a number of publications have appeared on the mathematical models for treatment planning. Experimental data from animal and clinical studies are at this stage relatively scarce. However, they validate the fundamental hypothesis that the special mode of delivering NTIRE discussed in this introduction produces unique results. The goal of this review will therefore be to bring a survey of experimental results and emphasize the unique attributes of the IRE technique.

## LIVER

In developing minimally invasive ablation techniques the liver is often studied first. Early results from the first in vivo IRE experiments in the rat liver have already demonstrated the unique aspects of IRE [6].

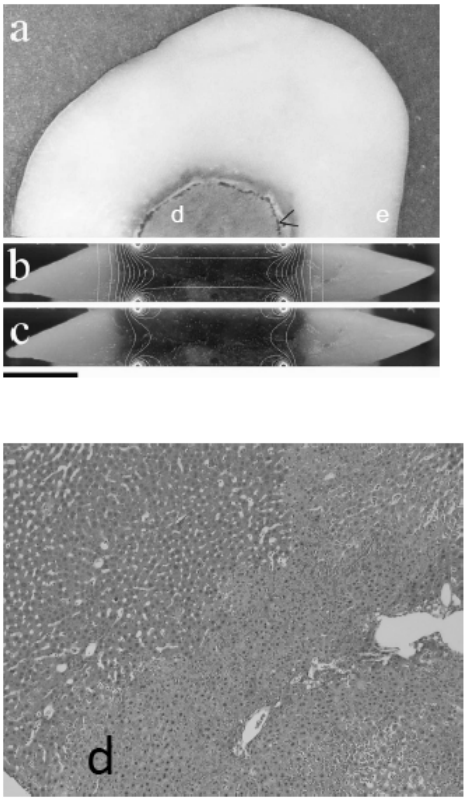


***Figure 1:*** Macroscopic (a,b,c) and microscopic images (d) of non thermal IRE treated liver. Note in (d) the patent blood vessel – middle right – within the IRE treated area.

Figure 1 shows a liver treated by clamping a lobe with two circular electrodes and delivering the IRE

pulses from these electrodes. The liver was flushed prior to embedding and H&E histology. The most interesting aspect is in panel d). It shows that while the sinusoids in the treated area of the liver are impacted with necrotic red blood cells, a major blood vessel has remained intact and is clear - meaning that it was successfully flushed. This ability of NTIRE to ablate cells but retain the mechanical integrity of blood vessels scaffold is unique and we will discuss its value.

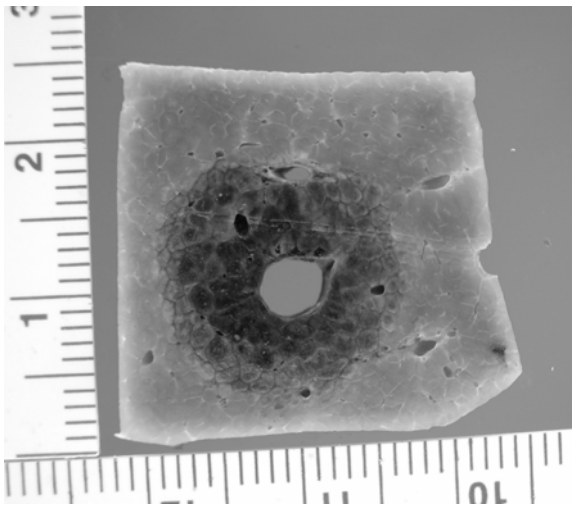

***Figure 2:*** NTIRE ablation of tissue around a 4 mm vein in the liver.

Large, long term, animal studies in the pig liver show another interesting outcome of the non-thermal IRE treatment, Fig 2. Because the blood vessels remain intact after non-thermal IRE it is possible to treat tumours in areas of the body that were not accessible to treatment before – such as in the vicinity of large ducts of the body. Furthermore, the fact that the large blood vessel structures remain intact leads to rapid access by the immune system to all the treated areas and an incredible fast disposal of the damaged tissue (two weeks with NTIRE versus six months with cryosurgery or radiotherapy)

## PROSTATE

Figure 3 illustrates our first results with the prostate [7]

The prostrate is an organ in which the collateral damage from treatment of the cancer is often as detrimental to the quality of life as the cancer itself. (A similar situation occurs with the pancreas or brain). This is why they are often left untreated in many parts of the world. Because the nerves in the prostate are covered by a sheath of myelin and because the ducts (urethra, rectum) and blood vessels remain intact when treated with NTIRE, it is possible to treat the cancer with IRE without any collateral damage.

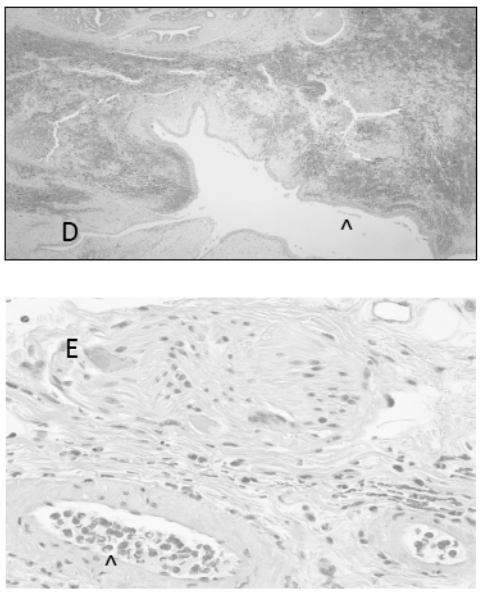


***Figure 3:*** Images from NTIRE treated dog prostate.

The figure shows in the top panel an intact urethra surrounded by NTIRE ablated tissue. The bottom panel shows an intact blood vessel and an intact nerve surrounded by treated tissue.

## BLOOD VESSELS

Another unique application made possible by the properties of NTIRE is the use on blood vessels, for restenosis for instance [8][9].

Figure 4 is a good illustration of the effects of NTIRE on blood vessels. The treatment ablates all the cells in the treated volume. However, the mechanical integrity of the blood vessel is retained and the extracellular scaffold remains. This makes it possible for the embryonic stem cells in the blood stream to attach to the extracellular matrix and regenerate an endothelial cover of the blood vessel. This inhibits restenosis.

## CLINICAL RESULTS

At this stage, over 20 sites are at various stages in the use of NTIRE for clinical treatment. I will bring here results from two that are at the most advanced stage.

Dr. Gary Onik has used NTIRE with 16 patients. The results will be presented at the meeting. The potency of the patients was not affected by the procedure. This is illustrated in Figure 5.

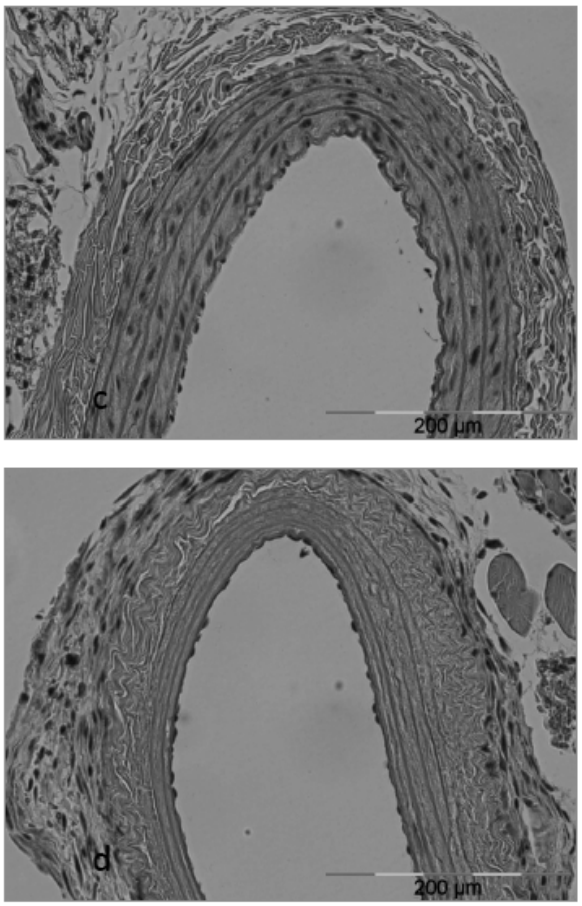


***Figure 4:*** Carotid artery treated by NTIRE. Top panel control. Bottom panel - one week after NTIRE. Notice the absence of smooth muscles and the regrowth of the endothelial cells.

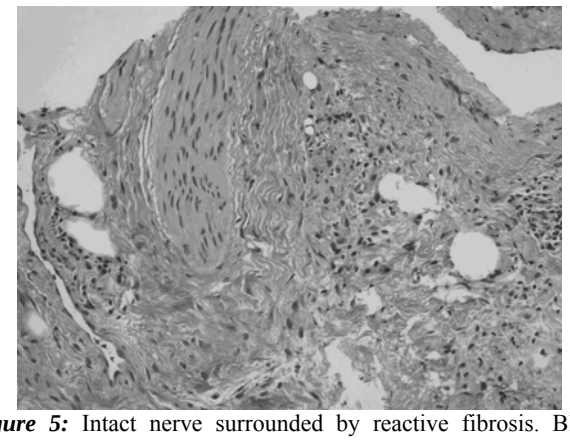

***Figure 5:*** Intact nerve surrounded by reactive fibrosis. Blood vessels are also intact. (Courtesy of Dr. Onik)

Dr. Kenneth Thomson has performed NTIRE in over 30 patients with cancers in the liver, kidney, lung and the lymph nodes. His results are consistent with the unique properties of NTIRE. He was able to treat tumors near large blood vessels (Figure 6)

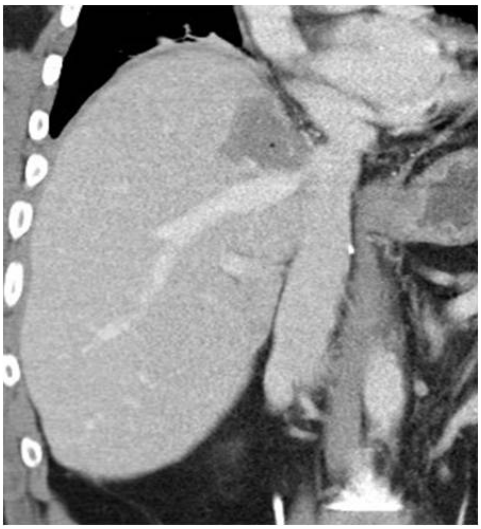

***Figure 6:*** Colorectal carcinoma near right atrium, diaphragm and hepatic vein IVC confluence. Successful procedure without damage to these structures and no post procedure pain. (Courtesy of Dr. Thomson)

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**NOTES**

# Safety issues in electrochemotherapy

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**Abstract:** The safety profile of any treatment is always important in terms of decision in favour of treatment, and it is also crucial to be able to inform patients adequately about potential side effects of a proposed treatment. The present paper evaluates the safety profile of electrochemotherapy. For electrochemotherapy several issues are important; 1) the application of anesthesia (local or general), 2) the administration of a cytotoxic drug (locally or systemically), 3) the application of electric pulses, 4) normal tissue damage in the treatment area, 5) tumor tissue response (necrosis) and risk of subsequent infection, 6) time to healing, and subsequent scarring, 7) possible damage to important structures such as nerves or vessels, and finally 8) possibilities for treatment concurrent with or after other treatments e.g. systemic chemotherapy and radiotherapy. Electrochemotherapy is now routinely in use for the treatment of tumors up to 3 cm. Furthermore a number of case reports also describe the treatment of larger tumors. Clinical trials systematically investigating treatment of larger tumors are underway, and in the near future treatment of tumors in internal organs is envisaged. This review will systematically evaluate the evidence from clinical practice concerning these different aspects, as well as speculate what future use of technology may touch upon.

## THE PATIENT REQUESTING TREATMENT

Electrochemotherapy (ECT) is generally used in the palliative setting. ECT has been shown to work for all cancer histologies. Presently ECT is being used for cutaneous and subcutaneous nodules, but treatment of metastases in internal organs is also envisaged in the not so distant future.

What is important to consider is, that when treating a patient in the palliative setting (i.e. a patient who has cancer at a stage where treatment will not cure the patient, but prolong life, relieve symptoms or both), there has to be;

a) a need for palliation. For example an ulcerated or bleeding metastasis.
b) the patients estimated life expectancy should be long enough for the patient to gain a benefit of the treatment, and the patient should feel that treating the area of metastases in question would indeed be important.

Often a patient will have several metastases, and it is a good idea to consult the patient, about which of these metastases are causing the main problems, so that treatment is directed at the problem.

Patients most often suffering from skin metastases are those with disseminated breast cancer or melanoma, although skin metastases can be seen occasionally in virtually all cancer types. A number of patients live for many years with disseminated disease, and sequential treatment with multiple modalities may help the patient optimise quality of life as well as prolong life. Electrochemotherapy is one of many modalities.

In the following, 8 important issues in relation to ECT will be evaluated.

## ANESTHESIA

ECT must be performed under anesthesia – local or geneal depending on the extent of the lesion(s) [1].

The pain associated with the procedure is associated to insertion of needle electrodes, as well as application of the electric pulse. Perceived pain is associated with the actual applied voltage. Thus, increasing electrode distance will lead to a higher applied voltage to keep the desired voltage to electrode distance ratio, and thus choosing an electrode with a small gap (e.g. 4 mm) may be part of the anesthetic strategy.

Local anesthesia can be performed when few or smaller metastases are to be treated, and when there is no periostal invasion into underlying bone. The few potential complications to local anesthesia include allergy (the question of which is addressed before the procedure), and intravascular injection (which is prevented by aspiration to control injection site).

General anesthesia is used when there are more or larger nodules. The time in general anesthesia is brief (app. 40 min.), so short acting anesthetics can be used. Although general anesthesia is considered a very safe procedure in modern day, the patient must be informed of risks before the procedure. It is important that the patient is stable after induction of anesthesia, with inspired oxygen of 30 % or less (in order to avoid lung complications caused by bleomycin).

## DRUG ADMINISTRATION

Two drugs have hitherto been used in electrochemotherapy, namely cisplatin and bleomycin. Cisplatin can be used for intratumoral administration

only, since the therapeutic window is smaller than for bleomycin (3-8 fold versus 300-700 fold).

For intratumoral use, the issues of drug distribution and normal tissue damage need to be addressed. Drug distribution will always be more varied with direct injection than with intravenous injection. This may lead to overdosing in parts of the injected area, which may again lead to a less predictable outcome for the normal tissue. Some studies have used 5 U/ml (5000 IU/ml) in the injection syringe, whilst we recommend 1 U/ml (1000 IU/ml), in order to reduce normal tissue necrosis. In a comparative study, using 1000 IU/ml was equally efficient to using intravenous administration [2], and thus this dosing seems relevant in terms of tumour response, yet in our experience the extent of necrosis of normal tissue is considerably smaller using the 1000 IU/ml dose than the 5000 IU/ml dose [3].

In the case of cisplatin, one dose has been used and recommended, for local injection only.

For the use of intravenous bleomycin, particular attention should be paid to the cumulative dose, and avoidance of overhydration and uncritical use of oxygen, as this may increase risk of lung toxicity. We are currently conducting a study on large breast cancer recurrences, where DLCO (lung diffusion capacity measured by carbon monoxide diffusion) will be carried out in between treatments, where up to 4 treatments are allowed. This may bring light to the actual effects of repeated intravenous bleomycin use under general anesthesia.

## APPLICATION OF ELECTRIC PULSES

Application of electric pulses will affect nearby nerve and muscle tissue. In a comparative study, surface anesthesia by topical application of anesthetic cream (lidocaine/prilocaine) was insufficient pain control for the pulsing procedure [2], leading to the conclusion that there is direct excitation of local nerve firing.

Although treatment of chest wall recurrence of breast cancer is a frequent indication, we have not encountered case reports with arrhythmias, with the current pulsing conditions used for ECT.

Muscle contractions may be unpleasant for the patient, and can be relieved by lifting the lesions being treated from the underlying muscle when possible. Advising the patient of the muscle contraction in underlying muscle groups is very important before the start of treatment.

When using general anesthesia, muscle contractions around the head/neck/shoulder area may prompt securing the airway passage so that the tube/mask does not move as a result of contraction. Patients may experience post treatment muscle soreness, in particular when a large number of pulses have been given in a specific area.

## NORMAL TISSUE DAMAGE IN THE TREATMENT AREA

Ironically it seems that iv injection actually may spare normal tissue better than intratumoral or local injection. Thus, one would expect that with intratumoral injection, normal tissue around could be spared. However, the margins need to be treated, and it seems from clinical experience that there is a therapeutic window allowing for preservation of normal tissue and necrosis of tumor tissue, at least when you are treating tumors in the skin [2;3].

Use of plate electrodes on skin may leave burn marks, as the insulating properties of the skin will cause large voltage drops (and thus fields) over the skin.

## TUMOR TISSUE NECROSIS

For treatment of small tumors, tumor necrosis is rarely a problem, because the tumor will often mummify and the crust will fall off when the tissue underneath has healed in.

However, in the treatment of large tumors, such as breast cancer recurrence, large areas may become necrotic and this may lead to opportunistic infections. Prevention by surgical debridement of necrotic areas, state of the art antibacterial wound dressings as well as monitoring infection parameters in the patient and applying antibiotic treatment when necessary, may be warranted.

## TIME TO HEALING – SUBSEQUENT SCARRING

For tumors up to 3 cm, a healing time of 6-10 weeks may be expected, with treatment in previously irradiated areas being the slowest to heal due to impaired fibroblast response.

For large tumors, month long healing times may be expected, in particular in cases where curatively intended radiotherapy has been performed previously [4].

It is important to inform the patient that although the ECT treatment procedure is quickly done, the healing time takes patience.

It is a general truth, that if cancer destroys normal tissue, and the cancer is treated – then there will be scarring rather than return to the situation when there was intact tissue. What has been destroyed by the cancerous growth can not readily be restored. Patients must be informed that even if the tumor is successfully treated, there may be scarring at the treatment site.

## POSSIBLE DAMAGE TO NERVES AND VESSELS AT TREATMENT SITE

So far the major body of evidence on treatment stems from ECT of small nodules in the skin. The development of the technology will eventually lead to a much wider use, and in this context, risk of damage to larger nerves and vessels become important.

Theoretically, walls of large vessels are acellular, and should therefore withstand treatment very well. Indeed, experimental evidence from intraluminal electropration show that there is no visible damage to the vessel walls, only to the intimal layer.

The cruel exception is when a tumor has invaded the vessel wall, so that successful eradication of the tumor will lead to profuse bleeding because the part of the vessel wall which was eroded by the tumor is no longer obliterated by the tumor.

So far no evidence regarding ECT in areas with large nerves (such as the femoral nerve, the axillary nerve bundle) has been presented. There is sporadic case reports about patients being treated on the chest wall, who experience neurogenic pain post-treatment. This may indeed be due to electroporation damage of nerves. From electrical injury studies, we know that nerve damage is a concern.

## RELATION TO OTHER TREATMENT MODALITIES

It has been shown that ECT can safely be administered in previously irradiated areas. This is actually an important indication, since radiation resistant ulcerated tumors are a particularly difficult problem. In previously irradiated skin, healing will be slower that in normal skin.

Bleomycin is used in combination with other chemotherapeutic drugs as the standard treatment of testicular cancer and lymphoma. Although the indication for ECT depends on other chemotherapeutic regimens to have failed, there are a number of cases where there is stable disease in some organs (e.g. bone), warranting continuation of systemic therapy, whilst there is progression in skin (warranting ECT). We believe that administering bleomycin with other drugs will in most cases be safe, since bleomycin does not exhibit myolotoxic properties in the same way as certain other chemotherapeutic agents. However, pending accumulation of evidence, it will be the decision of the physician what may be acceptable to combine with.

## CONCLUSION

ECT is generally a safe and very tolerable treatment. Patients must always be informed of potential risks and likely side effects, and this review evaluates the various parameters to be taken into account.

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**NOTES**

# Electroporation in Food Sanitization

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**Abstract:** Microbial growth as well as spore formation are the most frequent causes of food spoilage. Thus, the agro-food industry developed during the years preservation methods, mainly based on the use of heat, to inhibit the growth or inactivate microorganisms. However, even if thermal treatments are recognized and approved by regulatory authorities as reliable food processing technologies responding to all issues related to consumer safety and health insurance, the detrimental effect of pasteurization and sterilization on the quality of foods due to thermal damage is still matter of concern. In the last decades there was an increasing demand of consumers for fresh and minimally processed food products. This promoted the development of alternative novel technologies for food preservation based on the use of stresses different from heat to achieve microbial death. Among them, high hydrostatic pressure technology (HP), high pressure homogenization (HPH) and pulsed electric field technology (PEF) gained the attention of many researchers from the academic as well as from the industrial world and a very high number of scientific publications on their application to inactivate microorganisms in model and real foods is available in the literature.
One of the more promising non-thermal technology is PEF, consisting in the application of a high intensity electric field (10-50 kV/cm) as a train of short duration pulses (of the order of microseconds) to a liquid food flowing between two electrodes in a treatment chamber.
In this lecture, the mechanism of microbial inactivation due to PEF is presented and the effect of PEF electrical variables, processing conditions, microbial strain and suspending medium physicochemical characteristics on microbial inactivation is discussed. Experimental data reported demonstrate that the key electrical parameters are the electric field strength and total energy input but also microorganism characteristics and environmental variables, such us food composition and food additives, play an important role in determining the response of microorganisms to external stresses. Moreover, the simultaneous application of different stresses, i.e. the superimposition of several factors to reduce the microbial resistance and induce microbial inactivation, allows to reduce the intensity of the electric field applied, confirming that an hurdle approach in food preservation process design is sound and can be used to promote and extend the industrial utilization of PEF processing technology.

## INTRODUCTION

Microbial spoilage is one of the most important causes of food deterioration. Thus, in order to ensure food safety and increase the availability of products with an extended shelf life, food preservation methods have been set up through the years. The reduction of microbial contamination, in particular from pathogens, is traditionally obtained by means of thermal treatments. However, heat causes undesirable side-effects on the sensory, nutritional and functional properties of foodstuff, in contrast with the increasing consumer demand of fresh-like processed products. The latter requirement represented in the recent years the driving force for the development of non-thermal methods for microbial inactivation, either for improving food quality and safety or for the design of "new to the market" products. Non-thermal technologies encompass all preservation treatments that are effective at ambient or at sub-lethal temperatures, including antimicrobial additives, pH adjustment and modified atmospheres packaging. The term "non-thermal processing" identifies all those novel technologies, such as high hydrostatic pressure, pulsed electric field (PEF), high pressure homogenization, which are intended for application as microbial inactivating processes during food manufacture, and require the application of physical stresses different from heat. While HP process is already in use for some commercial application, only one PEF processed product is available on the market. This is due to some delay on the regulatory approval processes as well as to the lack of reliable mathematical models able to predict microbial inactivation at different processing conditions and accounting for the role played on inactivation by physical and chemical characteristics of foods. The PEF process consists in the application of a high intensity electric field (10-50 kV/cm) as a train of short duration pulses (of the order of microseconds) to a liquid food flowing between two electrodes in a treatment chamber.

The use of electric current for food preservation has been described since the beginning of the last century. A process called “Electropure” was patented as a method to pasteurize milk using an alternating electrical current, in the voltage range from 220 to 4200 V, discharged through the food placed between two carbon electrodes in a treatment chamber [1]. Indeed, in this case microbial inactivation has to be substantially attributed to the temperature increase of the product due to Joule effect, thus the “Electropure process“ can be regarded as a thermal treatment.

In the 1960s, Doevenspeck [2] and Sale and Hamilton [3,4] demonstrated the existence of a “non-thermal bactericidal effect” of electric current when it is applied as high voltage electric field pulses of very short duration (of the order of microseconds). In this case, the most commonly accepted theory assess that microbial inactivation by PEF is due to electroporation of cell membrane. Pulsed electric field treatments induce a permanent loss of the permeability of the microbial membrane causing cells death.

The effectiveness of PEF to inactivate yeasts and many bacteria in liquid foods strongly depends on process parameters, such as electric field strength and specific energy input, as well as microbial characteristics (microbial strain and growth phase) and chemical and physical properties of foods (pH, electrical conductivity, aw, etc.) [5-9].

In the last decade the application of PEF technology was successfully extended from inoculated buffer systems to real foods, such as fruit juice, milk, egg yolk, green pea soup [9] even if more detailed studies on the shelf-life of products are necessary to confirm the capability of PEF treatment to provide stable foods with high quality.

However, due to the large number of parameters affecting microbial inactivation by PEF, as well as to the wide range of experimental conditions investigated and type of equipment adopted the comparison of the results of various research groups and the formulation of a generally accepted model able to predict microbial inactivation in different process conditions and for different food product properties was not possible yet. Furthermore, only few papers are focused on the investigation of the crucial role that flow conditions play on the effectiveness and energy efficiency of PEF process [11]. Finally, since high operating costs represent one of the main drawbacks for the extended use of PEF in industrial applications, efforts should be also addressed to increase the energy efficiency of this technology.

## MECHANISM OF MICROORGANISMS INACTIVATION BY PEF

It is widely recognized that the observed reduction in microbial viability during PEF treatment is due to the permeabilisation of the cell membrane, due to electroporation and/or electrofusion, causing cell death. Cell membrane permeabilization is a reversible process if the electric field applied doesn’t exceed a threshold value of the electric field strength. As the electric field strength increases the electroporation is irreversible and the loss of cells viability occurs. The electroporation effect is at the basis of PEF applications in medicine for the controlled injection of substances, drugs, proteins or DNA in cells without detrimental effects on their viability. Genetic engineering applications of PEF, instead, utilize the electrofusion effect to increase the selectivity and control of fusion processes [12].

Zimmerman et al. [13], proposed a theory to explain the electropermeabilization of microbial cells and their inactivation caused by PEF treatments. Cell membrane is considered as a capacitor filled with a dielectric material of very low dielectric constant compared to the inside of the cell and the external environment. Due to the difference in dielectric constant, free charges accumulate on the two surfaces of the cell determining a transmembrane potential of the order of 10 V. Keeping the transmembrane potential is crucial to guarantee vital energy-linked process such as maintaining cell turgor and intracellular pH, allowing the entrance of substrates into the cell against concentration gradient and so on. When an external field is applied, transmembrane potential increases due to the additional free charges accumulating on the surfaces of the membrane. These charges of opposite sign attract each other causing the compression of the cell membrane and, consequently, the reduction of membrane thickness. Viscoelastic properties of the cell membrane counteract the compression forces, but as the the transmembrane potential reaches approximately 1 V, the electrocompressive forces exceed the viscoelastic properties determining the membrane breakdown. The electric field strength determining the electrical breakdown of the cell membrane is called threshold or critical electric field value. The number of pores and their size depend on electric field strength applied and treatment time. At electric field strength well above the critical value the permeabilization process is irreversible and the mechanical disruption of the cell occurs.

## FACTORS DETERMINING MICROBIAL INACTIVATION BY PEF

The effectiveness of PEF in determining microbial inactivation depends on many factors such as electrical parameters, as electric field strength, processing time and total specific energy input, processing parameters, as fluid flow rate, microorganism characteristics, as microbial strain and growth phase and suspending medium characteristics, as concentration of solutes, pH, electrical conductivity [5–8].

### Effect of electrical parameters

In PEF treatment the main electrical variables influencing microbial inactivation rate and effectiveness are the electric field strength E, and the cumulative treatment time t. The latter is calculated by multiplying the number of pulses discharged on the sample, n, times the pulse duration, τ. Experimental data on microbial inactivation are reported as microorganism survival fraction as a function of electric field strength or of cumulative treatment time. However, also the total specific energy input $W_T$ has been suggested as a suitable control parameter instead of treatment time, especially when exponentially decay pulses are applied [14]. Indeed, since $W_T$ is a function of the electric field strength, treatment time and electrical resistance of the treatment chamber, it also gives information on the process intensity. The microbial inactivation level in PEF processing increases with increasing the electric field strength as well as the total energy input and, thus, the treatment time. Data reported in Figures 1 show these findings for a strain of *Saccharomices Cerevisiae* yeast treated in a continuous flow PEF apparatus. Data available in the literature highlight that, whatever is the electric field intensity utilized, the microorganisms gradually become more pulse-resistant as the energy input increases, i.e. the inactivation rate during the pulse treatment decreases. This behaviour was observed for different microbial strains in batch as well as in continuous systems [3, 8, 11, 15, 16, 17]. The decrease of the inactivation rate with increasing the energy input could be attributed to the occurrence of different phenomena such as the natural distribution of resistance to the electric field strength within the microbial population [16], the uneven distribution of the electric field in the PEF treatment chamber [15], the non uniform distribution of the residence time of the product in the treatment chamber [18].

No effect of pulse width on microbial inactivation has been observed by several authors. On the contrary, the pulse shape and pulse polarity seems to affect the microbicidal action of PEF treatments. It has been reported that square pulses are more effective than exponential decay pulses and bipolar pulses are more efficient in determining cell membrane breakdown with respect to monopolar pulses [19]. The application of a train of short duration pulses (of the order of microseconds) characterizes PEF treatments.

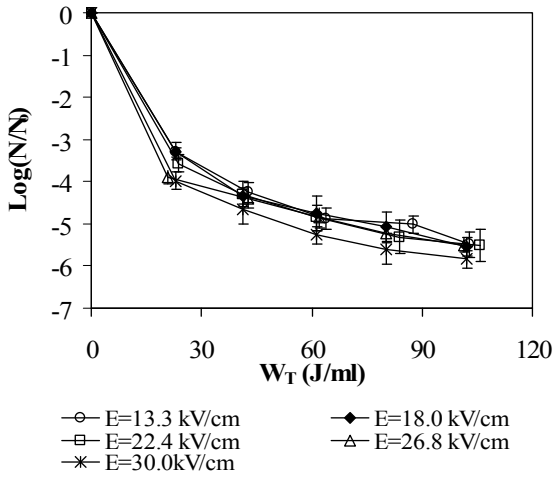


***Figure 1:*** Survival curves of *Saccharomices Cerevisiae* yeast vs. the total specific energy input, $W_T$ for different electric field strength. Flow rate = 2 l/h (G. Pataro and G. Ferrari, unpublished data).

The range of frequencies utilized to apply intermittently pulses is between 1 to 5000 Hz. It has been demonstrated that, provided that the treatment time is kept constant, the microbial inactivation level doesn't change with increasing the frequency. Even if the treatment time could be decreased with increasing the frequency, the main drawback of the frequency increasing is related to the undesired increase of the temperature of liquid samples due to Joule effect. In this case, microbial inactivation is not only due to PEF but also to a thermal treatment, with detrimental effects on the nutritional and sensory characteristics of foodstuff.

### Effect of process parameters

Only few authors investigated on the role that the flow conditions of liquid samples play on the effectiveness and energy efficiency of PEF process [11]. When a flow stream pass through a PEF chamber, not only the distribution of the electric field but also the residence time distribution of the product should be uniform to guarantee that each cells of the microbial population could receive the same lethal dose. It has been demonstrated that the degree of mixing of the liquid product in the treatment chamber strongly affect the effectiveness of the PEF process on microbial inactivation [15]. For this reason, turbulent flow condition through the chamber should be preferred. In Figure 2, the survival fractions of

suspensions of *Saccharomices Cerevisiae* yeast flowing in a continuous PEF treatment chamber at different flow rates, as a function of the total energy input, $W_T$, are reported. Experimental data show that with increasing the fluid flow rate, the microbial inactivation rate increases.

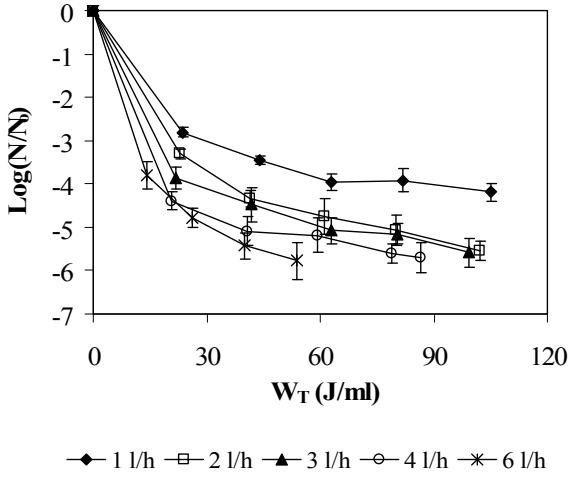


***Figure 2:*** Survival curves of *Saccharomices Cerevisiae* yeast vs. the total specific energy input, $W_T$ for different suspension flow rates. E = 18 kV/cm (G. Ferrari and G. Pataro, unpublished data).

To increase the lethality and energy efficiency of a PEF treatment using moderate field strength, as well as to reduce the ohmic heating of the product, the possibility of utilizing a multiple step process can be also investigated. In the multistep configuration of a PEF treatment the same microbial suspension flows several times through the treatment chamber. Thus, to gradually increase the total amount of the electric energy received without increasing the product temperature an intermediate cooling system should be located in the flow circuit. In Figure 3, the inactivation curves of *Saccharomices cerevisiae* yeast, at fixed field strength and suspension flow rate, as a function of the number of passes through the treatment chamber, $N_p$, and for different amount of total energy input per pass are reported.

Results show that the number of passes through the chamber is an important operating parameter strongly affecting microbial inactivation, whose effect is strictly related to the energy delivered in each pass, the lower the energy per pass, the higher the number of passes through the chamber necessary to achieve the same inactivation level.

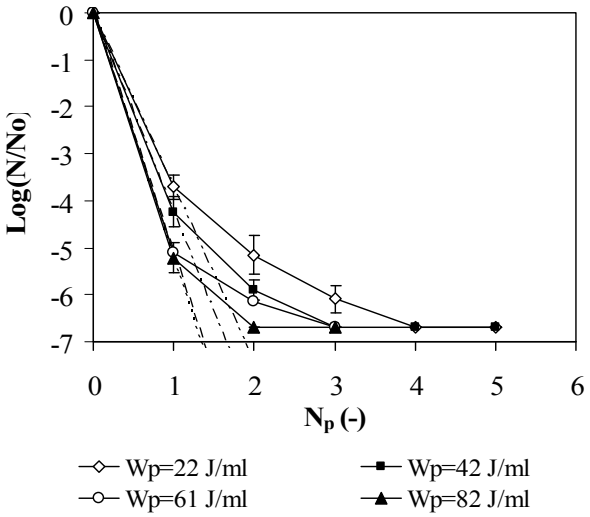


***Figure 3:*** Survival curves of *Saccharomices Cerevisiae* yeast vs. the number of passes trough the treatment chamber for different total specific energy input, $W_T$. E = 18 kV/cm; Flow rate = 2 l/h (G. Ferrari and G. Pataro, unpublished data).

**Effect of microorganism characteristics**

The effectiveness of PEF in determining microbial inactivation strongly depend on the type of microorganism, growth phase as well as cells concentration. Gram-negative bacteria are more sensitive to PEF than gram-positive bacteria [20], bacteria are more resistant than yeasts [3]. Data reported in Figure 4 confirm this hipothesis. Many authors found that bacterial spores and mold ascospores are resistant to PEF treatments [21, 22]. Thus, the application of PEF can be only envisaged to replace pasteurization and not sterilization of foods. Microorganisms in the exponential growth phase are more sensitive to PEF process than those in the stationary growth phase. The size and shape of microorganisms determine their sensitivity on PEF treatment. Cell size affect PEF resistance of microorganism trough its effect on transmembrane potential. With decreasing the cell size the transmembrane potential induced with increasing the electric field strength decreases, thus scarce or no effect on cell inactivation can be detected [13]. The general conclusions that should be made on the effects of microorganisms characteristics on PEF treatment effectiveness is that not only cell size and shape should be considered but also the structure and thickness of the cell membrane. The effect of cell concentration is still unclear. While some author reported that microbial inactivation during PEF treatment increases with decreasing the initial cell concentration, other authors showed that no influence of this parameter can be detected.

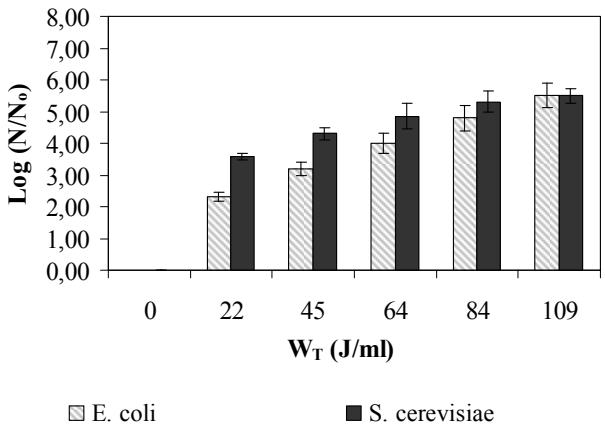


***Figure 4:*** Inactivation levels of *Saccharomices Cerevisiae* yeast and E. coli vs. total specific energy input. E = 18 kV/cm; Flow rate = 2 l/h (G. Ferrari and G. Pataro, unpublished data).

### Effects of product characteristics

The influence of the pH on the microbial inactivation by PEF treatment is still unclear. Results obtained by different authors showed that the sensitivity of *E. coli*, *S. cerevisiae*, *L. innocua*, *L. monocytogenes* to PEF treatment increases at acidic pH [23, 24]. Furthermore, the low pH of the treatment medium might prevent the repairing of cells sub-lethal injuries caused by electric field treatment. The influence of pH is also related to the type of microorganism under investigation. In fact, *S. enteretidis* and *S. senftemberg* seemed to be more resistant at acidic than at neutral pH [6, 25] while no significant effects of the pH of the treatment medium on PEF inactivation of *E. coli, B. subtilis* and *Y. enterocolitica* have been reported [3, 20, 26].

The influence of the water activity on PEF microbial inactivation has not been extensively investigated. Results obtained with *Enterobacter cloacae* [27], *E. coli* and *S. cerevisiae* [24], L. monocytogenes and *Y. enterocolitica* [26] are all in agreement and indicate that with decreasing the water activity of the suspending medium the microorganisms are inactivated by PEF treatment to a less extent. However, the protective effect of solute concentration against PEF strongly depend on the nature of the compound utilised to decrease the aw.

Different authors studied the influence of liquid medium electrical conductivity on microbial inactivation by PEF [5, 6, 7]. However, it is unclear if the conductivity affects the effectiveness of PEF treatment trough the influence exerted on the electric field strength and pulse width rather then that exerted by the electric field strength on the cell membrane. Most data published show that cells survival decreases with decreasing the conductivity of the suspension as a consequence of its influence on the membrane permeability. With decreasing the conductivity of the liquid medium the difference in ionic concentration between cytoplasm and the environment becomes larger, facilitating the increase of the flow of ionic substances across the cell membrane. This, in turn, weakens the membrane structure that become more sensitive to pulse application [5]. On the contrary, Alvarez et al. [7], demonstrated that inactivation rate increases with increasing the conductivity of the suspending medium.

## USE OF THE "HURDLE" TECHNOLOGIES APPROACH

In order to extend the application of non-thermal preservation methods in the food industry, the use of the "hurdle" technologies approach could be suggested. It implies the simultaneous utilization of several lethal hurdles (i.e. superimposition of the effects) with the purpose of reducing the level of the non-thermal stress applied (electric field strength , processing time) and, thus, to reduce the costs of the processing plant as well as operating costs. Different hurdles can be used in food processing design. The PEF treatment is applied in overlapping with mild heat treatment, acidification, antimicrobial or antioxidants compounds. This allows to reduce the level of the non-thermal stress applied to obtain shelf stable products and preserve their high nutritional and sensorial quality. Moreover the processed products can be stored in refrigerated condition and eventually packaged with active packaging materials. The synergistic effect of pulse processing of *E. coli* in presence of some bactericides, like ozone and $H_2O_2$, has been investigated [28]. Even though low concentration of bactericide compounds doesn't determine any cell death, the use of bactericides in combination with PEF gives rise to an increased level of microbial inactivation, due to cell membrane wounding. Because no residues of bactericides were detected in the sample after treatment, the utilization of combined PEF processing and bactericides in the food industry appears to be sound. However, due to the increasing consumer concerns on artificial additives, pulsed electric field treatment has been also used in combination with some natural antimicrobial such as nisin [29-31]. A synergistic effect on microbial inactivation was observed as the electric field strength, number of pulses and nisin concentration increased.

It is known that the effectiveness of pulse process increases with increasing the temperature of the liquid samples. However, the maximum temperature utilized should be well below the temperature causing the thermal death of the cells and, thus, food thermal damage. Several studies reported the synergistic effect

of moderate heating and PEF treatment on microbial inactivation rate [17, 28, 32]. The increase of PEF process effectiveness can be probably related to the phase transition of the phospholipid molecules, from gel to liquid-crystalline phase, which determines the reduction of the bi-layer thickness of the cell membrane. Thus, at a relatively high temperature, cell membrane breakdown occurs at low field strength [33]. Moreover, energy consumption during PEF treatment at moderate temperatures (35–65°C) could be reduced leading to a drastic reduction of the operating costs [32].

## CONCLUSIONS

The effect of the main processing variables and environmental factors on the effectiveness of PEF treatments of liquid foods is discussed in this lecture. This can allow a better understanding of potentialities and limits of the utilization of PEF technology for food sanitization with the aim of preserving nutritional and sensorial quality of products.

The electric field strength and treatment time are the main electrical parameters affecting microbial inactivation. Moreover, while bacteria and yeasts are sensitive to the PEF treatments, bacterial spores and mold ascospores are PEF resistant species. Thus, PEF can only be regarded as a suitable technology for food pasteurization. Also the physical and chemical characteristics of the liquid food play an important role in determining the effectiveness of PEF treatment.

It is also important to underline that the simultaneous application of different lethal agents, i.e. the “hurdle” technology approach” or, alternatively, the use of a multistep process is a possible way to achieve food sanitization with lower fixed and operating costs.

However, despite the numerous scientific papers available, more research efforts are needed to clarify the mechanisms of microbial inactivation by PEF and to better understand if microorganisms stress adaptation and sub-lethal injury can occur. Both aspects are of great relevance in view of a wide industrial utilization of PEF technology. Moreover, the set up of reliable mathematical models based on physically meaningful parameters represents an useful way for tuning the treatment conditions and for predicting the lethality of the process. The model should also take into account the effect of the properties of the environment on the effectiveness of the PEF treatment in order to envisage the level of the microbial inactivation that can be achieved by changing the chemical and physical properties of the suspending medium.

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## NOTES

# STUDENTS' ABSTRACTS

# Electropermeabilization of mixed lipid bilayers (PC:PS) in the presence of calcium

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## INTRODUCTION

Molecular dynamics (MD) simulations of systems containing calcium and phosphatidylcholine (PC) [1] and mixed phosphatidylcholine-phosphatidylserine (PC:PS) lipid bilayers [2], are consistent with experimental observations [3]. Extending that work, we have assembled PC and PC:PS systems with varying calcium ion concentrations to study the effect of $Ca^{2+}$ on electric field-induced pore formation and pore lifetime.

## METHODS

MD simulations were performed using GROMACS 3.3.3 as previously reported [4]. Each system contained 128 phospholipids and 8958 water molecules (70 waters per lipid). In PC:PS systems, 20 PC lipids were replaced in a single leaflet by PS and sodium counter-ions. Calcium and chloride ions were added in the bulk water region on both sides of the membrane. Each system was equilibrated for 200 ns before an electric field was applied. Pore formation times were measured by taking the time at which the phosphorus-atom group in one leaflet merged with the phosphorus-atom group in the opposing leaflet. A group is a set of atoms, each separated by a maximum distance of 1.2 nm. Pore lifetimes were measured from a different set of simulations in which pores were created with varying electric fields in the absence of calcium. When a pore formed, calcium was inserted into the system, the field was removed, and the system was monitored until two separate leaflets reformed.

## RESULTS AND CONCLUSIONS

Preliminary evidence suggests that the time to pore formation increases in systems containing calcium, and this effect is magnified in PC:PS systems (Table 1).

**Table 1**: **Mean Pore Formation Times (ns)**

| Field (MV/m) | PC | PC+Ca | PC:PS | PC:PS+Ca |
|---|---|---|---|---|
| 300 | >25 | >25 | >25 | >25 |
| 400 | 12.19 | >25 | >25 | >25 |
| 500 | 4.08 | 8.10 | 15.37 | 11.98 |
| 600 | 1.36 | 1.78 | 6.71 | 11.89 |

Calcium alone appears to have little effect on pore lifetime, but PC:PS systems have reduced pore lifetimes when compared to pure PC systems (Figure 1). We also report binding isotherms for calcium and PC:PS bilayers, a new metric for the validity of simulations of phospholipid bilayer systems containing calcium.

## FIGURES

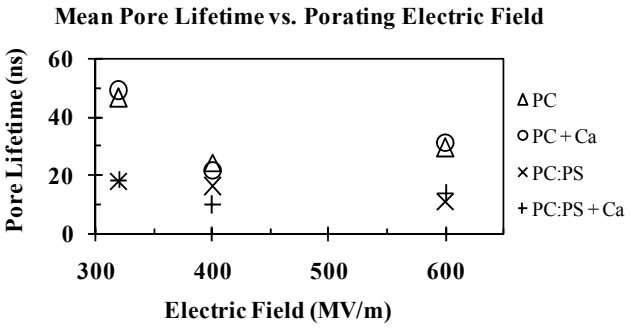


**Figure 1**: Pore lifetime in PC:PS systems are shorter than those in PC systems.

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# Interaction between like-charged membrane surfaces mediated by positively and negatively charged nanoparticles of blood plasma - Experiments

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## INTRODUCTION

Our recent results using functional density theory and MC simulations show that spatial and orientational ordering of charged spherical nanoparticles, containing two negative/positive charges separated by a nanoparticle diameter (dimeric internal charge distribution), may give rise to an attractive interaction between negatively charged membrane surfaces. In this way plasma-derived nanoparticles with dimeric internal charge distribution, such as β2-glycoprotein I (β2-GPI) and IgG antibodies may mediate the adhesion of daughter buds to the mother membrane surface thereby suppressing membrane microvesiculation (Fig. 1A). In this work the experiments were put forward in an *in vitro* model of giant phospholipid vesicles (GPVs) to assess the attractive interactions (adhesion) between negatively charged phospholipid membranes in the presence of isolated human plasma proteins or healthy donor plasma samples. The number of microvesicles (MVs), isolated from healthy donor plasma samples was correlated with the extent of adhesion between GPVs in the presence of plasma samples.

## MATERIALS and METHODS

Negatively charged GPVs containing cardiolipin or phosphatidylserine were prepared by the modified electroformation method and were observed by the phase-contrast microscope Zeiss Axiovert 200. The extent of GPV adhesion in the presence of human plasma-derived β2-GPI, human serum-derived IgG antibodies or healthy donor plasma samples was determined semi-quantitatively as an average effective angle of contact between adhering GPVs (Fig.1E) using Image J software. MVs were isolated from healthy donor peripheral blood by differential centrifugation and were analysed by Altra Flow Cytometer.

## RESULTS AND CONCLUSIONS

As shown in Fig.1B β2-GPI mediates, in a temperature-induced GPV budding, an adhesion of a daughter bud to the negatively charged mother GPV membrane thus suppressing its exovesiculation. β2-GPI (Fig.1C) and IgG antibodies (Fig.1D), plasma nanoparticles with dimeric internal charge distribution, induce in a dose-dependent manner the adhesion of negatively charged GPVs through juxtaposed membrane cross-linking (inserts in Figs.1C and 1D).

Negatively charged GPVs adhere in the presence of healthy donor plasma, which contains negatively and positively charged nanoparticles. The extent of GPV adhesion in the presence of plasma samples inversely correlates with the number of MVs per platelet in plasma samples (Fig.1F). Our results indicate that plasma-derived nanoparticles, e.g. β2-GPI and IgG antibodies, with dimeric internal charge distribution may induce the suppression of microvesiculation through mediating the attractive interactions between like-charged membranes.

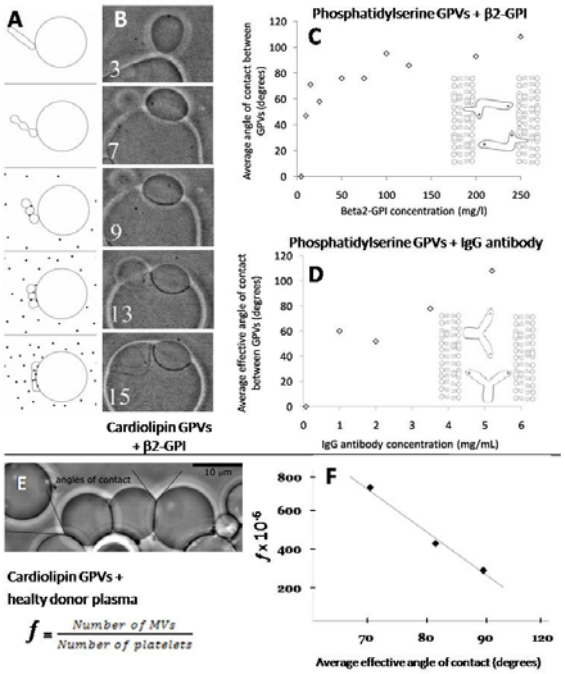


**Figure 1**. Adhesion of a bud to negatively charged mother membrane: model (A), β2-GPI (B). β2-GPI (C) or IgG antibody (D)-mediated adhesion between phosphatidylserine GPVs. Adhesion of cardiolipin GPVs in the presence of healthy donor plasma (E) negatively correlates with the number of MV per platelet in healthy donor plasma (F).

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# Use of Giant Unilamellar Vesicles as a model to study electropermeabilization

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## INTRODUCTION

The application of electrical pulses on a cell membrane induces a transient state of permeabilization of the bilayer. This phenomenon is a physical process that allows the entry of different size of molecules. This property gives to these technique a great potential in cancer treatment and gene therapy [1,2].

Here we study the electrical parameters allowing the entry in a model membrane (Giant Unilamellar Vesicles) of fluorescent molecules of different size.

## MEMBRANE MODEL

In front of the complexity of the cell membrane, we need to work on membrane model to control the different physical and chemical parameters involved in the process of electropermeabilization. We choose to work on the system of giant unilamellar vesicles (GUV). Indeed, the size of these models is close to those of cells and they can be observed directly under microscope which provides a good spatial resolution. Furthermore, electropermeabilzation is activated during the application of the electrical pulse. This property provides a good temporal resolution.

## ELECTROPERMEABILIZATION OF MEMBRANE SYTEMS

Cell membranes are not permeable to hydrophilic molecules and are weakly permeable to ions.

It has already been shown that the application of an external electric field on cells induces their permeabilization [1,3] and if they are in close contact, their fusion [4]. When a membrane system in suspension is put inside a homogeneous electric field, this system disrupts the field lines which induce an accumulation of the electric charges on the membrane surface. As a result, a membrane potential is created and is added to the resting membrane potential.

## RESULTS

Four different methods have been used to study the critical electric field required for electropermeabilzation of GUV.

The first uses the technique of phase contrast. We visualize here the decrease of the GUV's size induced by a strong perméabilisation as previously reported [5].

Then, we observe the entry or the exit of molecules. GUVs are charged with Calcium Green 2, fluorescent molecule when it forms a complex with calcium. First, the exit of Calcium Green 2 is observed, and then the entries of EDTA and manganese inside the GUVs are studied. As EDTA enters spontaneously inside GUVs, it is difficult to determine a critical electric field. With manganese we can determine more precisely this critical electric field (Figure 1) than with the exit of Calcium Green 2, which is a bigger molecule or with the study of the decrease of the GUV's size, which needs stronger electric fields.

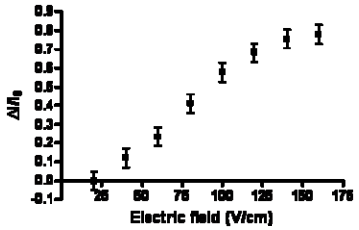


**Figure 1**: Differences of fluorescence intensity of a GUV as a function of the applied electric field.

## CONCLUSIONS

Here we compared different methods for the study of the critical electric field required for the electropermeabilization of GUV.

First of all, we observed that the choice of the method is critical to obtain results close to the reality. The one that give the best resolution is the method using manganese because these ions are the smallest one.

Furthermore, we can conclude here that the critical electric field required for the electropermeabilzation is lower when the diameter of GUV increases.

Knowing the best parameters for electropermeabilization will facilitate electrofusion between GUVs which we plan to study.

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# Interaction between like-charged membranes surfaces mediated by positively and negatively charged nanoparticles of blood plasma - theory

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## INTRODUCTION

Our recent experiments indicated that blood plasma which contains positively and negatively charged nanoparticles promotes the adhesion between like-charged membrane surfaces and in this way cause the membrane buds to adhere to the mother membrane, i.e. suppress the microvesiculation process. In this work the theory was put forward to describe the blood plasma mediated attractive interaction between negatively charged membrane surfaces. The attractive interaction between negatively charged membrane surfaces mediated by negatively and positively charged spherical nanoparticles of blood plasma is explained by using functional density theory and Monte Carlo simulations (MC). The spatial distribution of charge within a single nanoparticle is taken into account. Both, the rigorous solution of the variational problem and the MC simulations show that spatial and orientational ordering of charged nanoparticles in blood plasma may gives rise to an attractive interaction between negatively charged membrane surfaces and this way suppress membrane vesiculation.

## THEORY

In this work we use functional density approach [1] to describe an aqueous solution containing positively and negatively charged spheroidal nanoparticles with internal charge distribution. In the model the charge of single nanoparticle is composed of two negative or positive elementary charges (each of the valency $Z$), separated by a diameter of the nanoparticle. The solution of nanoparticles is sandwiched between two large, planar like-charged surfaces with the surface charge density σ and distance $D$. The normalized free energy of the system:

$$F^* = \int_0^D L\,\mathrm{d}x = \frac{1}{8\pi l_B}\int_0^D \Psi'(x)^2\,\mathrm{d}x + \int_0^D \left[\sum_{i=\pm} n_i(x)\ln\frac{n_i(x)}{n_0} + 2n_0 - \sum_{i=\pm} n_i(x)\right]\mathrm{d}x + \int_0^D \sum_{i=\pm} n_i(x)\frac{1}{l}\int_{-l/2}^{l/2} p_i(s|x)[\ln p_i(s|x) + U(x)]\,\mathrm{d}x \qquad (1)$$

is composed of the energy stored in the electrostatic field as well as the translational and orientational entropy of the nanoparticles. The system equilibrium configuration was solved by variating of the functional in Eq. (1), where ψ is the reduced electrostatic potential, $p_i(s|x)$ is conditional probability distribution of the charge for certain orientation, $n_i$ is the number density of the positive and negative nanoparticles, $n_0$ is the bulk concentration of positively and negatively charged spheroidal nanoparticles, $l_B$ is the Bjerrum length, $e_0$ is the elementary charge, $U(x)$ is a potential of the hard wall and ε is the dielectric constant of water.

## RESULTS AND CONCLUSIONS

Fig. 1 shows the positions $D=D_{eq}$ where the free energy $F/AkT$ exhibits an absolute minimum as a function of the normalized sphere diameter $l^*$. At large diameters the equilibrium distance $D = D_{eq}$ is approximately equal to the diameters of spheres. The results of our study indicate that charged blood plasma nanoparticles with internal charge distribution can induce attractive interactions between negatively like-charged membranes.

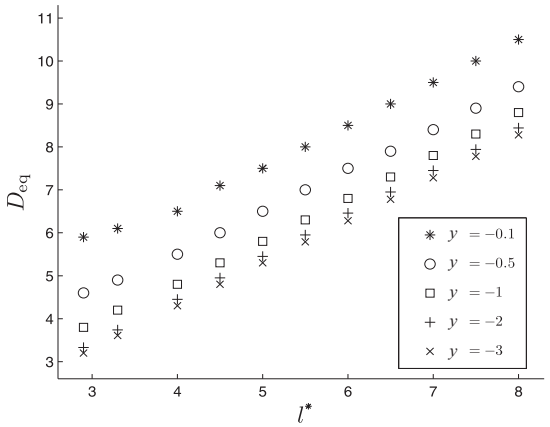


**Figure 1**: Normalized stable equilibrium distances between the charged surfaces $D_{eq}$ as a function of normalized sphere diameter $l^*$ for different $y = 2\pi l_B l_D \sigma e_0^{-1}$ and $Z = 2$, where $l_D$ is Debye length.

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# Development of a CARS microscope for visualisation of molecular changes induced on cell's membrane by application of nanosecond Pulsed Electric Fields.

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## INTRODUCTION

Different groups are presently studying the interactions between cells and nanosecond Pulsed Electric Fields (nsPEF, or nanopulses) which are extremely short (from 10 to 300 ns) and intense (from 10 to 150 kV/cm, 1 to 15 MV/m) electric pulses. Among the biological effects that have been observed, we can mention: permeabilisation of the plasma membrane to small molecules, liberation of intracellular calcium or transgene expression enhancement [1-4].

## THEORETICAL BACKGROUND

CARS microscopy relies on a non-linear optical phenomenon. Images are generated due to the intrinsic vibration of molecules of the sample. Two lasers are simultaneously focused on the sample: the first one is called Pump and the second one is called Stokes. Their frequency $w_p$ and $w_s$ are chosen so that they force molecules to vibrate in phase. When the difference $w_p$ - $w_s$ corresponds to the RAMAN frequency of vibration of a given type of chemical bond, an intense signal is generated in a known direction and at a known frequency ($w_{as} = 2w_p - w_s$) which is called the anti-stokes frequency (figure 1).

This technique allows observation of biological samples without the introduction of any marker or probe. Among the interesting molecules we are planning to image are the phospholipids which should produce an intense and specific Raman vibration [5-6] . This technique also benefits from a great temporal dynamic. Indeed, an image can be acquired in 2 ns only. This should in principal allow to image cells during the application of nanopulses.

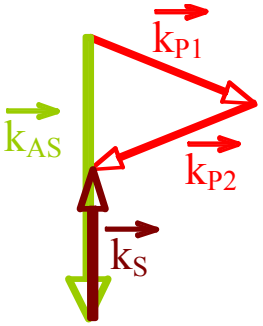


**Figure 1**: Phase vectors of Pump, Stokes and Anti-stokes photons.

## PRESENT ACHIEVEMENTS

The development of the microscope is made using polystyrene beads as the object to image. This allows us to test different optical combinations to improve the global size of the images, their spatial resolution and also the intensity of signal (to make sure one single laser shot will provide enough photons to be detected on the camera).

At present, we have already fixed excitation wavelength panel to get a maximum signal to noise ratio.

The size of the objet that we can see is still low (around 6 nm of diameter) but we expect we will reach 20 nm of diameter so that we will be able to see a whole cell in one shot, that is in an illumination of 2 – 3 ns.

## CONCLUSION

Most of the studies exploring the effects of nanopulses on cells use biological techniques. With this study, we aim at proposing a new kind of approach based on both microscopy and biochemistry. It should at some point give us information at the molecular level and thus it should be pertinent in finding which structures in the cell are mostly and initially affected.

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# A numerical study of water-micelle system exposed to high E-field nano-pusles

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## INTRODUCTION

Recently, colloidal nanocarrier systems as micelles and liposomes have been receiving much attention in the field of drug delivery and gene targeting because of their high loading capacity for chemicals [1]. Intelligent drug delivery systems could be possibly based on stimuli-responsive polymers, which sense a change in a specific variable (as for example an electric field) and activate the delivery [2]. The objective of the present work is to understand, by means of molecular simulations, if electric field nano-pulses can possibly interact with a specific molecular nano-carrier, a zwitterionic micelle, evaluating how the presence of an exogenous field modifies its behavior. The main purpose is to obtain a detailed description of such small carrier system to potentially utilize its main microscopic characteristics for relevant macroscopic applications as nanomedicine.

## MATERIALS AND METHODS

Molecular dynamic (MD) simulations of a water-micelle system have been performed using GROMACS MD package [3]. The model system consisted in a single zwitterionic micelle made up of 55 lipids (TDDNO: N,N-dimethyl-tetradecylamine-N-oxide) and 14123 water molecules (considered as SPC: Simple Point Charge). NVT (constant number of force centers, volume and temperature) simulations have been performed in a 3-dimensional periodic cubic box, at T=300K with the Berendsen temperature coupling with a time-step of 2 fs.

Simulations were carried out for 50 ns at different external electric field conditions, ranging from zero-field up to $10^7$ V/m, to compare our theoretical approach to experimental micro and nano-pulses applications. In order to obtain a direct evaluation of possible effects induced by nano-pulses two main approaches are adopted: 1) the static electric field of $10^6$ V/m is switched off after reaching thermodinamical equilibrium conditions (50 ns), 2) starting from a non exposed simulation, a static E-field is applied (i.e. zero rise-time, $10^7$ V/m) and is switched off after 10 ns.

## RESULTS AND FIGURES

Simulations with static E-fields show a statistically significant variation in density profile of water molecules near the inner boundary of the micelle (distances < 1.8 nm from micelle centre of mass) when an exogenous field of sufficient strength is imposed ($\geq 10^4$ V/m). The introduction of instantaneous switches in field intensities (from zero to several V/m or the reverse case) gives us deeper understanding of the characteristic times in which water density profile reach a thermodynamical stable condition consequently to high-intensity pulse. From figure 1, which refers to the first approach, we note that after 40 ns from the E-field switching-off water molecules tend to return inside micelle core. If we simulate a shorter pulse, following the second approach, we observe (data not shown) an initial significant decrease of water density profile (well matching previous static field simulations) during at least 30 ns, indicating that the recovery time exceeds 30ns.

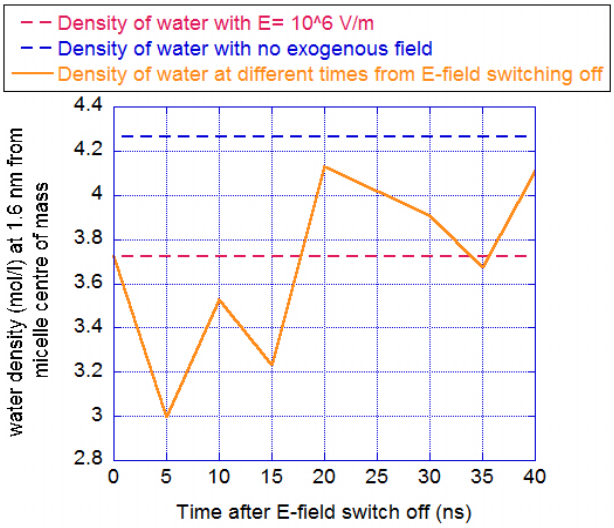


**Figure 1**: Density of water molecules at different times from pulse switch-off.

## CONCLUSIONS

Results seem to show a reversible and non-destructive effect of high energy electric pulses ($10^6$ V/m - $10^7$ V/m) on micelle system. Further (and longer) simulations are needed to reach suitable times and statistics to validate our preliminary results.

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# Modified Blumlein Pulse Forming Networks for Bioelectrical Applications

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## INTRODUCTION

High voltage nanosecond pulsed electric fields (nsPEFs) have been shown to induce, on intracellular structures, interesting effects dependent on pulse length and amplitude, and on the number of pulses in a pulse train.

Changes in intracellular [$Ca^{2+}$] as response to nsPEFs of 60ns and 25, 50 and 100kV/cm were investigated [1]. The nsPEFs induced increase of $[Ca^{2+}]_i$ was dependent on field intensity and, for 100kV/cm pulses, also dependent on the presence or absence of extracellular $Ca^{2+}$. With shorter electric field, apoptosis has been observed when the pulse amplitude exceeded a certain threshold. These effects have been observed to depend on pulse duration and cell type, and not depend on pulse energy and cell morphology [2].

Such experimental evidences suggest the importance of high flexible nsPEFs generation systems to be available. The possibility to change the length and amplitude of pulses is a basic requirement to be able to modulate effects, such as intracellular $Ca^{2+}$ release (which is involved in important cell functions) and apoptosis (which could lead to the use of nsPEFs as a way to treat tumors without chemotherapeutic agents). A widely used high voltage PEF generator is the Blumlein Pulse Forming Network (BPFN), which is composed by two identical transmission lines, and is able to form square voltage pulses, with a value equal to the charging voltage, delivered into a load matching twice the characteristic impedance of the single line. In the classical configuration of the BPFN, using a single closing switch, the pulse duration is strictly dependent on the electrical and physical properties of the transmission line. A new, more flexible BPFN configuration, called the Modified BPFN, has been proposed [3]. It uses two independently controllable switches to terminate the circuit on each side. In such a way, it is possible to change pulse duration and polarity simply varying the closing delay and order between the switches.

## MATERIALS AND METHODS

Two types of modified BPFNs have been designed. The first one is based on coaxial cable transmission lines and MOSFET switches, and is aimed to generate pulses up to 200ns and 100kV/cm on cells suspended in microscope slides (100μm gap between the electrodes). The second one is a compact, spiral stripline network (Fig.1) able to generate pulses up to 100ns and 150kV/cm (assuming to use for the stripline, as dielectric element, materials with a dielectric constant $\varepsilon_r \approx 10$, like $Al_2O_3$) on cells suspended into electroporation cuvettes (1mm gap between the electrodes). In this case, the line requires high voltage switches, such as spark gaps. The spiral stripline was modelled and simulated by FI (Finite Integration) method with CST Microwave Studio®5 (kindly provided by Prof. R. Massa, from the University of Naples, Federico II).

## RESULTS AND CONCLUSIONS

Figures 2-4 show some numerical results relative to the pulse generation through the stripline modified Blumlein.

The Modified BPFNs presented here allow flexibility in generating high voltage nsPEFs, varying pulses duration and polarity without physical changes of the line. They have been designed to match two different pulse delivery systems: microscope slides and electroporation cuvettes.

Thus, it will be possible to carry out complementary assays on cellular suspensions, and to observe various kinds of bioelectrical effects.

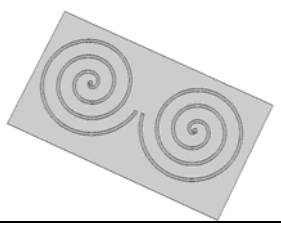

**Figure 1**: Spiral stripline modified Blumlein PFN

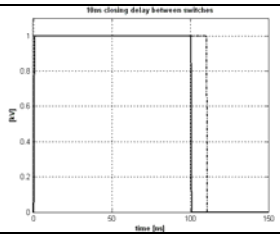

**Figure 2:** 10ns closing delay between switches

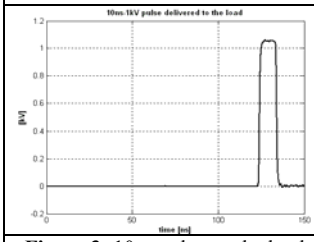

**Figure 3:** 10ns pulse on the load

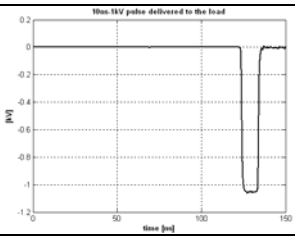

**Figure 4:** Inverted closing order of switches

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# Subcellular effects of nanosecond, high field electrical pulses

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## INTRODUCTION

Nanosecond, megavolt-per meter, pulsed electric fields (nanoelectropulses) interact with intracellular structures and stimulate a variety of responses such as DNA damage, phosphatidylserine (PS) translocation, caspase activation, and loss of mitochondrial transmembrane potential. These effects are hallmarks of apoptosis and may be key factors in nanoelectropulse cancer therapy.

Mitochondria, which play a crucial role in apoptosis, release several apoptosis-inducing factors into the cytoplasm, presumably through the mitochondrial permeability transition pore (MTP). Under certain conditions, nanoelectropulses can manipulate mitochondrial structure and permeabilize the mitochondrial membrane without permanent damage to the cell membrane. In this work we investigate the effects of nanoelectropulses on mitochondrial permeabilization, assess changes in mitochondrial transmembrane potential, and monitor plasma membrane integrity under the same pulse conditions.

## METHOD

The 4 ns hybrid core pulse generator used for these studies was designed and assembled in the Pulsed Power Group at the University of Southern California. The electrical pulses were applied to cell suspensions in a hand-assembled microchamber with a 100 μm gap between two Pt electrodes on a glass microscope slide. Human Jurkat T lymphoblasts (ATCC TIB-152) were cultured in RPMI 1640 at 37 °C under 5% $CO_2$ and concentrated to $2x10^7$ cells/mL before pulse treatment.

Changes in mitochondrial transmembrane potential were evaluated with rhodamine 123 (R123), a lipophilic cationic fluorescent dye that is accumulated within mitochondria. Cells were incubated with R123 for 45 minutes and washed once before pulse treatment.

For assessing MTP opening, a cobalt-quenched calcein quenching method was used. Calcein-AM is an anionic fluorochrome that enters cells freely and labels cytoplasmic as well as mitochondrial regions following removal of the acetoxymethyl (AM) group. When working with cobalt, which cannot readily pass through the mitochondrial membrane, mitochondria can be specifically identified by the quenching of cytoplasmic calcein by $Co^{2+}$, and MTP opening can be recognized by the decrease of calcein fluorescence within mitochondria. Cells were loaded with calcein-AM in the presence of $CoCl_2$ for optimum quenching. Before pulse treatment, cells were washed and resuspended in fresh medium.

Cell membrane integrity was evaluated with propidium iodide (PI), which is excluded from the cell interior by an intact plasma membrane. When the cell membrane is permeabilized, PI enters the cell, binds to double-stranded nucleic acid molecules, and exhibits red fluorescence.

Fluorescence microscopy was performed using a Zeiss Axiovert 200M fluorescence microscope, and Hamamatsu ImageEM EM-CCD camera. Fluorescence images were analyzed with the Hamamatsu software SimplePCI.

## RESULTS

The effects of different pulse amplitudes and pulse numbers on mitochondrial membrane permeability will be reported, providing a framework for an analysis of pulse doses and exposure conditions which lead to mitochondrial modifications while minimizing effects on the plasma membrane. We will discuss also the interpretation of data obtained from fluorescence microscopic imaging analysis using R123 and cobalt-quenched intracellular calcein fluorescence intensity and the influx of PI.

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# Dielectrophoresis as a method for separation of biological cells by their physical properties

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## INTRODUCTION

Dielectrophoresis is a method for separation of small particles on the basis of differences in their electrical or geometrical properties. Several studies have shown that it is efficient in separating viable cells from nonviable ones, as they differ considerably in their electrical conductivity of the membrane and cytoplasm [1]. Electroporated cells also differ from nonporated ones in the conductivity of their membrane, and provided that poration is performed in a low-conductivity medium, also in the conductivity of the cytoplasm [2]. In this study, we analyze theoretically the dielectrophoretic spectra of cells with different membrane and cytoplasmic conductivities with the aim to find for optimal parameters for successful separation of porated and nonporated mammalian cells. Force on a cell in inhomogeneous field was calculated analytically and numerically.

## METHODS

Dielectroforetic force on a spherical object is given by the equation

$$\boldsymbol{F} = \pi\varepsilon_0\varepsilon_{\mathrm{m}} r^3 \,\mathrm{Re}\left[f_{\mathrm{CM}}(\omega)\right]\nabla\left|\boldsymbol{E}_0\right|^2 \quad (1)$$

*where* $f_{\mathrm{CM}}$ is called Clausius-Mossotti factor and is calculated from complex permittivity of the medium and cell

$$f_{CM}(\omega) = \frac{\varepsilon'_{\mathrm{p}} - \varepsilon'_{\mathrm{e}}}{\varepsilon'_{\mathrm{p}} + 2\varepsilon'_{\mathrm{e}}} \quad (2)$$

A numerical model was built in Comsol Multiphysics, a programme for finite-element numerical computations, to calculate dielectrophoretic force on the electroporated cell. Force was calculated using Maxwell stress tensor.

Experiments were performed on Chinese hamster ovary cells in a KPB medium which was diluted to reach electrical conductivity of 12 mS/m and isoosmotic pressure [2]. Cells were porated with Cliniporator (Igea, Italy) at 1250 V/cm and 8×100 µs square pulses. Castellated prototype electrodes were used for dielectrophoresis. Distance between the electrodes was 100 µm and voltage applied was 5 $V_{pp}$.

## RESULTS AND FIGURES

Decrease of medium conductivity improves the efficiency of dielectrophoretic separation, since in low conducting media the crossover frequency of Clausius-Mossotti factor is lower and conductivity change results in a larger change on that frequency than in normal conductive media. The drawback of using low conducting media is a decrease in cell viability, particularly with prolonged incubation of cells is such medium.

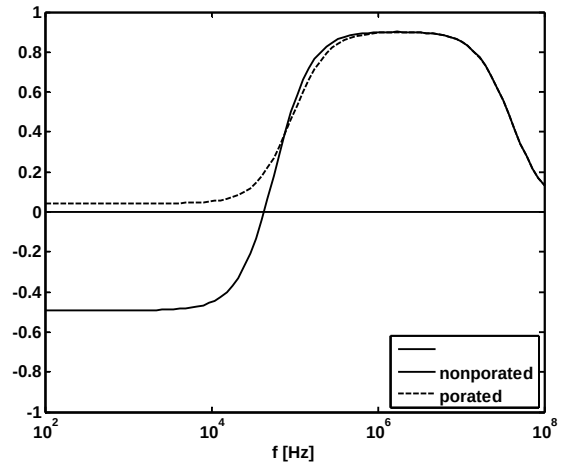


**Figure 1**: Clausius-Mossotti factor of nonporated and porated cell

In theory, negative dielectrophoresis acts on nonporated cells at low frequencies up to 35 kHz and positive at higher frequencies. Positive dielectrophoresis acts on electroporated cells at all frequencies (Figure1).

Experiments on CHO cells showed that the crossover frequency of nonporated cells is between 25 and 35 kHz, while porated cells have a crossover frequency between 20 and 30 kHz.

Difference between theoretical predictions and experiment on porated cells is most probably due to rapid resealing after the electroporation. High membrane conductivity lasts just a few minutes after electroporation, so dielectrophoretic separation must be performed as soon as possible after the electroporation.

## CONCLUSIONS

Separation of porated from nonporated cells is difficult in principle but feasible in a properly designed microchamber, with a short exposure and optimised medium, provided that delay between electroporation and the start of dielectrophoresis is sufficiently short.

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# Enhanced uptake of noncytotoxic superparamagnetic iron oxide nanoparticles in the presence of an external magnetic field in different cell lines *in vitro*

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## INTRODUCTION

The fast development of magnetic nanotechnology and its implication to medicine require strict physical and chemical properties, low toxicity and increased cell internalization of magnetic nanoparticles.

According to these concerns, silica - coated maghemite nanoparticles were prepared and dispersed in a physiological medium before testing for cytotoxicity in different cell lines *in vitro*. Hence, the aim of our study was to prepare biocompatible superparamagnetic iron oxide nanoparticles (SPIONs) and to evaluate their cellular internalization in the absence and presence of an external magnetic field of two different flux densities.

## METHODS

Silica ($SiO_2$) - coated maghemite ($\gamma$-$Fe_2O_3$) nanoparticles were synthesized by precipitation in the microemulsion system *water/SDS,1-butanol/cyclohexane*, dispersed in a 0.9% sodium chloride (NaCl) solution and autoclaved [1]. pH of the dispersion was adjusted to physiological range with 0.1 M citric acid ($C_6H_8O_7$).

The size and morphology of the nanoparticles were evaluated using transmission electron microscopy and X-ray diffractometry (XRD). Mean maghemite crystallite size was calculated according to the broadening of the X-ray diffraction pattern using the Debye-Scherrer formula. Using a magnetometer, mass magnetization of the prepared samples was measured and a hysteresis loop was obtained.

The potential cytotoxicity of SPIONs to human mesothelial (MeT-5A), mouse fibroblast (L929) and human melanoma (SK-MEL-28) cells was assessed by the MTS assay and clonogenic assay. The cells were exposed to permanent magnets of two different flux densities for 15 minutes. The cellular uptake and trafficking pathways of SPIONs were quantitatively evaluated by inductively coupled plasma atomic emission absorption spectrometry (ICP-AES) measuring the iron content.

## RESULTS

The results of transmission electron microscopy analysis demonstrated monodispersity of core - shell type nanoparticles without changes in the properties of the autoclaved material. The measured pH was 6.9 and 7.0 directly after the preparation of dispersion and was still in the physiological range after a period of approximately 4 months when the pH value was 7.1 and 7.4, respectively.

According to the X-ray diffraction's characteristic peak of the spinel structure of maghemite, the mean crystallite size was 7.8 nm. The hysteresis loop obtained by the magnetometer demonstrated superparamagnetic behaviour of the nanoparticles with mass magnetization of 37.9 $Am^2/kg$.

No cytotoxicity to L929, SK-MEL-28 and MeT-5A cells exposed to different concentrations of nanoparticles dispersion was observed according to the MTS assay results whereas the clonogenic assay demonstrated decreased cell survival of MeT-5A cells in comparison to the untreated control cells when exposed to medium and high concentrations of the nanoparticles dispersion.

The iron content in the cells, indicating the cellular uptake of SPIONs, was enhanced in the presence of an external magnetic field of two different flux densities (Figure 1).

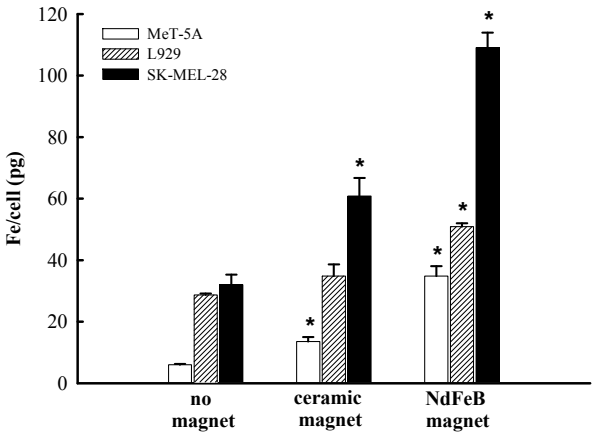


**Figure 1:** Internalization of SPIONs after 15 minute cell incubation. Barrs represent mean ± standard error ($p \leq 0{,}04$ vs. no magnet).

## CONCLUSIONS

This study demonstrates that in the physiological medium stable silica - coated maghemite nanoparticles, synthesized by the precipitation in microemulsion system *water/SDS,1-butanol/cyclohexane,* possess principal properties required for application to biological materials, being noncytotoxic and having enhanced cellular internalization in the presence of an external magnetic field.

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# A microdosimetric study on nanosecond pulsed electric field

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## INTRODUCTION

Exposure of cell lines and tissues to nanosecond pulsed electric fields (nsPEF) has been associated with a number of biological relevant phenomena, suggesting the plasma membrane as one of the main interaction targets. Consequently in this paper a microdosimetric analysis is performed on a single cell constituted by: the extra-cellular medium, the plasma membrane, and the cytoplasm. The study aims to assess the role played by Debye model as well as to compare different membrane dielectric parameters on trans-membrane potential (TMP) and pore number distribution (N(t)). The reliable knowledge of these quantities is of fundamental importance in modelling bio-physical phenomena related to nsPEF.

## METHODS

To take into account the frequency content of the incident pulse a frequency dependent dielectric description of each cell compartment is unavoidable. Therefore the cell dielectric model adopted takes into account the dispersive properties of each cell layer through the Debye equation. The Debye membrane parameters have been obtained through the quantitative assessment described in detail in [1, 2]. The estimate of the membrane parameters is performed starting from permittivity measurements on liposome suspensions in [1] while erythrocytes solutions have been used in [2]. Trapezoidal pulses have been considered with duration of 10 and 3 ns. For the EM solution, a quasi-static approach (Laplace equation) has been adopted and a proper algorithm has been applied to take into account the wide nsPEF spectral content, as reported in [3]. To obtain the pore number distribution a simplified electroporation model suggested by Neu and Krassowska [4] is used, which does not take into account the effects of pore expansions, as pore creation dominates the electrical response on this time scale.

## RESULTS

The TMP obtained for the model as in [2] considering or disregarding the Debye equation are shown in Figure 1 a) for the 3 ns pulse. Disregarding the Debye model (dotted line) an error of about 70% is obtained during the signal rise time where the pulse high frequency components prevail. This suggests that the development of pore number can be underestimated if Debye equation is not considered. In fact, pore number strictly depends on the TMP intensity. The spatial distribution of the pores number for the same signal is further reported in Figure 1 b) disregarding or including Debye modelling. It is evident that Debye model affects this quantity with variation of about two orders of magnitude. Further the TMP time course (3 ns signal) for the dielectric model as in [1] is reported in Figure 1 a) (full gray line). Wide time course variation, due to the adoption of different models, are clearly demonstrated with differences in the peak value of 35 % and 30 % for the 3 ns (Figure 1 a)) and 10 ns signal (data not shown) respectively.

## DISCUSSION AND CONCLUSIONS

In order to approach nsPEF microdosimetry a wide band analytical solution on a three-layered sphere has been proposed. The TMP values obtained have been further coupled with an asymptotic model of electroporation. In this context the role of Debye dielectric modelling of cell membrane as well as the adoption of different dielectric models have been discussed. Particularly, the introduction of Debye equation in the characterization of cell membrane and the proper determination of dielectric model are recommended for accurate quantification of TMP and pore number distribution.

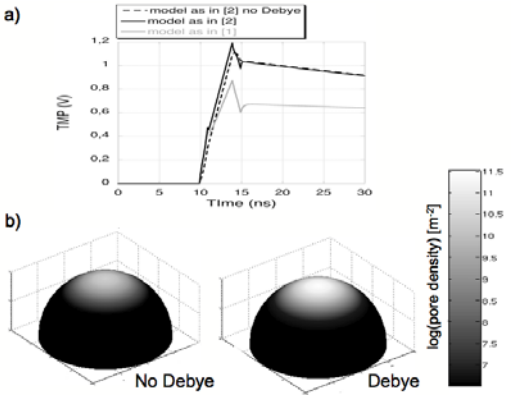


**Figure 1**: a) TMP for different dielectric models as in [1, 2] and b) spatial pores number distribution with and without Debye modelling.

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# Pulsed EM-Induced Membrane Heating in a Living Cell with Frequency-Dependent Membrane Capacitance.

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## INTRODUCTION

The specific capacitance of living cell membranes is known to be frequency-dependent [1]. In spectral bands where the membrane specific capacitance drops significantly, most of the absorbed power can be dissipated in the plasma membrane. For pulsed fields acting on timescales (much) shorter than those of thermal diffusion across the cell, this may result into rapid increase of the local membrane temperature up to possible biological damage, even at low perhaps non-thermal field levels.

In order to analyze this scenario, a semi-analytic solution of the coupled electromagnetic and heat-diffusion problems for a homogeneous spherical cell with (linear, dispersive) membrane capacitance embedded in a homogeneous lossy half-space and exposed to a pulsed-electromagnetic field is derived.

## METHODS

We consider an insulated spherical cell with radius $R$ embedded in a uniform unbounded medium exposed to a $\hat{z}$ - polarized time-harmonic electromagnetic field (Fig. 1a). In the quasi-static approximation the average power dissipated in the membrane ($P_m$ ) and cytoplasm ($P_c$) is given by [2]:

$$P_m(\omega) = \frac{3\pi}{2} R^4 \left| \frac{\sigma(\omega) E^{(inc.)}(\omega)}{\sigma(\omega) + (3/2) R \Upsilon_m(\omega)} \right|^2 \mathrm{Re}[\Upsilon_m], \qquad (1)$$

and

$$P_C(\omega) = \frac{2\pi}{3} \sigma(\omega) R^3 \left| 1 - \frac{\sigma(\omega)}{\sigma(\omega) + (3/2) R \Upsilon_m(\omega)} \right|^2 \left| E^{(inc)} \right|^2, \qquad (2)$$

where $\Upsilon_m(\omega) \cong \sigma_m(\omega)\delta_m^{-1} + j\omega\tilde{C}_m(\omega)$ is the cell membrane complex specific admittance, $\delta_m$ the membrane thickness, $\sigma_m(\omega)$ and $\sigma(\omega)$ the cell membrane and cytoplasm conductivity, respectively. For the membrane (specific) capacitance we adopt the following toy model:

$$\tilde{C}_m(\omega) = \tilde{C}_\infty - \frac{1}{2}\left(\tilde{C}_\infty - \tilde{C}_0\right) Erfc\left[\frac{\omega - \omega_0}{\Delta\omega}\right], \quad \omega \geq 0 \qquad (3)$$

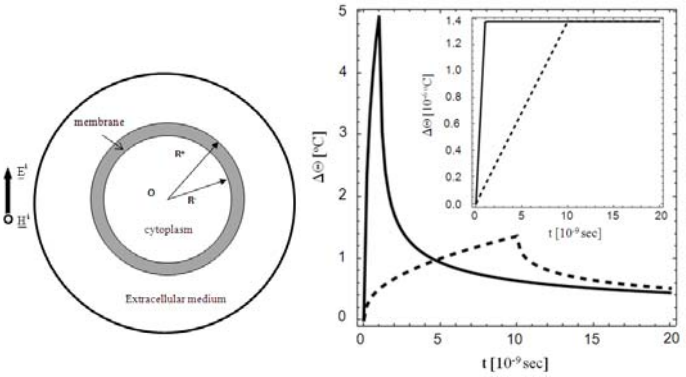
$$\tilde{C}_m(\omega) = \tilde{C}_m(-\omega) \quad \omega < 0$$

where $\omega_0$ is the center frequency of the dispersion band, $\tilde{C}_\infty$ and $\tilde{C}_0$ are the asymptotic (minimum and maximum) values of the membrane specific capacitance, corresponding to $\omega \to \infty$ and $\omega \to 0$, respectively, and $\Delta\omega$ measures the width of the dispersion window.

We focus on the case where the impressed field is a pulse-modulated carrier, whose duration is short compared to the cell thermal relaxation time (ns), and long compared to the carrier oscillation period. Under these conditions, the ratio between the energy delivered to the membrane and cytoplasm can be a *large* number if $|\sigma(\omega)| >> R|\Upsilon_m(\omega)|$ [2].

## SIMULATIONS AND RESULTS

We solve numerically the (forced) heat diffusion equation starting from fixed-temperature initial conditions, continuity of temperature and heat flux across the cytoplasm-membrane and membrane-outer medium boundaries, and enforcing a further adiabatic condition on the outer boundary of the extracellular medium.




(a) (b)

**Figure 1**: Problem's geometry (a); Heating of membrane and cytoplasm (b).

In Fig.1b we show the time evolution of the temperature variation in the membrane and cytoplasm (inset), for a fixed value of the electromagnetic specific absorbed dose of *1J/kg*, and pulse durations of 1 and 10*ns* (solid and dashed line, respectively). The cell radius and membrane thickness are 1mm and 10nm. It is interesting to note that increasing the pulse duration at constant pulse energy produces a slower temperature raise.

## CONCLUSIONS

Our results suggest that whenever the pulse duration is small compared to the involved thermal relaxation constants one may observe a significant increase in the membrane temperature, the average (cytoplasm) temperature being essentially unaffected. It remains to understand to what extent this may relate to some of the observed effects of fields on living cells.

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# Electroporation potentiates cytotoxic effect of the anticancer ruthenium compound KP1339 *in vivo*

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## INTRODUCTION

Several metal-based compounds are being synthesised with the purpose to find new efficient anticancer agents. Among them, ruthenium-based compounds are extensively studied. Two compounds from the group, NAMI-A [ImH] *trans*-[$RuCl_4$(Im)(dmso-S)] (Im = imidazole) and KP1019 [IndH] *trans*-[$RuCl_4(Ind)_2$] (Ind = indazole) are already being studied in clinical trials. It has been shown that electroporation potentiates cytotoxic effect of anticancer agents bleomycin and cisplatin *in vitro*, *in vivo* and in clinics [1]. Up to now, NAMI-A is the only ruthenium-based anticancer agent which has been tested in combination with electroporation *in vitro* [2]. As electroporation significantly potentiates cytotoxic effect of NAMI-A, we continued our study with another ruthenium compound, namely KP1339, [Na] *trans*-[$RuCl_4(Ind)_2$] (Ind = indazole). KP1339 is sodium salt of KP1019 with improved solubility and comparable activity *in vitro* [3]. We tested the influence of electroporation on cytotoxicity of KP1339 *in vitro* and *in vivo*. Additionally, we measured intratumoral ruthenium content to correlate KP1339 concentration in tumors with tumor growth delay.

## MATERIALS AND METHODS

Two cell lines, CHO and SA-1, were tested *in vitro*. Cells were treated with KP1339 (0-200 μM) for 15 or 60 min with or without electroporation (8 pulses, 800 V/cm, 100 μs, 1 Hz). Cell viability was measured 48 h after treatment using MTS-based assay.

SA-1 murine tumor model was used for experiments *in vivo*. Mice were treated with KP1339 injected *i.v.* (10.5, 21.0, 42.0 mg/kg) and with or without electroporation (2x4 pulses, 1300 V/cm, 100 μs, 1 Hz). Tumor growth was measured every second day for 18 days. Intratumoral ruthenium content was measured by inductively coupled plasma mass spectrometry from tumors excised at various time points (4 min-48 h) after treatment with KP1339 alone or in combination with electroporation. The dose of KP1339 was 21.0 mg/kg.

## RESULTS

Our results show that electroporation does not potentiate cytotoxicity of KP1339 *in vitro* but significantly potentiates antitumor effectiveness *in vivo*. Tumor growth was delayed due to electroporation for 5.2 days at the lowest dose tested and for 7.3 days at the highest. Ruthenium content in both groups of tumors was rising continuously for at least 48 h. Electroporation enhanced ruthenium uptake immediately after treatment which lasted for 1 h, consequently causing persistent higher intratumoral ruthenium content.

## CONCLUSIONS

Electroporation significantly potentiates antitumor effect of ruthenium compound KP1339 *in vivo*, though no potentiation is obtained *in vitro*.

Intratumoral uptake of KP1339 was enhanced by electroporation for at least 1 h. Ruthenium concentration in electroporated tumors was higher for at least 48 h.

Observed effect is probably due to combination of unique biochemical features of KP1339 and tumor blood flow changes induced by electroporation [4].

Cell viability assay *in vitro* as a first screen for anticancer drugs is not always enough for extrapolation to *in vivo* cytotoxic effect [5].

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# Resealing Process of CHO Cells After Electroporation at Room Temperature in Isotonic and Hypotonic Buffers

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## INTRODUCTION

Electroporation or electropermeabilization, causes significant increase in the electrical conductivity and permeability of the cell plasma membrane. Electroporation is usually used in molecular biology as a way of introducing substance into a cell [1]. Increased permeability of the electroporated cell membranes is transient and disappears with time if pulsing conditions are not too drastic [2]. Resealing process in cells is a biological process [3]. It depends on the cell metabolism and it's speed is strongly controlled by the post pulse temperature. At room temperature, the quantitative analysis of resealing shows that it is a first order process with its rate constant under the control of the cumulated pulse duration [2]. Under the same electric field conditions, no difference in the kinetics of resealing of cells electropermeabilized at different ionic strengths is observed [4]. The aim of our study was to determine cell membrane resealing of plated and suspended CHO cells and to determine the effect of electric pulse amplitudes and electroporation buffer tonicity on the process of resealing in cells in suspension.

## MATERIALS AND METHODS

Chinese hamster ovary cells (CHO) were grown in Petri dishes as a monolayer in a culture medium at 37°C and 5 % $CO_2$. Resealing was studied on plated cells in isotonic electroporation buffer (pH 7.2, composed of 10 mM $K_2HPO_4$ / $KH_2PO_4$, 1 mM $MgCl_2$ and 250 mM sucrose, 260 mOsm). Cells were grown on multiwells in concentration $10^5$ cells per ml. Culture medium was replaced by electroporation buffer which was followed by electroporation. Electric pulse parameters were: 8×100 μs, 1 Hz, 1000 V/cm. Two parallel Pt/Ir electrodes with a 4 mm distance between them were used. The pulses were generated using a Cliniporator device. The resealing was monitored from 5 to 30 minutes in 5 minutes intervals at room temperature by propidium iodide (PI) uptake. After 3 minutes of incubation with PI fluorescence intensity was measured on Tecan spectrofluorometer (Ex/Em: 535/617 nm). All measurements were done in 4 replicates. Every experiment was repeated 3 times. From the data obtained the percentage of resealed cells was calculated. The results are presented as mean values ± standard deviation.

Similar experiments were repeated for cellular suspension in concentration $5\times10^6$ cells per ml. The parameters were the same as for plated cells only the resealing was monitored after 1 - 30 minutes incubation and the pulse amplitudes were higher; 1400 and 1600 V/cm. For electroporation of cells in suspension 4 mm electroporation cuvettes were used. Resealing was studied in isotonic and hypotonic buffers (pH 7.2, composed of 10 mM $K_2HPO_4$ / $KH_2PO_4$, 1 mM $MgCl_2$ and 75 mM sucrose, 93 mOsm). All measurements were done in 5 replicates and every experiment was repeated 3 times.

## RESULTS

Results show that the speed of the resealing process of plated cells and cells in suspension was fast as 70 – 80 % of the cells were resealed after 5 minutes of incubation at room temperature. However resealing was faster in cell suspension than in plated cells. After 5 minutes 70 % of plated cells were resealed, which stayed unchanged through the experiment until the end when 85 % of plated cells were resealed. In cells in suspension after 5 minutes 80 % of cells were resealed and reached 90 % after 30 minutes. In addition for cells in suspension we tested the effect of two pulse amplitudes and electroporation buffer tonicity. Neither pulse amplitude nor electroporation buffer tonicity affected resealing process in cells in suspension.

## CONCLUSIONS

Our results show that the resealing process is slower in plated cells than in cells in suspension. For cells in suspension there is no significant difference in the resealing process for different pulse amplitudes 1400 and 1600 V/cm and electroporation buffer tonicities. The results from our previous studies show that at chosen amplitudes electropermeabilization is 100 % and 24 hours after the treatment more than 60 % of the cells survive in hypotonic buffer [5].

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# Evaluation of the efficiency of DNA electrotransfer *in vitro* using high voltage and low voltage pulses

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## INTRODUCTION

The effectiveness of gene electrotransfer *in vivo* can be increased by using two types of electric pulses [1]. First one is of short duration high voltage pulse (HV) that is mainly responsible for cell electroporation. The second one is of longer duration low voltage pulse (LV). It was shown *in vivo* that LV pulse governs DNA electrotransfer efficiency, presumably by exerting electrophoretic forces that can interact with DNA and facilitate its translocation across the membrane into porated cells.

*In vitro* importance of electrophoretic forces on DNA electrotransfer using HV and LV pulses is somehow ambiguous and not yet fully understood. In this study we used GFP and luciferase coding plasmids to further evaluate importance of LV pulse for the efficiency of DNA electrotransfer.

## METHODS

Chinese hamster ovary (CHO) cells were used in experiments. The cells were grown in DMEM complete medium. After trypsinization cells were suspended in electroporation medium (0.25 M glucose, 1 mM $MgCl_2$, 10 mM $Na_2HPO_4$). For the experiments 50 μl of the suspension containing 5 μg of luciferase or green fluorescent protein (GFP) coding plasmid was placed between plate electrodes and subjected to the specific treatment with electric pulses. Cell viability as well as GFP positive cells were evaluated by using fluorescent microscopy. Luciferase activity in the treated cells was quantified using luminometer (Tecan).

## RESULTS

In order to reveal importance of LV pulse for DNA electrotransfer into CHO cells *in vitro* we set up our experiments to evaluate the efficiency of DNA electrotransfer by measuring percentage of transfected cells (GFP positive cells) as well as to quantify luciferace activity in the cell lysate in dependence of LV pulse parameters (Fig. 1). As it is seen from the figure, stepwise increase of LV pulse strength resulted in gradual decrease of cell viability. Irrespectively of that, rate of GFP positive cells remained nearly constant and levelled near 10%. Quantification of luciferase activity in transfected cells showed no difference between HV and all of the HV+LV groups (Fig. 1).

Recently it has been proposed that importance of the LV pulses for the DNA electrotransfer *in vitro* can be masked by the excess of plasmid in cell suspension [2]. Therefore, in further studies we tested importance of LV pulse by performing experiments with 100-fold reduced concentration the plasmid (1 μg/ml) Several LV pulse parameters have been tested. Results showed that at all these parameters luciferase activity in HV+LV groups was significantly larger than in HV group (Fig. 2).

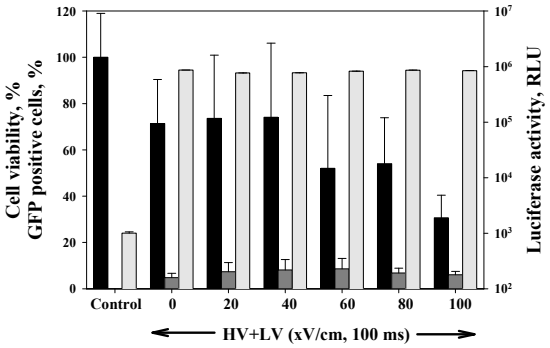


**Figure 1:** Cell viability (black bars), rate of GFP positive cells (dark grey bars) and luciferace activity (grey bars) after cell treatment with various HV+LV pulse combinations.

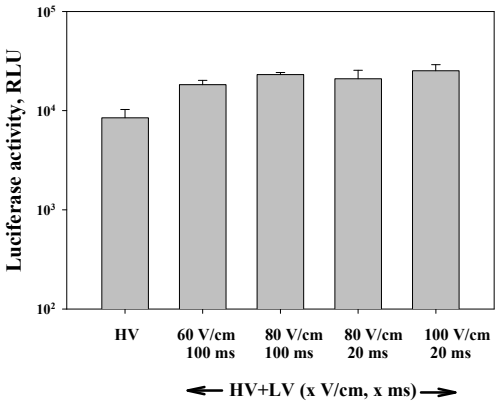


**Figure 2:** Luciferace activity after cell treatment with HV pulse and various HV+LV pulse combinations in cell suspension at reduced (1 μg/ml) plasmid concentration.

## CONCLUSIONS

Our result show that LV pulse importance for DNA electrotransfer *in vitro* can be observed and examined by reducing plasmid concentration. Further experiments are set up to examine these results.

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# The Effects of High Frequency Electric Pulses on Tumor Blood Flow *in vivo*

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## INTRODUCTION

Electrochemotherapy at high repetition frequency reduces the number of individual muscle contractions and painful sensation, whereas the effectiveness remains at the same level as at the frequency of 1 Hz. Higher electric pulse repetition frequency also decreases the time of therapy session and is better tolerated by the patients in comparison to the standard 1 Hz frequency [1]. As electrochemotherapy with higher repetition frequencies inhibits tumor growth and similar efficiency of treatment regardless of the pulse frequency examined, 1 Hz and 5 kHz, in vivo in mice [2-3] but there has been no research in electroporation that would clearly demonstrate tumor perfusion changes after the application of electric pulses at 5 kHz.

The aim of this study is evaluating the effect of 5 kHz repetition frequency electric pulses in comparison to the standard 1 Hz frequency on blood flow of invasive ducal carcinoma tumor in Blab/C mice.

## METHODS

Spontaneous mouse mammary tumor (SMMT) that is an invasive ductal carcinoma, spontaneously developed in female Balb/c mice. Tumor was induced implanting a 4 mm3 fragment into the right flank of anesthetized mice. Eight square-wave electric pulses of 1000 Vcm-1, 100 µs duration at repetition frequency of 1 Hz and 5 kHz were delivered to the mice (by ECT-SBDC electroporation). The blood flow changes in the tumors were measured by laser Doppler flowmetry. Monitoring initially started before the application of electric pulses and data capture restarted immediately after the application of electric pulses with 5 min interval up to 40 min.

## RESULTS

The variation of tumor blood flow result as can be seen in Figure.1, is a rapid reduction immeaditaly after the application of the electric pulses of 1 Hz and 5 kHz repetition frequencies. The rapid reduction in blood flow of the tumors was followed by gradual reperfusion. At frequency of 5 kHz, rapid reduction in tumor perfusion immediately after the application of electric pulses is higher than at a 1 Hz frequency and with slower restoration of blood flow in tumors.

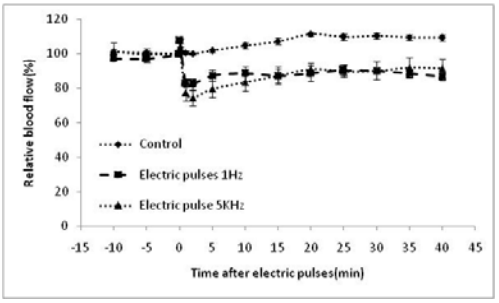


**Figure 1:** Blood flow changes at different intervals following the application of electric pulses at different frequencies. The results are presented as mean±S.E. of mean.

## CONCLUSIONS

The findings showed that electric pulse at these two pulse frequencies, 1 Hz and 5 kHz, induces significant changes in blood flow of SMMT in Balb/C mice and a higher pulse frequency 5 kHz has a comparable effect to a 1 Hz frequency on tumor blood flow.

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# Is the actin cytoskeleton involved in the *in vitro* gene delivery mediated by electric field?

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## INTRODUCTION

*In vitro* gene electrotransfer is a method based on the application of electric field to cells in the presence of exogenous plasmid DNA [1]. Many models have been proposed to explain the mechanisms involved but it is generally accepted that very little is known. Gene expression is a multi-step process, which requires (i) DNA/membrane interaction, (ii) the translocation and the migration of the plasmid in the cytoplasm and (iii) its passage through the nuclear envelope [2,3]. We investigated on a potential partner of the membrane crossing and transport in cytoplasm: the actin cytoskeleton.

## METHODS

Two strategies have been performed in order to determine the eventual role of the actin cytoskeleton in the gene delivery by electroporation. The first is the use of a drug that depolymerises actin filaments, the latrunculin B, and the observation of the DNA/membrane interactions thanks to the labelling of the DNA. The second is to label actin and observe the cytoskeleton organization following the electric pulses application. Both methods consist in the visualization of local phenomenon at the single cell level *via* the use of fluorescence microscopy.

## RESULTS

The DNA/membrane interaction shows changes after 0.1 µM latrunculin B incubation for 1h at 37°C. The DNA accumulation is clearly less visible but still present in aggregates form in the treated cells (Figure 1A). This is associated with a significant decrease of the fluorescence intensity of the DNA aggregates. Indeed, on the treated cells, 49% of the aggregates has fluorescence intensity below 4 000 au and 7% up to 16 000 au, while for the control cells we measure resp. 2% and 24% in these ranges (Figure 1B).

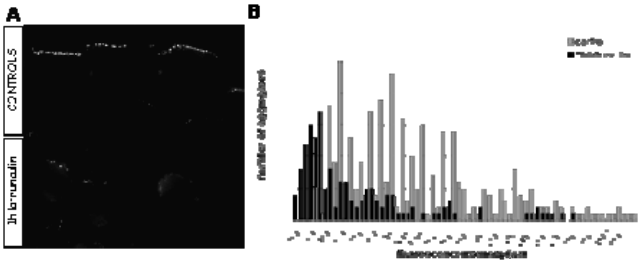


**Figure 1**: DNA/membrane interaction visualization (A) and distribution of the fluorescence intensity associated (B) on CHO cells.

The labelling of the actin reveals, for the first time, the formation of actin patches similar in place, time, size, number as the DNA aggregates occurrence (Figure 2A-B). These patches are observable only when DNA is present and electric field applied. These structures begin to be visible 5 min after the electric pulses and persist until 40 min.

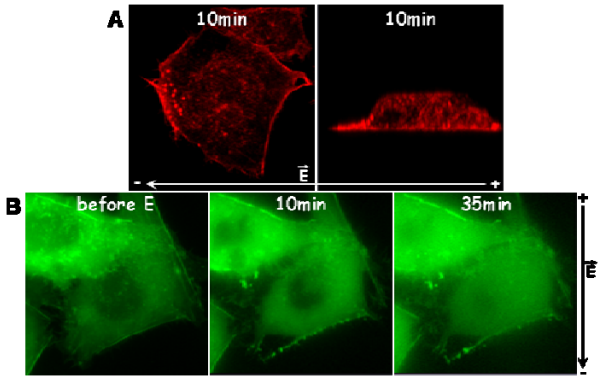


**Figure 2**: Actin patches formed at the plasma membrane of CHO cells after DNA electrotransfer. (A) 3D reconstruction of cell stained with phalloidin-rhodamine123 10 min after electrotransfer, (B) Cell transiently expressing eGFP-actin observed at different time after electric field application.

## CONCLUSIONS

This study tends to show that actin is involved in the mechanism of DNA electrotransfer in CHO cells. Actin seems to be necessary to the accumulation of DNA on the membrane. It also seems to be a partner of the DNA crossing through the plasma membrane by forming patches similar to the DNA aggregates.

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# PNA delivery by electroporation using a muscle model

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## INTRODUCTION

Peptide nucleic acid (PNA) was discovered by Nielsen et al. in 1991 [1]. PNA is a DNA mimic in which a synthetic peptide backbone replaces the sugar-phosphate backbone. Despite the radical change in structure, PNA is capable of sequence-specific binding to DNA and RNA, leading to the formation of extremely stable complexes with complementary DNA or RNA. PNA is original a non-ionic molecule, but can be designed with different charges and conjugated with various peptides. Unlike DNA and RNA, PNA show great resistance to both nucleases and proteases [2]. Together, these properties make PNA very promising antisense candidates. Cellular delivery, however, has been a major problem in particular in vivo, where only a few attempts have been reported.

Duchenne muscular dystrophy (DMD) is the most common and severe form of muscular dystrophy, caused by mutations in the DMD gene that abolish the production of functional dystrophin protein. Antisense-mediated exon-skipping is one of the most promising therapeutic approaches for DMD. The idea is to splice out selected exons containing stop codons from the pre-mRNA, in order to restore the reading frame and express functional dystrophin. Mdx mice, the animal model of DMD, will be used as a muscle model in the present project. We will combine our long experience with electroporation-mediated delivery of molecules to muscle tissue with the chemistry of PNA; aiming at developing an efficient in vivo PNA delivery system.

## METHODS

PNA electrotransfer will be performed in NMRI and mdx mice. The tibialis cranialis (TC) muscle of anaesthetized mice will be injected intramuscularly with 20 µl PNA solution. Different charged PNA will be tested. Plate electrodes will be fitted around the muscle. Electric parameters will be selected based on our experience with pulse optimization for small drug delivery and DNA electrotransfer. Transfer of fluorescent-labelled PNA (PNA congugated with Alexa Fluor 680) will elucidate the spatial distribution of the PNA within the muscle. In vivo Bio-Imaging (Optix MX-2 Optical Molecular Image System, Advanced Research Technologies, Canada) and histological techniques will be applied. RT-PCR will be performed to test for exon skipping in PNA treated muscles. Positive dystrophin fibers of mdx mice will be identified by immunohistochemistry and western blotting.

## RESULTS

In our preliminary studies (enrolled as a PhD student the 1st of August 2009), we have shown delivery of PNA to muscle tissue using electroporation. In these studies the charge of the PNA was important for efficient transfer as positive charged PNA were more easily electrotransferred than neutral charged PNA.

## CONCLUSION AND PERSPECTIVES

Electroporation shows great promises as a delivery method for PNA antisense molecules. Not only in the treatment of DMD, but also in other diseases. This study will provide a systematic investigation of PNA delivery by electroporation, with the aim of bringing PNA-mediated antisense treatment to the clinic.

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# Mechanism of MD-2/Toll-like Receptor 4 Activation by Endotoxin

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## INTRODUCTION

Innate immune system utilizes several families of pattern-recognition receptors that recognize conserved microbial molecular patterns. The recognition of such molecules (*e.g.* lipopolysaccharide, flagellin, CpG DNA, *etc.*) is a prerequisite first step in the activation of an appropriate innate and adaptive immune response.

Toll-like receptors (TLRs) are a family of pattern-recognition receptors expressed on the plasma membrane or on the membrane of the endosomal compartments. After the binding of ligand they dimerise and trigger an intracellular signalling cascade leading to the activation of the NF-kappaB transcription factor that starts the transcription of several important inflammatory cytokines and thus immune response (1).

Toll-like receptor 4 (TLR4) together with co-receptor MD-2 recognizes Gram-negative bacterial endotoxin (i.e. lipopolysaccharide (LPS)), which is one of the most potent stimulants of the innate immune system (2). TLR4 is found on the surface of several different immune cells (e.g. macrophages, dendritic cells). The signaling through TLR4 receptor is implicated in several diseases, including sepsis, cancer, rheumatoid arthritis and Crohn's disease.

## METHODS

We studied the importance of specific amino acid residues in MD-2 and TLR4 by site-directed mutagenesis. The mutants were tested for their activity in HEK293 cells using Luciferase reporter assay to measure NF-kappaB activation. The ability of MD-2 mutants to bind LPS was measured using size exclusion chromatography. The structural models of the complex were calculated using the program HADDOCK.

## RESULTS

We investigated the mechanism of MD-2/TLR4 activation by hexaacylated lipid A (the active LPS moiety). We identified residues within the hairpin of MD-2 that influence activation of TLR4 by lipid A. We show that hydrophobic residues at positions 82, 85, and 87 of MD-2 are essential both for transfer of endotoxin from CD14 (LPS-binding protein also involved in MD-2/TLR4 activation) to monomeric MD-2 and for TLR4 activation.

We also identified conserved hydrophobic residues (Phe-440 and Phe-463) in the TLR4 ectodomain, which are essential for activation of TLR4 by LPS. Charge reversal of neighboring cationic groups in the TLR4 ectodomain (Lys-388 and Lys-435), in contrast, did not affect cell activation.

Based on our mutagenesis studies we propose a molecular model in which Val-82, Met-85, and Leu-87 in MD-2 and distal portions of an acyl chain of hexaacylated lipid A that do not fit into the hydrophobic binding pocket of MD-2 form a hydrophobic surface that interacts with Phe-440 and Phe-463 on a neighbouring TLR4/MD-2/LPS complex, driving TLR4 activation (3).

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# The Effect of Electric Pulse Number in Electrochemotherapy by Low Voltage and High Frequency

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## INTRODUCTION

Electrochemotherapy (ECT) is an effective approach for treatment of solid tumors. The conventional electric pulses used in ECT are eight electric pulses of 1000-1300 V/cm with pulse duration of 100 µs and pulse repetition frequency of 1 Hz [1]. Besides the advantages, there are also some side effects of this standard protocol including unpleasant sensation to the patient due to muscle contraction and also the high amplitude of electric pulses that is essential for effective electropermeabilization of tumor cell are dangerous for clinic [2]. However, it would be possible to reduce the number of individual muscle contractions by increasing of pulse repetition frequency to a value over the frequency of titanic contraction. In vivo studies showed that ECT with higher repetition frequencies (5 kHz) inhibits tumor growth and with almost same efficiency of low frequency treatment (1 Hz) [2]. In previous studies it was shown that applying electric pulses with low pulse amplitudes (50 -150 V/cm) is an effective method for treatment of solid tumors [3-5]. Therefore, the aim of this study was to investigate the efficiency of combination of low pulse amplitude (100 V/cm) and high repetition frequency (5 kHz) with different number of pulses for treatment of animal model tumor (invasive ductal carcinoma) and comparison with high frequency conventional ECT.

## METHODS

Tumor was induced implanting a fragment of invasive ductal carcinoma into flank of Balb/c female mice. When the size of tumor reached 5 mm, bleomycin was injected intratumoral. Two minutes after the injection, the electric pulses were applied. Two different protocols were investigated in this study. In the first protocol, eight pulses with high amplitude of 1000 V/cm and duration of 100 µs and repetition frequencies of 5 kHz were delivered. In the second protocol, different number of pulses with low amplitude of 100 V/cm and duration of 100 µs with frequency of 5 kHz were delivered. The number of applied pulses in the second protocol were 500 (1 pulse 50 millisecond duration), 2000 (4 pulse with 50 millisecond duration), 4000 (8 pulse with 50 millisecond duration), 5000 (10 pulse with 50 millisecond duration).

## RESULTS

As it can be seen in Figure 1, applying 5 kHz electric pulses with low amplitude to the tumors can significantly inhibit tumor growth. As it is shown in the figure, these kinds of pulses are even more effective than ECT with 5 kHz pulse repetition frequency. Based on the results best antitumor effect was obtained with 4000 low voltage pulses with high frequency ($P < 0.05$). The rate of inhibition tumor growth was statistically increased with number of electric higher than 2000. In this study, we showed that for the number of low voltage pulses more than 2000 (up to 4000), the electric pulse treatment is effective for inhibition.

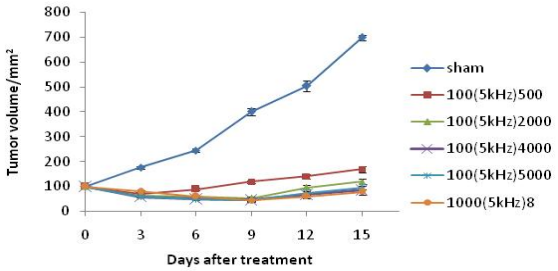


**Figure 1:** Tumor growth after applying of electric pulses with different parameters to the tumors in mice. The results are presented as mean±S.E. of mean.

## CONCLUSIONS

The finding showed that high frequency low voltage electric pulse treatment of tumors has a comparable effect to high frequency ECT.

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# Safety and efficacy of intratumoural electrotransfer of plasmid AMEP in patients suffering from advanced or metastatic melanoma: an open phase 1 trial

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## INTRODUCTION

Morbidity and mortality of disseminated cancer are high, and side effects of established treatments continue to be a problem, thus new treatment and treatment modalities are warranted.

After initial set-backs gene therapy is now gaining new momentum, due to both increased clinical success rates and better understanding of the technology [1]. Generally viral vectors result in high gene transfer efficiency but also have safety issues with systemic toxicity, host immune response and uncontrolled integration into the host genome. Direct injection of DNA into tissue, although safer than viral vectors, suffers from low gene transfer efficiency. Several in vivo studies have shown that electroporation significantly enhances gene transfer efficiency [2] and thus it is possible to retain the safety advantages of plasmid DNA.

Angiogenesis and cell motility are involved in tumour growth and formation of metastasis. The therapeutic peptide AMEP (for Antiangiogenic METargidin Peptide) is the recombinant disintegrin domain of metargidin (also known as ADAM-15). This domain contains a RGD (Arg-Gly-Asp) sequence which specifically binds to the *alpha*v*beta*3 and *alpha*5*beta*1 integrins. It is a novel anti-cancer agent with proven anti-angiogenic and anti-proliferative properties [3].

BioAlliance Pharma (Paris, France) has developed a plasmid coding for the human AMEP cDNA. The plasmid is devoid of any antibiotic resistance gene in order to be used for humans, and is currently being produce according to GMP (Good Manufacturing Practice).

## METHODS

Under the ANGIOSKIN project EU 6th framework program we will perform a phase 1 dose escalation study of electrotransfer of plasmid AMEP in patients suffering from advanced or metastatic melanoma. Successive cohorts of 3 patients will receive increasing doses of plasmid AMEP. Up to four dose levels (1 mg, 2 mg, 4 mg and 6 mg) will be evaluated, and patients will receive treatment on day 1 and day 8. Treatment consists of injection of plasmid AMEP into non-necrotic cutaneous melanoma lesions immediately followed by electrotransfer. A linear needle electrode (0.4 cm between needle arrays) is inserted into the tumour and a combination of a short (100 µsec) high-voltage (1250 V/cm) pulse followed by a long (400 msec) low-voltage pulse (140 V/cm) will be delivered. Primary endpoint of this phase 1 study will be safety; secondary endpoints will be tumour response, determination of pharmacokinetic parameters for plasmid AMEP and acceptability of the treatment procedure. Pre- and post-treatment biopsies will be obtained at defined time points for detailed histologic evaluation including *alpha*v*beta*3 and *alpha*5*beta*1 integrins.

## RESULTS

The study is a joint study encompassing three centres: Department of Oncology, Copenhagen University Hospital Herlev, Denmark, Institut Gustave-Roussy, Villejuif, France and Institute of Oncology Ljubljana, Slovenia.

Enrolment is planned to start in the fall of 2009. Expected end date is spring 2011.

## CONCLUSION AND PERSPECTIVE

Recently the first human trial on gene electrotransfer was reported [4]. Here plasmid IL-12 was injected into melanoma nodules and followed by electrotransfer. Daud et al found this modality to be safe, effective and reproducible. Once the safety profile of plasmid AMEP has been established, future clinical trials using electrotransfer of plasmid AMEP are anticipated. One perspective is electrotransfer of plasmid AMEP not only to tumours but to muscle tissue in order to obtain long term expression, thus using muscle tissue as a production facility for cancer medicine.

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# Electrochemotherapy: The Lisbon experience

Victor Farricha[1,2], Hugo Vasques[1], Sara Carvalhal[1],José Crespo Mendes de Almeida[1,2]; *[1] Portuguese Oncological Institute of Lisbon – Melanoma and Sarcoma Unit, Rua Professor Lima Basto 1099-023 Lisbon PORTUGAL, [2] Faculty of Medicine – University of Lisbon, Av Professor Egas Moniz 1649-028 Lisbon PORTUGAL.*

## INTRODUCTION

We performed Electrochemotherapy (ECT) in Lisbon since February 2008. We initially treated mainly melanoma patients but with the growing knowledge on this technique we treat now different kind of presentations in pathologies of the skin, of the mucosa and even intraabdominal progression of oncological diseases.

In our point of view the surgical management of cutaneous and subcutaneous tumours, especially metastatic lesions, can be difficult for the surgeon. The treatment may be complex regarding the type, the location or even the number of these lesions. In this cases ECT is a good option and is now a standard procedure in our Institute, giving us the chance to treat less aggressively the patient when we talk about advanced skin cancer or skin involvement by other types of tumours.

## MATERIAL AND METHODS

Since February of 2008 we selected 76 patients for the procedure. We perform ECT according to ESOPE protocol. The patients were treated by electrochemotherapy using either bleomycin or cisplatin in low doses followed by application of electric pulses to the tumours by the CE labelled electric pulse generator Cliniporator$^{TM}$ (IGEA S.r.l., Carpi, Italy), in order to potentiate cytotoxicity of the

chemotherapeutics using plate or needle electrodes. We used the N-50-5I finger prototype electrode for mucosal approach.

## RESULTS

We have already performed 103 sessions of ECT since that (figure 1).

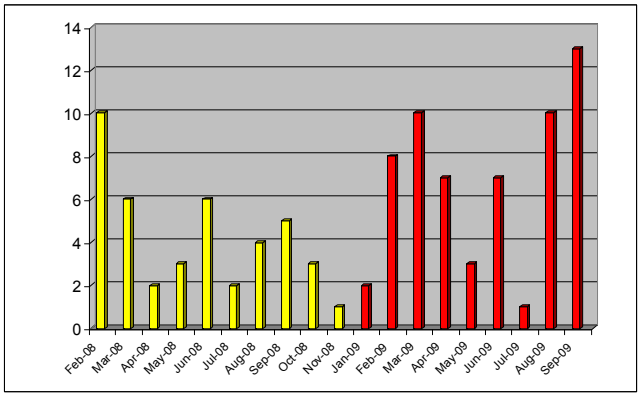


**Figure 1**: Number of ECT procedures per month.

We have made 3674 electroporations with a maximum of 124 applications in a single sessions and a maximum of 7 sessions in the same patient.. The ECT treatments were mainly for lower limbs and trunk disease (84% of all treatments). We perform mainly palliative management (84%). The mucosal patients that we treated (a primary melanoma of the anal canal and a recurrence of a vulvar melanoma) allow us to avoid amputation surgeries. One of the patients that we treat performs intraabdominal ECT. He now has a follow up of 5 month with an acceptable control of the disease.

The complete response rate was 88% in our series with an overall response rate of 97% (figure 2).

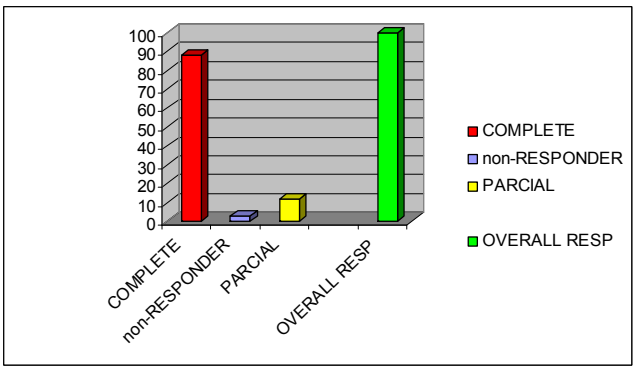


**Figure 2**: ECT Response rate.

During the follow up we only confirm the histological response in the mucosal lesions. The response of the intraabdominal patient treated was evaluated by PET-scan at week 4 and 10. We had 16,5% of complications (pain-4% and scar-12,5) that we think are related with some over treated lesions.

## RESULTS

ECT is a simple and effective technique and allows us to reduce the necessity of multiple surgeries in this group of patients.

The complication rate is low.

We think that the application of the technique in other areas than the skin is possible and could be an option in the future in combination with other surgical and medical approaches.

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# Bleomycin treatment of brain tumors: an evaluation

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## INTRODUCTION

Bleomycin has been used in the treatment of brain tumors for over 30 years. Currently, we are evaluating electrochemotherapy (the use of electric pulses to enhance uptake of bleomycin) for patients with secondary brain tumors. We, therefore, reviewed the literature with specific reference to the tolerability and toxicity of bleomycin.

## METHODS

Using the keywords 'brain' and 'bleomycin', a database search without date restriction was performed and over 500 articles were found. Twenty-five articles were used for this study based on relevance determined by (i) clinical studies, (ii) use of bleomycin, and (iii) direct injection into brain tissue or cysts.

## RESULTS

There were two main indications for the use of bleomycin directly into the brain: (i) cystic tumors in the form of craniopharyngiomas and (ii) solid brain tumors such as glioblastomas and astrocytomas.

The most frequent adverse effects reported were transient fever, headaches, nausea and vomiting, lethargy, and peritumoral edema. Out of 189 patients treated from 1973 to 2007, only five patients (3 %) had severe and six patients (3%) had moderate adverse effects.

**Table 3**: Five cases with severe complications after intracerebral bleomycin related to dose.

| Studies | Dose | Severe adverse effects |
|---|---|---|
| Savas et al [1] | 100 U in 8 days (56 mg) | Generalised brain edema leading to death |
| Mottolese et al [2] | 1 incorrectly diluted dose | Blindness |
| Belen et al [3] | 141 U in 5 weeks (75 mg) | Blind, one eye |
| Broggi et al [4] | Single dose 9 U (15 mg) | Deafness |
| Frank et al [5] | Unknown | Deafness |

One death was directly related to this treatment, where very high doses were used. Two patients developed loss of vision and two patients had hearing loss because of the treatment.

## CONCLUSIONS

All cases with severe and moderate adverse effects except one were patients with craniopharyngiomas and probably because of tumor localization in the deep brain. In conclusion, bleomycin injection into the brain has been a fairly well tolerated at doses much higher than that used in electrochemotherapy.

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# Treatment Planning for Electrochemoterapy Treatment of Deep-Lying Tumors

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## INTRODUCTION

Electrochemoterapy relies on strong pulsed electric fields causing cell plasma membrane permeabilization, which allows chemical agents to enter the cell and cause cell death. To ensure successful treatment, the whole tumor needs to be covered with sufficiently strong electric field while preferably keeping field in neighboring healthy tissue as low as possible. At the same time, constraints from the electroporation device, such as maximum voltage and current, have to be taken into account.

Placement and shape of electrodes has a strong influence on the distribution of electric fields in surrounding tissues. Together with difficulties in positioning them precisely near deep-lying tumors, this calls for in advance planning. Treatment planning was done by running an optimization algorithm on a numerical finite element model of the tumor and surrounding tissues.

## METHODS

First step in treatment planning is the construction of an sufficiently detailed model of the anatomy. Medical images of the region of interest were segmented, with the tumor to be treated and nearby organs being the most important. Especially tissues with high or low electrical conductivities are important as the ratio of tumor conductivity versus surrounding tissue conductivities has a great effect on the distribution of the electric field and total current needed.

In the model presented, tumor, muscle and adipose tissues were included and their conductivities were taken from the literature (0.2 S/m, 0.02 S/m, 0.135 S/m and 0.75 S/m for tumor, adipose, muscle in xy direction and muscle in z direction, respectively)[1]. To perform optimization, an algorithm in Matlab was constructed that assigned conductivity values to each pixel of segmented image and these values were then imported into COMSOL Multiphysics as a 3-dimentional spatial conductivity data.

During the application of electroporation pulses, the tissue conductivity changes significantly [2,3], and this in turn affects electric field distribution, therefore current and electric field distributions were checked with a non-linear conductivity dependency on electric field.

## RESULTS

Optimization was performed for a four and five-electrode configuration. In the five-electrode configuration, one electrode was allowed to penetrate the tumor, while the remaining four electrodes were constrained to only touching the tumor. In the four-electrode configuration, all electrodes were constrained to remain outside the tumor. In both cases, results of current calculations were below the 50 A current and 3000 V voltage limit of the Cliniporator™ electroporation device.

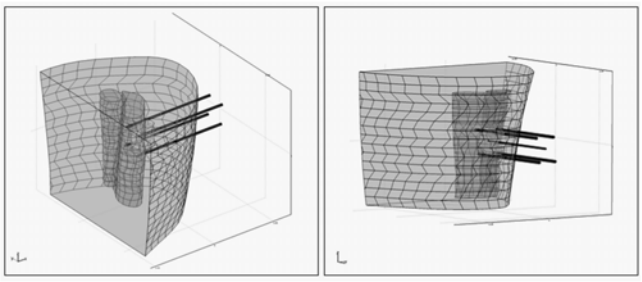

**Figure 1**: Optimized electrode positions for four- and five-electrode configuration

| 4 electrodes | 5 electrodes |
|---|---|
| 1-2: 2000V | 1-2: 2000V |
| 1-3: 2700 V | 1-3: 2500 V |
| 2-4: 2700 V | 2-4: 2500 V |
| 3-4: 2000 V | 3-4: 2000 V |
| 1-4: 3000 V | 1-5: 1700 V |
| 2-3: 2900 V | 2-5: 1700 V |
| | 3-5: 1700 V |
| | 4-5: 1700 V |

**Table 1:** Optimized electrode voltages. Electrode nr. 5 denotes the central electrode in the 5-electrode case.

## CONCLUSIONS

Results of the optimization procedure show that electroporation is a viable treatment method even for deep-lying tumors. In both cases investigated, it was shown that electrode positions need to be as close to the tumor as possible and minimum voltages for each electrode pair were proposed.

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# Comparison of electropermeabilization and gene electrotransfer on plated cells and cells in a suspension for two cell lines

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## INTRODUCTION

Electroporation - a method of inserting foreign material into cells by using electric pulses was described first almost three decades ago [1]. Since then various applications using electroporation were developed, such as electrochemotherapy and gene electrotransfer. Gene electrotransfer has a great potential as an alternative to virus transfection for gene therapy, however for more efficient transfection, understanding of the mechanisms and optimization of protocols are needed [2, 3]. One of the prerequisites for successful gene transfection is the use of adequate electric pulse parameters at which induced transmembrane voltage reaches value ($ITV_c$) required for membrane permeabilization.

In our in-vitro study chinese hamster ovary cells (CHO) and mouse melanoma cells (B16F1) were compared for gene transfection and permeabilization using square pulses. Furthermore, the size of cells was measured and $ITV_c$ was calculated.

The aim of this study was to analyze in parallel viability, gene electrotransfer, electropermeabilization and critical induced transmembrane voltage ($ITV_c$) for both plated cells and cells in suspension.

## MATERIALS AND METHODS

CHO and B16F1 cell lines in a plated state and in a suspension were used. To determine efficiency of gene electrotransfer GFP in concentration 10 µg/ml for plated cells and 40 µg/ml for cells in suspension was used whereas electropermeabilization was determined by means of 150 µM PI. GFP expression, on plated cells was evaluated under inverted fluorescence microscope (Axiovert 2000, Zeiss, Germany) whereas for cells in suspension, percent of fluorescent cells was determined by flow cytometry (Coulter EPICS Altra flow cytometer, Beckman Coulter Electronics). For permeabilization experiments, uptake of PI in cells was evaluated with spectrofluorometer (Tecan Infinite M200, Tecan Austria GmbH).

For gene electrotransfer on plated cells pulse amplitudes were: 0.3 kV/cm - 1.4 kV/cm, while for permeabilization 0.2 kV/cm - 1.6 kV/cm were used. For cells in suspension we used pulse amplitudes: 0.6 kV/cm - to 1.6 kV/cm for gene electrotransfer, and 0.3 kV/cm - 1.6 kV/cm for electropermeabilization. Electric pulse parameters were: length: 200 µs, frequency: 1 Hz, number of pulses: 4.

The average size of a cell was determined by analyzing phase contrast images of non-pulsed cells from each experiment. Using Schwan equation for spherical (cells in a suspension) and spheroidal (plated cells) cells critical induced transmembrane voltage was calculated.

## RESULTS

For gene transfer on plated cells GFP expression is increasing up to 1.2 kV/cm where it reaches its maximum around 20 % and 23 % for CHO and B16F1 cells. PI uptake is also similar for both cell lines, however maximum permeabilization of 80 % is reached in B16F1 cells at 0.8 kV/cm and for CHO cells at 1.0 kV/cm [4].

For gene transfer in cells in suspension the highest GFP expression of 70 % for CHO cells is obtained at the pulse amplitude of 1.6 kV/cm whereas B16F1 transfection is around 30 % at the same amplitude. CHO cells show the highest uptake of PI of 60 % at 1.2 kV where B16F1 cells are already at the saturation level.

## CONCLUSIONS

Our results indicate that there is no simple rule, which would enable us to extrapolate results from cells in a suspension to plated cells. When comparing CHO and B16F1 cell lines we obtained similar transfection and permeabilization on plated cells while in suspension CHO cells exhibited higher transfection efficiency even though permeabilization was similar for both cell lines. Calculation of critical ITV in suspension showed that for permeabilization as well as for transfection CHO cells have lower $ITV_c$, while on plated cells the distribution of sizes is so large that we can only estimate that $ITV_c$ are similar.

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# Electrodenaturation treatment of tumours using a one-probe two - electrodes device

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The electrochemical treatment of tumours (EChT) consists in the passage of a direct electric current through two or more electrodes inserted locally in the tumour tissue. Electrochemical reactions induce extreme pH changes and, consequently, protein electrodenaturation fronts intimately related to tumour destruction. In the classical EChT electrodes configuration experiment, from an initial uniform condition two electrodenaturation fronts evolve expanding towards each other, leaving the possible existence of a biological pH region between them [1]. Here we introduce a one-probe two-electrodes device (OPTED) containing the cathode and the anode very close to each other. Upon application of the OPTED, two electrodenaturation half spherical fronts, one basic the other acid, expand from the electrodes towards the periphery conforming a full sphere. Electrodenaturation front tracking reveals a time scaling close to $t^{1/2}$, signature of a diffusion-controlled process. Based on this evidence it is possible, in principle, to predict the necessary time needed for total tumour destruction without compromising healthy tissue. The main advantages of the OPTED are the insertion of "one electrode" rather than two or more thus minimizing tissue intrusion, the passage of uncontrolled current is constrained to a very small region between electrodes, high acidity/alcalinity regions are localized in one tiny zone around electrodes, the possible existence of a biological pH region between electrodes is suppressed, and the likelihood of reaching tumours beyond possibilities of conventional surgery. These results could have significant implications in EChT optimal operative conditions and dose planning, in particular, in the way in which the evolving electrodenaturation spherical fronts cover the active cancer cells spherical casket. Moreover, they may have potential advantages in the application of electrochemotherapy (ECT).

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# Comparison of plate electrode and pin electrode for skin gene electrotransfer using numerical models

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## INTRODUCTION

The skin consists of different layers representing different functions and electrical properties. Stratum corneum, the outmost layer of the skin, is known to play a major role when voltage pulses are applied to the skin [1]. Two types of electrodes ('pin' and 'plate'), have been modeled using numerical techniques and compared in terms of electric field distribution, to find support for experimental data from in vivo gene transfection in pig skin.

## METHODS

The electric field distributions were calculated using finite element method software, COMSOL Multiphysics 3.5a, on a 64-bit Linux platform. Due to large uncertainties as regards the electrical parameters of skin and in the determination of thicknesses of the skin layers, a fairly simple model was preferred.

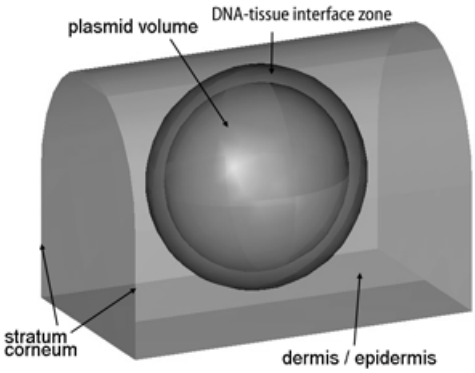


**Figure 1:** The model geometry used for numerical calculations of the plate electrode condition.

The geometry consists of one physical layer only (epidermis/dermis). The injected plasmid volume was modeled as an ellipsoid and treated as a separate compartment in the epidermis/dermis layer. The stratum corneum was modeled as a boundary condition to avoid calculations errors, due to the small layer thickness. A 0.5 mm zone between the plasmid volume and the dermis/epidermis cells consisting of dermis/epidermis cells mixed in plasmid solution was defined as the DNA-tissue interface zone. It is suggested that this zone is the primary location for gene transfection.

## RESULTS

The results are shown and compared based on central cross sections of the electric field distributions from both high voltage (HV) and low voltage (LV) pulses (fig. 2). Two different distributions were calculated for the plate electrode in the high voltage situation (not shown), representing two different conductivity states of the stratum corneum [2].

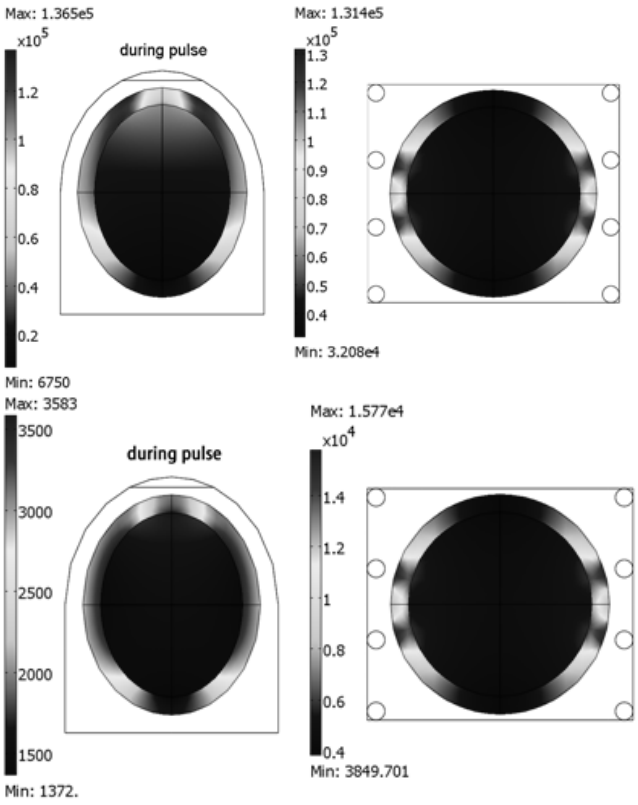


**Figure 2:** Left side: Plate electrode HV (top), LV (bottom). Right side: pin electrode HV (top), LV (bottom).

## CONCLUSIONS

The plate electrode and the pin electrode generate comparable electric field intensities during the high voltage pulse. This suggests that using a protocol with high voltage pulses only, plate electrode and the pin electrode will perform equally. In the case of one high voltage and one low voltage pulse, the pin electrode is a better choice.

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# Irreversible Electroporation for Microbial Control of Drugs in Solution*

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## INTRODUCTION

The problem of eye infections by microorganisms in topical ophthalmic medications has not been adequately solved yet.

The prevalence of bacterial presence in anti hypertensive glaucoma eye drops in the community setting was determined by various studies. Geyer (1995) reported finding bacteria in more than 28% of the bottles[1] . There was a significant relationship between the contamination rate and the time since opening the container, with 40% of those used for more than 3 three months contaminated. Similar rates were found by Schein (1992) in eye drops used by patients with ocular surface diseases[2].

The danger of growth of microorganisms in ophthalmic medication is reduced by the inclusion of preservatives, principally benzalkonium chloride (BAK), to the solution. However, not only were the contamination rates cited above determined in the presence of preservatives, all preservatives cause considerable side effects, particularly when used chronically. For instance, BAK is harmful to the surface of the eye and probably accounts well over half treated glaucoma patients suffering from symptoms and signs of dry eyes[3]. It is not only the superficial eye tissues that are damaged by BAK – it seems that chronic administration of BAK damage the trabecular meshwork and thus may counteract the anti-ocular hypertension drugs it helps sterilize[4].

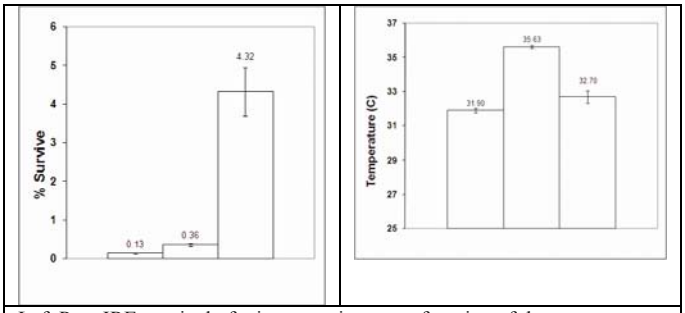
The goal of this study is to explore the feasibility of using irreversible electroporation(IRE), formally showed as effective for reduction of microbial load in foods [5], for the non-chemical sterilization of liquid drugs; with particular emphasize on ways to deliver the pulses without altering the drugs. Alteration could be from thermal or chemical effects of the IRE and is not acceptable when treating drugs.

## METHODS.

Commercially available HYLO-COMOD® Sodium Hyaluronate 0.1% Preservative-free drop solution was contaminated with E.coli bacteria to $10^6$ CFU/ml level. Electroporation with 100μsec length 1 second separated square pulses was performed applying 5.4kV/cm, 7.2kV/cm or 10kV/cm electric field. Ten and 20 pulses were tested. The 20 pulses were applied continuously or dividing into 4 sets, 5 pulses each with 1 minute interval between sets. Spread plate counting method was used to determine the survival percentage after the treatment. The impact of treatment on temperature and pH was monitored.

## RESULTS

The increase from 10 pulses to 20 pulses caused more than 10 fold reduction in bacterial survival from 4.33% to 0.37%, with only about 3 $^0$C difference in the sample temperature. The separating of 20 pulses to 4 sets of 5 pulses each with 1 minute pause between the pulses resulted in additional 3 times decrease of number of surviving microorganisms from 0.37% to 0.14%, (Left hand figure) while the temperature increase was the lowest (Right hand figure). Figure 1B. In all experiments the pH of the samples after treatment was unchanged at 7.5.




Left Post-IRE survival of microorganisms as a function of the number and sequence of electroporation pulses for an electrical field of 10 kV/cm. A-10 kV 20 pulses (four sets × 5), B-10 kV 20 pulses (one set), C-10 kV ten pulses (one set). Error bars represent 1 standard deviation.
Right Post-IRE temperature of the solution as a function of the number and sequence of electroporation pulses for an electrical field of 10 kV/cm. A-10 kV 20 pulses (four sets × 5), B-10 kV 20 pulses (one set), C-10 kV ten pulses (one set). Error bars represent 1 standard deviation

## CONCLUSIONS.

In this work we performed experiments to evaluate the feasibility of using non-thermal and non-chemical IRE in the field of drug sterilization. We demonstrated the feasibility and identified several ways to deliver the IRE pulses so as to minimize thermal and chemical effects.

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# Tumor Treatment Using Irreversible Electroporation with a Minimally Invasive Single Needle Electrode

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## INTRODUCTION

Irreversible electroporation (IRE) is an effective focal ablation technique that increases the permeability of cell membranes by exposing the cell to electric pulses [1]. When the electric pulses are beyond a critical level, the cell cannot repair itself and dies in a non-thermal manner that spares the surrounding extracellular matrix and sensitive structures. IRE has been shown to be a predictable and advantageous method for ablation of benign tissues [2-3]. This technique has been applied to effectively treat aggressive cutaneous tumors using plate electrodes [4]. We present the results here of using a new, minimally invasive needle electrode to treat orthotopically implanted tumors.

## METHODS

The preliminary study approved by the Institutional Animal Care and Use Committee used a single needle electrode, similar in dimension to those used for cryoablation, to deliver a series of electric pulses directly into the tumors of 7 female Nu/Nu mice. Tumors were grown from injections of MDA-MB231 human mammary carcinoma cells injected into the fourth inguinal mammary fat pad. A 5 mm incision was made to ensure proper electrode placement within the center of the tumor. The experimental setup may be seen in Figure 1 (A).

After electrode placement, a series of 100 pulses, in 3 second intervals, each 100 µs long, were delivered, alternating polarity every 25 pulses, with an initial applied voltage set to 1300 V. Due to changes in tissue conductivity that occurred during treatment, the voltage was reduced in 100 V increments at any sign of electrical arcing between the anode and cathode of the electrode. The minimum voltage reached was 1100 V. Tumor volume was measured using calipers.

In addition to the experiments conducted, numerical simulations were performed representing the treatment for a conductive tumor ($\sigma_t$ = 0.25 S/m) embedded within a fatty tissue matrix ($\sigma_p$ = 0.02 S/m) placed over muscle tissue.

## RESULTS

All mice tolerated the treatment well and healed well, showing no signs of pain. Complete tumor regression was observed in 5 out of the 7 tumors treated, while all 4 sham controls, where the electrode was placed but no pulses were delivered, showed continued tumor growth.

The model setup and output may be seen in Figure 1 (C,D). Correlating the treatment with the model, it appears that the minimum electric field to which the tumor was exposed is roughly 1000 V/cm, a value similar to one found in previous *in vitro* experiments on the same cell line [5].

These results show that IRE can effectively treat tumors using a single needle electrode design. Due to the shape of the electric field resulting from such a configuration, it is desirable to use a customizable electrode design where the diameters and exposure lengths may be adjusted to tailor the electric field distribution to the shape of the targeted region.

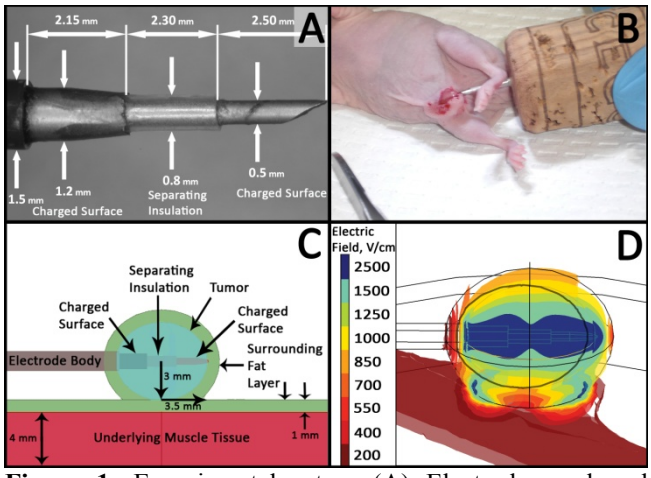


**Figure 1**: Experimental setup: (A) Electrode used and dimensions. (B) Insertion into mice. (C) Model setup. (D) Model output.

## CONCLUSION

This study provides evidence that IRE may be used to successfully treat tumors located within their physiologic location using a clinically translatable electrode and treatment protocol. These findings suggest that IRE may prove to be an effective method for the therapeutic treatment of pathologic tissues.

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# Bubble radius dynamics induced by ultrasound

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## INTRODUCTION

In recent years, the use of low-frequency ultrasound sources (30-200 kHz) has become increasingly common for non-invasive localized fat tissue removal [1,2]. The efficiency of the therapy varies with the region of treatment. After single treatment of the abdomen, the waist circumference reduces for 2-3 cm and the thickness of abdominal wall fat tissue reduces for 1-2 cm. Since cavitation has been shown to be responsible for many biophysical effects of ultrasound on cells, it is speculated to be the primary mechanism also in these applications. Cavitation is the creation, expansion, contraction and disappearance of gas bubbles in liquids exposed to acoustic pressure waves.

As the therapy based on ultrasound frequencies round 30 kHz was shown to be effective, we examined oscillations of differently sized bubbles due to acoustic pressure excitation of 30 kHz.

## METHODS

The structure of biological tissue on microscopic and macroscopic levels is complex. The rough approximation of biological tissue can be water, which represents a large part of the tissue. Therefore we studied the cavitation phenomenon in water. The dynamic behavior of a single spherical gas-bubble immersed in water can be modeled by Rayleigh-Plesset differential equation (RP). The equation relates pressure changes in the surrounding liquid to the changes of bubble radius, while the amplitude of pressure changes, the initial radius of the bubble, and the properties of the gas and liquid should be known.

The amplitude of pressure changes caused by a 30-kHz prototype ultrasound source was measured by a spherical hydrophone. Amplitudes up to 100 kPa were obtained and used as excitation amplitude in RP equation. The RP equation was then solved for different initial bubble radius sizes (in the range between 1 μm and 1 mm) and for low ultrasound frequencies (under 200 kHz).

Numerical solutions for the RP equation were obtained using Matlab (Mathworks, CA, USA) software.

## RESULTS

A single bubble behavior depends on ultrasound frequency and initial bubble radius. Harmonic pressure waves excite only bubbles of a distinct size. Dynamic changes in the bubble's radius are greater when the excitation frequency is close to the bubble's main resonance frequency. This frequency can be derived analytically as the Minnaert frequency [3]. A bubble with an initial radius of 0.1 mm has a resonance frequency of approximately 30 kHz. Bubbles with smaller (0.01 mm) or greater (1 mm) initial radius exhibit smaller changes in radius in response to identical ultrasound waves. The RP equation characterizes bubbles with an initial radius between 1 mm and 1 μm as stable. Therefore, there are no bubble implosions and thus no unstable cavitations.

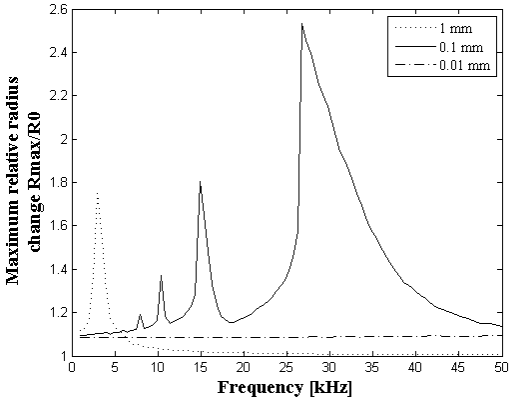


**Figure 1**: Maximal relative radius change as a function of excitation frequency. Initial values of bubble radius: 1 mm, 0.1 mm and 0.01 mm. Amplitude of harmonic pressure waves was 30 kPa, observation time 1 ms.

## DISCUSSION

The behavior of a single bubble due to harmonic pressure changes in the surrounding liquid depends on initial radius of the bubble. Our results show that in the water ultrasound at frequency of 30 kHz excites bubbles with initial radius of about 0.1 mm much more then bubbles with smaller and greater initial radius. Applying results on biological tissue, we can speculate, that ultrasound at these frequencies and amplitudes could also have greatest effects on structures of specific size. The nature of these structures however has yet to be determined.

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# Intracranial Non-Thermal Irreversible Electroporation: Electrical Conductivity Analysis

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## INTRODUCTION

Non-thermal irreversible electroporation (N-TIRE) is a promising new technique for the ablation of tissue and tumors [1]. This minimally invasive procedure involves placing electrodes into or around a targeted area and delivering a series of short and intense electric pulses to induce irrecoverable structural changes in cell membranes. To achieve N-TIRE, the electric field in the targeted region needs to be above a critical value, which is dependent on a variety of conditions such as tissue properties, electrode geometry and pulse parameters. The electrical conductivity, σ, is significant because it determines the electric field distribution, current, and thermal effects from a procedure. A method of computing the electrical conductivity from *in vivo* data is presented in this paper.

## METHODS

The pilot study was approved by the Institutional Animal Care and Use Committee and performed in a Good Laboratory Practices (GLP) compliant facility. Focal ablative N-TIRE lesions were created in the ectosylvian gyrus using the NanoKnife® generator (Angiodynamics, Queensbury, NY USA), and blunt tip probes in 3 canine subjects. The applied voltage and resulting current were monitored during the treatments (Table 1). A linear relationship between current and electrical conductivity was determined using numerical models (Comsol Multiphysics, v.3.5a, Stockholm, Sweden) for the electrode geometry and pulse parameters used in our study [2]. The electrical conductivity was computed from the *in vivo* data.

## RESULTS AND FIGURES

Post-operatively, no adverse clinical effects were observed in the animals. Three linear relations ($R^2 = 1$) between current and electrical conductivity were determined from the numerical models by integrating the current density over the electrodes surfaces. The resulting slopes from the linear relations were 5.851, 3.573 and 1.786 A/S/m for the single (1600V), dual (1000 V) and dual (500 V) probes respectively (Figure 1).

The computed electrical conductivity ranged between 0.202-0.435 S/m and is given in Table 1. These results confirm that there is variability between subjects and probe location. The electrical conductivity changes that result from a multiple pulse electroporation treatment was not investigated but will be incorporated in future work [3].

**Table 1:** Computed σ using intracranial N-TIRE data.

| Subject | Probe | Voltage [V] | Current [A] | σ [S/m] |
|---|---|---|---|---|
| 1 | Single | 1600 | 2.547 | 0.435 |
| 2 | Dual | 1000 | 1.951 | 0.546 |
| 3 | Dual | 500 | *0.361* | *0.202* |

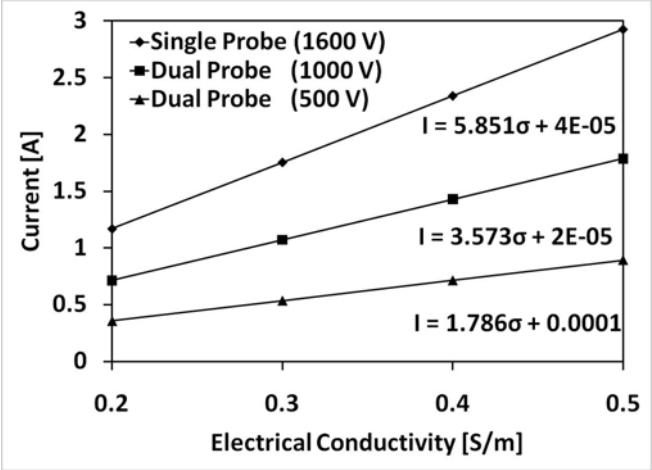


**Figure 1**: Graph of current as a function of electrical conductivity for a specific electrode geometry and voltage.

## CONCLUSION

We present a method of computing the electrical conductivity based on measured current and numerical methods. Due to the dependency of σ on individual subjects and treatment location, the electrical conductivity should be monitored before, during, and after an N-TIRE procedure. In this manner, one can ensure that the treatment planning is accurate and that damage beyond the volume of intended ablation is prevented.

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# Endoscopic Gene and Drug Delivery for Gastrointestinal Tissues and Tumours: A New Development

M.G. Bourke, M. Sadadcharam, P. Forde, M. de Kruijf, W. Byrne, L. Cogan, D. Soden, G.C. O'Sullivan. *Cork Cancer Research Centre, Biosciences Institute, University College Cork, Ireland.*

To date, electrochemotherapy (ECT) has been limited in application to the treatment of skin-based cancers. The development of ECT as an efficacious, less-invasive treatment strategy for the management of solid tumours has the potential to greatly enhance patient quality of life. Reduced hospitalisation costs, due to shorter treatment duration and less ICU requirements, are also a possibility. In addition, there is significant potential to treat patients who are presently considered unsuitable for surgery, systemic chemotherapy or radiotherapy. We have developed an endoscopic system that enables the extension of ECT to the treatment of intra-luminal gastrointestinal tumours. Studies conducted on spontaneous canine colorectal tumours have yielded very promising results. We report our initial experience with this system.

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